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Nitrate controls testa rupture and water content during release of physiological dormancy in seeds of *Sisymbrium officinale* (L.) Scop.

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Abstract

Seeds of *Sisymbrium officinale* display physiological dormancy and require nitrate to germinate. Rupture of the testa precedes radicle protrusion through the endosperm (germination *sensu stricto*). While both endosperm rupture and testa rupture (TR) required nitrate, endosperm rupture was fully inhibited by abscisic acid (ABA) but TR was not inhibited. The gibberellic acid (GA)-synthesis inhibitor paclobutrazol prevented TR, which was reverted by exogenous GA_4 but not by nitrate. The orientation of TR was transverse, which prompted the question whether seeds elongate prior to radicle protrusion, concurrent with an increase in water content. Between 9 h and 1 d no increase in length or water content was observed. During incubation in ABA the length of imbibed seeds without TR did not increase between 1 and 5 d, whereas nitrate added to ABA induced TR and a 94% increase in length. At the same time the water content of seeds without TR increased by 18%, while the water content of seeds with TR increased by 38%. Length and water content were correlated in a single-seed analysis for seeds with TR, but not for seeds without TR. Increased length was also observed in Arabidopsis seeds with nitrate-induced TR. These results indicate that prior to endosperm rupture dormancy release by nitrate is accompanied by TR, seed elongation and an increase in water content. A new multiphasic model is proposed for the imbibition curve, where the second phase of the classical triphasic curve is split into three sub-phases, of which phases IIB and IIC are associated with TR.

Keywords: Arabidopsis, imbibition phases, nitrate, physiological dormancy, seed imbibition, *Sisymbrium officinale*

Introduction

Most dry seeds readily imbibe when exposed to ample water, due to their negative water potential, whether physiological dormancy is present or not. During imbibition, three phases have been distinguished: phase I, marking a rapid uptake of water due to the low water potential of the seed; followed by phase II, in which the water content does not change substantially in the intact seed prior to radicle protrusion; and phase III, when rupture of the covering layers (testa and endosperm) allows growth of the collet and the protruded radicle becomes visible (Bewley and Black, 1994; Sliwinska *et al.*, 2009, 2012). Phases I and II represent the intact seed, while phase III represents the germinated seed with a visibly growing radicle. In this context radicle protrusion is usually the only developmental change that is observed.

Viable seeds that cannot complete germination when exposed to optimal germination conditions are considered to be dormant (Bewley and Black, 1994). Physiological dormancy is the most common type of dormancy (Baskin and Baskin, 1998), and this form of dormancy can be released through a range of environmental factors that form a requirement for the completion of germination (Cadman *et al.*, 2006). In most temperate species dormancy is lost by dry afterripening at higher temperatures or wet chilling (Hilhorst, 1995). Nitrate is an additional natural environmental component that is capable of releasing dormancy in several species (reviewed by Benech-Arnold *et al.*, 2000). While the nitrate content of seeds seems to play a limited role, the sensitivity to nitrate is of ecological significance (Bouwmeester *et al.*, 1994). Nitrate has been described as a way to release dormancy in a range of Brassicaceae species, including *Arabidopsis thaliana* and *Sisymbrium officinale* (Hilhorst *et al.*, 1986; Ali-Rachedi *et al.*, 2004; Finch-Savage *et al.*, 2007; Iglesias- Fernandez *et al.*, 2007; Toorop *et al.*, 2012; Carrillo-Barral *et al.*, 2014). Nitrate reduction seemed to play no role in dormancy release (Hilhorst

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and Karssen, 1989). Instead, in Arabidopsis, nitrate was thought to interfere with the degradation of abscisic acid (ABA) during imbibition (Matakiadis *et al.*, 2009). Seeds provided with nitrate still required light for radicle protrusion, indicating that nitrate alone is not sufficient for the completion of germination.

Completion of germination has been described as the product of two forces: embryo growth potential and the restraint of the covering layers (Ni and Bradford, 1993). Exogenous ABA was found to inhibit embryo growth potential during germination of *Brassica napus* (Schopfer and Plachy 1985), *Lycopersicon esculentum* (Toorop *et al.*, 2000) and *Coffea arabica* (da Silva *et al.*, 2004, 2008). The endosperm cap or micropylar endosperm is the tissue that weakens during germination, prior to radicle protrusion (Watkins and Cantliffe, 1983; Sanchez *et al.*, 1986; Welbaum *et al.*, 1995; Toorop *et al.*, 2000; da Silva *et al.* 2004; Müller *et al.*, 2006; Pinto *et al*, 2007). Exogenous ABA inhibits radicle protrusion and contributes to the inhibition of germination. In tomato seeds, ABA completely inhibits germination by inhibiting the second step in the weakening of the covering layers (Toorop *et al.*, 2000). ABA also inhibits the second step in the endosperm weakening of *C. arabica* (da Silva *et al.*, 2004), *Solanum lycocarpum* (Pinto *et al.*, 2007) and *Genipa americana* (Queiroz *et al.*, 2012); and delays the onset of endosperm weakening in *Lepidium sativum* (Müller *et al.*, 2006). Endosperm from dormant Arabidopsis seeds produces and releases ABA, maintained by high expression of *RGL2* (*RGA-LIKE2*), inhibiting embryo growth through ABI5 (*ABA-INSENSITIVE5*; Lee *et al.*, 2010). The expression of ABI5 is localized to the micropylar endosperm (Penfield *et al.*, 2006), which makes it likely that endogenous ABA plays a similar role as exogenous ABA in weakening of the endosperm.

Rupture of the testa has been described to precede rupture of the endosperm (reviewed by Weitbrecht *et al.*, 2011) in *Chenopodium album* (Karssen, 1976); *Trollius ledebouri* (Hepher and Roberts, 1985); *Nicotiana tabacum* (Leubner-Metzger *et al.*, 1995); *Phacelia tanacetifolia* (Serrato-Valenti *et al.*, 2000); *Nicotiana attenuata* (Krock *et al.*, 2002); *Petunia hybrida*, *Nicotiana sylvestris* and *Nicotiana plumbaginifolia* (Petruzelli *et al.*, 2003); *A. thaliana* and *Lepidium sativum* (Liu *et al.*, 2005; Müller *et al.*, 2006); *S. lycocarpum* (Pinto *et al.*, 2007); and *S. officinale* (Iglesias-Fernandez and Matilla, 2010). ABA did not alter the testa rupture of non-dormant *Cestroideae*-type seeds in the Solanaceae family (Petruzelli *et al.*, 2003), nor of *Lepidium*, Arabidopsis or *S. officinale* in the Brassicaceae family (Müller *et al.*, 2006; Carrillo-Barral *et al.*, 2014). On the other hand, nitrate stimulated the testa rupture of *S. officinale* (Carrillo-Barral *et al.*, 2014). Observations of testa rupture in this wide range of species suggest that this phenomenon is widespread in the plant kingdom.

In *S. lycocarpum*, testa rupture was accompanied by an increase in the water content of the embryo and a split in the testa that envelops the endosperm cap (Pinto *et al.*, 2007). In Arabidopsis it was noted that 'the endosperm elongates and emerges from the opened testa' upon testa rupture (Liu *et al.*, 2005), which typically is parallel to the axis. No detailed observations of changes in the seed water content or morphology have been described in relation to testa rupture. However, it seems likely that rupture of the testa, which is dead tissue, facilitates swelling of underlying tissues. The hypothesis is tested here that testa rupture in *S. officinale* seeds concurs with an increase in water content prior to endosperm rupture, and facilitates an increase in the seed dimensions.

Materials and methods

Seed material

Sisymbrium officinale (L.) Scop. seeds were collected in a field near Wageningen, The Netherlands, in the autumn of 2004, cleaned and stored. Plants of the *Arabidopsis thaliana* (Arabidopsis) accession Cape Verdi Islands (Cvi; accession number N8580) were grown as described by Cadman *et al.* (2006). Seeds were cleaned, dried and subsequently stored at 15% relative humidity (RH) and 15°C.

Germination

Seeds of *S. officinale* were sown in a 9-cm Petri dish on two layers of filter paper (Whatman No.1) and imbibed with 5 ml water or a solution containing (a combination of) 10 μM abscisic acid (racemic mixture, Sigma-Aldrich, Gillingham, Kent, UK), 50 μM paclobutrazol (Sigma-Aldrich), 20 mM KCl, 20 mM KNO_3 or 20 mM $NaNO_3$ (Fisher Chemicals, Loughborough, Leics, UK). Seeds were incubated in an incubator at 30°C with 8 h white light per day; Petri dishes were placed in a closed box lined with moist paper towel to avoid evaporation. For germination tests, three replicates of at least 50 seeds were used. Seeds were considered to have completed germination when 1-mm protrusion of covering layers by the gravitropically bent radicle was observed with a binocular microscope at 10× magnification. For optimization of germination temperature, dishes were placed at 1, 5, 10, 15, 20, 25, 30 or 35°C; germination was scored until a stable plateau was reached (up to 491, 281, 157, 144, 54, 25, 51 and 51 d, respectively). The germination rate was calculated for individual dishes as the reciprocal value of the time until 50% endosperm rupture, using the Boltzmann equation (Origin v6.1; http://www.originlab.com/). Occasional fungal infection observed during very long incubations was removed by rinsing the seeds briefly in water and transferring to a new dish; no fungal contamination was observed within 7 d of incubation.

Single-seed analysis of water content and length

Single seeds were weighed on a seven-place balance (model UMT2. Mettler Toledo, Leicester, UK) with a 0.1 μg readability, before, as well as various intervals after, imbibition in 5-cm Petri dishes on two layers of filter paper (Whatman No.1) on 10 μM ABA, to facilitate single-seed analysis of water content and length upon testa rupture but prior to endosperm rupture, with or without 20 mM KNO_3 and incubation at 30°C, as above. Water content was calculated by comparing data for dry and imbibed seeds, using 12 seeds per sampling time and treatment, and was expressed as the increase in water content after correction for the dry weight. Before recording the weight upon imbibition, single seeds were blotted briefly on two filter papers wetted with 2 ml demineralised water, to remove the excess water. Testa rupture was recorded for each seed. Prior to weighing, digital images were taken of individual seeds at ×200 magnification with a USB microscope (Dino-Lite AM413T; Absolute Data Services, Hemel Hempstead, Bucks, UK) connected to a computer. These images were used to measure the length of seeds, expressed as pixel number. Seed length and water content of individual seeds were used for single-seed analysis. The small size of Arabidopsis seeds and the presence of much mucilage formed an obstacle to performing single-seed analysis. Hence, only seed length was recorded for this species. The length of Arabidopsis seeds was measured 21 d after imbibition and incubation at 20°C on 100 μM ABA, 100 μM ABA and 10 mM KCl, or 100 μM ABA and 10 mM KNO_3. Testa rupture was recorded for each seed. All single-seed experiments were conducted in duplicate.

Statistical analysis

Data were analysed for two replicate experiments using GenStat (v.14.2; http://www.vsni.co.uk/). Since data did not follow a normal distribution and were not homoscedastic, Kruskal–Wallis analysis of variance (ANOVA) was applied to test differences between treatments, and Mann–Whitney *U* tests for *post-hoc* comparison of sets of two treatments within each group. A Spearman's rank correlation was applied to the increased seed length and water content.

Results

Germination of *S. officinale* seeds showed a bimodal response to temperature, with low germination in water at temperatures between 10 and 30°C and high germination at both lower (1 and 5°C) and higher (35°C) temperatures (Fig. 1). KNO_3 at 20 mM increased germination between 5 and 35°C, although germination at 15–25°C was less stimulated than at 10 or 30°C. The germination rate (GR) showed a single peak around 30°C and was extremely slow in water at low and high temperatures, with 98 d until half-maximal germination at 5°C and 26 d at 35°C (Fig. 1). When taking into account both the GR and the maximum germination in KNO_3, 30°C was considered the optimum temperature for germination. The low germination response in water at this temperature allowed investigation of the role of nitrate in the release of physiological dormancy.

KNO_3 was more efficient than $NaNO_3$ in facilitating endosperm rupture, while the KCl treatment confirmed the specificity of nitrate (Fig. 2). Testa rupture preceded endosperm rupture in all treatments. Both testa rupture and endosperm rupture were fully inhibited by tetcyclacis (data not shown) and paclobutrazol, two inhibitors of gibberellic acid (GA) synthesis, and KNO_3 could not reverse this inhibition; in contrast, exogenous GA_4 fully restored testa rupture and endosperm rupture in paclobutrazol, although the rate was low (Fig. 2). Constant darkness also fully inhibited endosperm rupture and testa rupture, despite the presence of nitrate (data not shown). ABA at a concentration of 10 μM fully inhibited endosperm rupture in the presence of KNO_3 but only delayed testa rupture, increasing the time until half-maximal testa rupture from 20 ± 2 to 38 ± 2 h in KNO_3. Multiple testa ruptures were commonly observed and the orientation of these testa ruptures was transverse to the axis (Fig. 3). This observation prompted the hypothesis that elongation of seeds

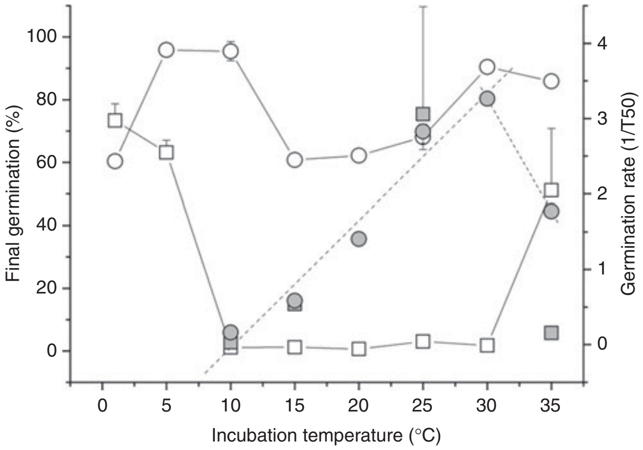

Figure 1. Final germination (open symbols) and germination rates (grey symbols) of *Sisymbrium officinale* seeds in water (squares) or 20 mM KNO_3 (circles). Data are means of three replicates with at least 50 seeds per plate; error bars are standard error.

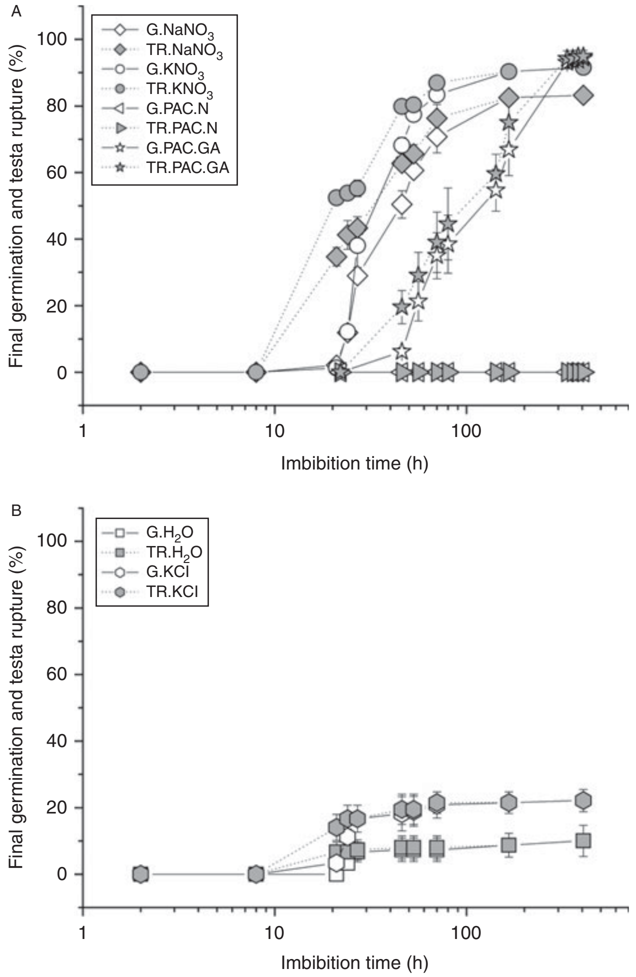

Figure 2. Germination (G, or endosperm rupture: closed symbols, solid lines) and testa rupture (TR: open symbols, dotted lines) of *S. officinale* seeds at 30°C in white light in 20 mM NaNO$_3$ (diamonds), 20 mM KNO$_3$ (circles), 50 μM paclobutrazol with 20 mM KNO$_3$ (triangles) or 50 μM paclobutrazol with 10 μM GA$_4$ (stars) (A), and in water (squares) or 20 mM KCl (hexagons) (B). Data are means of three replicates with at least 50 seeds per plate, error bars are standard error.

and a concurrent increase in water content takes place prior to endosperm rupture, facilitated by testa rupture. To test this, ABA was applied to inhibit endosperm rupture, facilitating the measurement of seed length prior to the completion of germination.

Seeds imbibed on ABA for 7 d displayed 88 ± 1% testa rupture in the presence of KNO$_3$, similar to the 94 ± 5% in the absence of ABA (data not shown). Seed length increase and water content increase differed for the following groups: imbibition on ABA without testa rupture, imbibition on ABA and KNO$_3$ without testa rupture, and imbibition on ABA and KNO$_3$ with testa rupture ($P < 0.001$). These parameters also differed for the sampling times 4, 9 and 14 h and 1, 3, 5 and 7 d ($P < 0.001$; Fig. 4). Because, after 4 h, seeds seemed not to be fully imbibed, this time point was excluded from further analysis, to restrict data to those that appeared fully imbibed. Seeds that did not display testa rupture, imbibed on ABA or ABA with KNO$_3$, did not show differences in length increase ($P = 0.419$). Although the water content increase of these seeds differed significantly ($P = 0.004$), the water content increase of the seeds imbibed on ABA with KNO$_3$ was only 5% more than those imbibed on ABA. Moreover, the separate comparisons of sampling times for these two treatments did not differ significantly. Therefore, seeds without testa rupture, imbibed on ABA and imbibed on ABA with KNO$_3$, were grouped and compared with seeds that displayed testa rupture and imbibed on ABA with KNO$_3$. The resulting groups (seeds with and seeds without testa rupture) differed in seed length increase ($P < 0.001$) and water content increase ($P < 0.001$), while sampling times also differed. When considering only the group of seeds without testa rupture, no differences were observed for the length increase between the sampling times ($P = 0.184$). Water content increase of seeds without

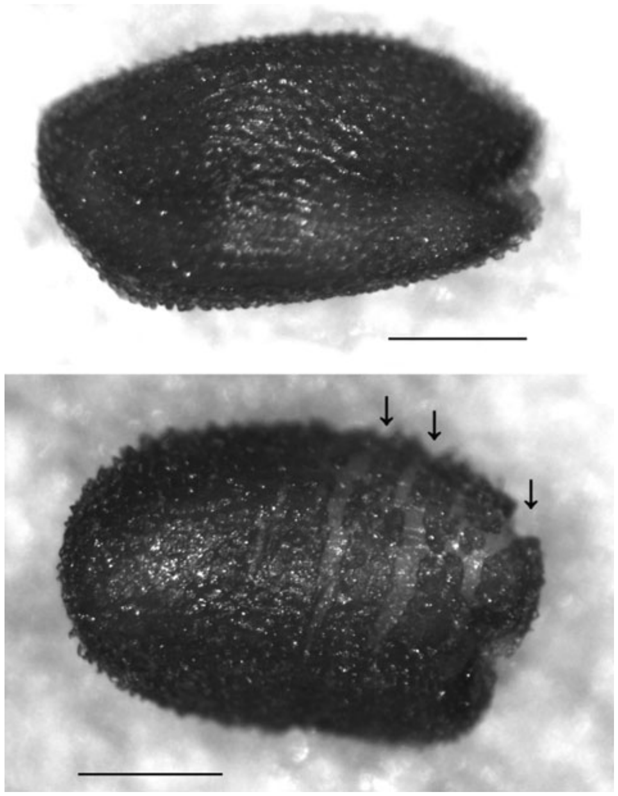

Figure 3. *Sisymbrium officinale* seeds after 7 d incubation in 100 μM ABA plus 20 mM KCl (top) or in 100 μM ABA plus 20 mM KNO$_3$ (bottom). Testa rupture was observed in the presence of nitrate, indicated by arrows. Typically, multiple ruptures occur which are transverse to the seed axis, wider at the axis side and narrower at the cotyledon side. Scale bar = 0.5 mm.

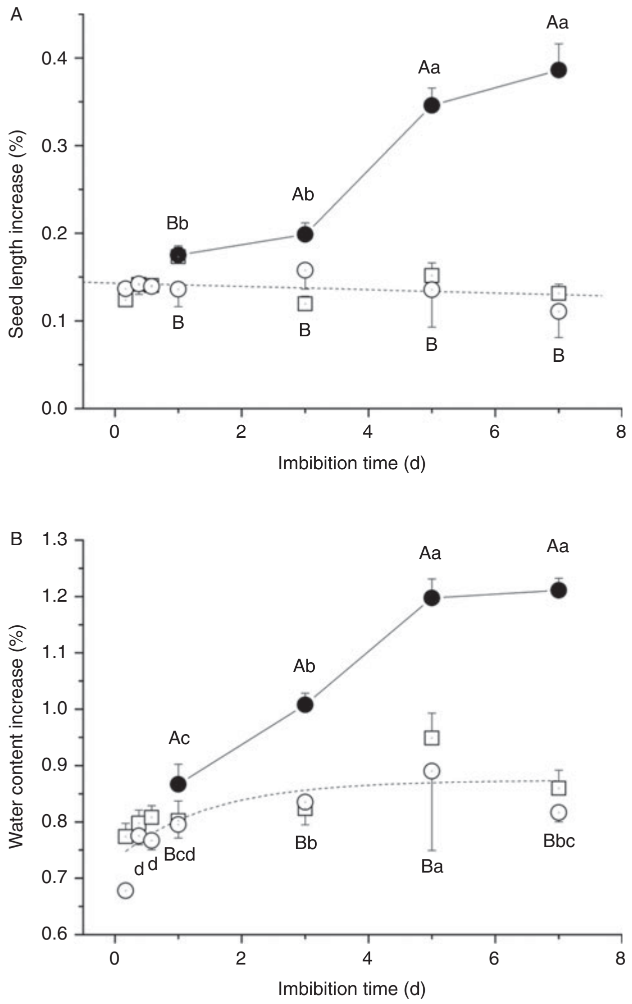

Figure 4. Increase in length (A) and water content (B) of *S. officinale* seeds upon imbibition on 10 μM ABA without nitrate showing no testa rupture (open squares), with 20 mM KNO_3 but showing no testa rupture (open circles), and with 20 mM KNO_3 showing testa rupture (closed circles). Identical capital letters indicate no differences for data points representing seeds with and without testa rupture (TR) at each sampling time; identical small letters indicate no differences for data points between sampling times within treatments (TR, no TR).

testa rupture differed between the sampling times ($P < 0.001$), displaying an 18% increase in the water content between incubations of 1 and 5 d (Fig. 4). The sampling times 9 h, 14 h and 1 d did not differ, indicating a plateau in water content between 9 h and 1 d. In contrast, the seeds with testa rupture differed in length increase ($P < 0.001$) and water content increase ($P < 0.001$) for 1 d and subsequent sampling times, displaying a 38% increase in the water content after 5 d (49% compared with seeds without testa rupture after 1 d) and a 94% increase in the length for the same interval (Fig. 4). The length and water content increase of seeds imbibed on paclobutrazol with or without KNO_3 remained at a similar level as that of seeds in ABA without KNO_3 (data not shown). Seeds without testa rupture, restricted to incubations of 1 d and longer to provide for a proper comparison of testa rupture, did not show a significant correlation for water content increase and length increase ($P = 0.192$), while seeds with testa rupture showed a significant correlation ($R = 0.654$, $P < 0.001$; Fig. 5).

Since testa rupture also preceded endosperm rupture in Arabidopsis Cvi seeds and ABA was incapable of inhibiting testa rupture, the effect of nitrate on length was also investigated in this species, which similarly displayed nitrate-stimulated dormancy release (12% germination in water, 100% in 10 mM KNO_3). ABA fully inhibited endosperm rupture of Arabidopsis seeds (not shown), but not testa rupture in the presence of KNO_3. In ABA, and ABA with KCl, Arabidopsis seeds with testa rupture were 6% longer than seeds without testa rupture (Fig. 6). In ABA with KNO_3, seeds with testa rupture were 16% longer than seeds in ABA without testa rupture (Fig. 6).

A conceptual model that explains the current results is presented (Fig. 7). In this model, the classical phase II of the triphasic imbibition model is split into three sub-classes: phase IIA is identical to the classical phase II, phase IIB is associated with testa rupture, while the transition between phase IIC and phase III marks endosperm rupture and radicle protrusion. This multiphasic model distinguishes two steps in water uptake, associated with dormancy and the absence of testa rupture (step 1) and with dormancy release with concomitant testa rupture (step 2).

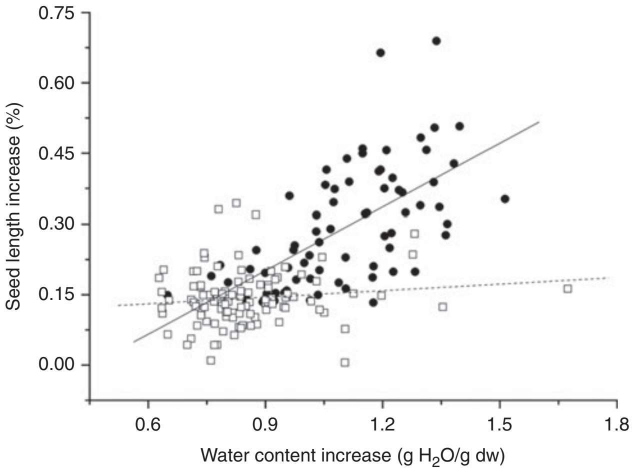

Figure 5. Water content increase versus length increase of *S. officinale* seeds, determined in a single-seed assay sampled 1, 3, 5 and 7 d after the start of imbibition. Treatments are: seeds without testa rupture (open squares, dotted line, $y = 0.103 + 0.047x$) and seeds with testa rupture (closed circles, solid line, $y = -0.205 + 0.451x$). Lines and equations represent linear regressions fitted separately for the groups.

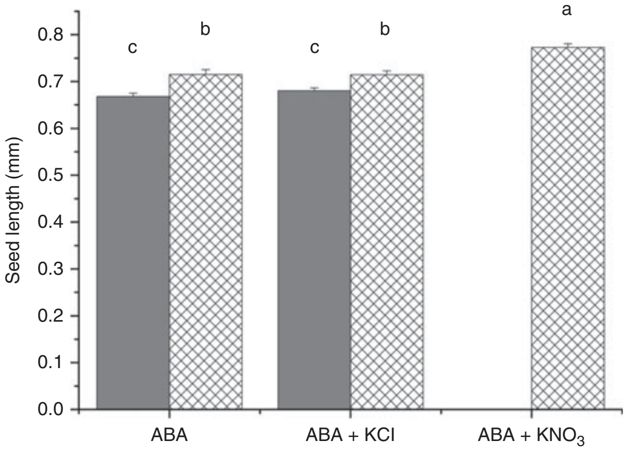

Figure 6. Seed length of Arabidopsis Cvi seeds after 21 d incubation in 10 μM ABA, 10 μM ABA with 10 mM KCl, and 10 μM ABA with 10 mM KNO_3. Cross-hatched bars represent seeds with testa rupture (TR), filled bars seeds without testa rupture. Identical letters indicate no differences between treatments and state of TR. Data are means of at least 20 seeds; error bars are standard error.

Discussion

Nitrate-induced testa rupture caused additional uptake of water and seed length increase. Isodiametric growth of embryo cells followed by cell elongation was described for coffee embryos upon full imbibition during germination (da Silva *et al.*, 2008). Elongation of the coffee embryo cells resulted in the appearance of a protuberance, since no restraining testa was present in these seeds, and in elongation of the seed later during imbibition. The current results are in agreement with this report, showing no continuing increase in length for seeds without testa rupture between 1 and 5 d, while elongation was detected upon testa rupture only after 5 d. The relatively short axis compared with the cotyledons in these seeds is probably the reason why elongation is observed only late after testa rupture compared with the increased water uptake, requiring a longer lag time until the axis extends beyond the dimensions of the cotyledons. Unlike coffee seeds, the rupturing of the testa precedes that of the endosperm in *S. officinale* and Arabidopsis (Linkies & Leubner-Metzger, 2012), two species with similar seed structure, and forms a prerequisite for seed elongation in both species. Although testa rupture in Arabidopsis is typically parallel to the axis (e.g. Liu *et al.*, 2005), unlike the transverse testa rupture pattern in *S. officinale*, seed elongation is still induced by nitrate, albeit after a longer time. Multiple ruptures were commonly observed in *S. officinale* seeds and the gaps of these ruptures were typically wider on the side of the axis than on the side of the cotyledons (Fig. 3, lower panel). This indicates that seed elongation and increased water content is a result of the swelling of the collet, the lower hypocotyl cells that elongate during radicle protrusion (Sliwinska *et al.*, 2009, 2012). The observed delay in testa rupture in the presence of ABA suggests that exogenous ABA modifies the embryo growth potential, possibly by delaying the swelling of the collet. Testa rupture of *S. lycocarpum* seeds appears to be limited to the micropylar region, and is typically observed as splitting along the plane of symmetry (Pinto *et al.*, 2007). A split testa in these seeds would allow expansion of the underlying tissues; although such observations have not been described. No increased water content of the collet has been reported prior to radicle protrusion in any species. However, it seems highly likely that this occurs shortly before endosperm rupture, thus driving the protrusion of the radicle through the endosperm cap. Even despite a growing protuberance and the absence of a rigid testa during germination, no increase in water content was reported for coffee seeds (da Silva *et al.*, 2004). The most likely reason for overlooking this detail in this and other species is that imbibition is typically described as an increase in mass instead of water content, resulting in large variation; and that single-seed analysis is not usually applied.

Water uptake of *S. officinale* seeds in the presence of potassium nitrate was described as being faster in

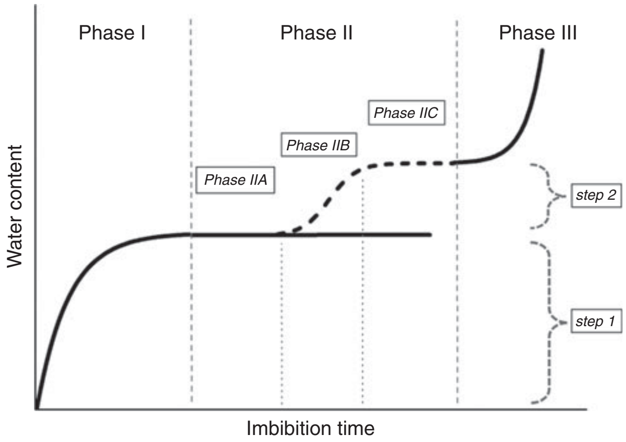

Figure 7. A new dormancy-dependent conceptual model for multiphasic imbibition of seeds that display testa rupture preceding endosperm rupture. Of the three phases in the classical model, delineated by the vertical dashed lines, the second phase is replaced by three sub-phases indicated with boxed italic text and separated by vertical dotted lines. Phase IIA concurs with the classical phase II. Phase IIB is associated with the onset of testa rupture, and phase IIC represents the elongated seed with testa rupture but without endosperm rupture. The dashed curve indicates phases IIB and IIC. The transition between phase IIC and phase III now marks endosperm rupture and radicle protrusion. The brackets indicate the two steps in water uptake prior to endosperm rupture. Step 1 is observed in dormant seeds without testa rupture, while in addition step 2 is observed in seeds with released dormancy and displaying testa rupture.

non-dormant afterripened seeds than in dormant seeds; nevertheless, phase II was reached after 5 h of imbibition at 30°C and remained stable up to 9 h (Iglesias-Fernandez and Matilla, 2009). The current data are in agreement, and confirmed that a plateau in water content was extended to 1 d. The complete dependence on nitrate for testa rupture, concurring with nitrate-dependent dormancy release, allowed further investigation of the role of nitrate in the present study. The limited continuing increase in water content in *S. officinale* seeds without testa rupture is possibly caused by radial swelling. Robert *et al.* (2008) observed a stronger early increase in radial swelling (termed short diameter in this reference) than elongation (termed long diameter) in Arabidopsis seeds, with further elongation between 3 and 24 h but no further radial swelling. No testa ruptures were reported in that study, and it is assumed that this did not take place within the first 24 h of imbibition. Although increased water content was described in Arabidopsis upon testa rupture but prior to endosperm rupture (fig. 1C in Dekkers *et al.*, 2013), this feature was not tested or described in detail. Hypothetically, the different water content for seeds with and without testa rupture may have contributed to the observed differences in gene expression (Dekkers *et al.*, 2013). Increased water content upon testa rupture was reported for tobacco seeds, which have highly noticeable elongation of the structures within prior to endosperm rupture (fig. 2B in Manz *et al.*, 2005). The current results demonstrate that testa rupture also facilitates elongation in both Arabidopsis and *S. officinale*, and increased water uptake in *S. officinale*. An increase in water content in phase II of the imbibition curve, concurring with testa rupture, has been described for *B. napus* (Schopfer and Plachy, 1984). However, this species is not endospermic and, since the testa forms only a barrier for swelling and growth of the embryo, this increase in water content is to be expected. In contrast, both *S. officinale* and Arabidopsis seeds are endospermic. The proposed new phases IIB and IIC were not observed in dormant seeds in the absence of nitrate. Therefore, it must be concluded that imbibition differs between dormant and non-dormant seeds.

Carrillo-Barral *et al.* (2013) reported that, in the presence of nitrate, in dormant *S. officinale* seeds the gibberellin synthesis inhibitor paclobutrazol allowed an intermediate degree of testa rupture, while in the absence of nitrate, testa rupture was fully inhibited; ABA also nearly completely inhibited testa rupture in these seeds. Testa rupture of afterripened seeds, on the other hand, was not sensitive to paclobutrazol (Carrillo-Barral *et al.* 2014). In contrast, our results demonstrated that, in the presence of nitrate, paclobutrazol completely inhibited testa rupture as well as endosperm rupture, indicating that gibberellin synthesis is required for testa rupture. This was further supported by the absence of testa rupture in the dark with or without nitrate. The accession that was used in the current study is considered to have stronger dormancy, since both testa rupture and endosperm rupture depended completely on exogenous nitrate. Moreover, the accession used here did not display afterripening despite several years of dry storage, providing further evidence for the deeply dormant status. Paclobutrazol was reported to inhibit transcription of *GA20ox2* and *GA3ox2*, two members of gene families involved in the synthesis of GA_4 during seed germination (Ogawa *et al.*, 2003; Iglesias-Fernandez and Matilla, 2010), while light at ambient temperature induced *GA3ox1* expression in the axis (Yamauchi *et al.*, 2004). When GA synthesis is inhibited, testa rupture in Arabidopsis is repressed by RGL2, which confers sensitivity to gibberellins; exogenous ABA allows *RGL2* expression and consequently does not repress testa rupture, which explained why ABA completely allowed testa rupture (Fig. 2; Piskurewicz *et al.*, 2008). *RGL3* also controls testa rupture but its contribution is minor compared with that of *RGL2*, with both genes acting redundantly in the inhibition of testa rupture (Piskurewicz and Lopez-Molina, 2009). It is possible that differences in GA signalling, which is possibly part of the nature of the different provenances, explain the different observations between the *S. officinale* accessions used here and those used by Carrillo-Barral *et al.* (2013, 2014).

The current results demonstrate an association of testa rupture, controlled by nitrate and associated with dormancy release, with an increase in seed length prior to endosperm rupture. A new multiphasic imbibition model is proposed to describe this phenomenon, in line with earlier observations by Manz *et al.* (2005). In this model, new elements are phase IIB, which is associated with the onset of testa rupture, and phase IIC, which forms a plateau prior to endosperm rupture, indicated by the transition between phases IIC and III. The duration of each phase varies with species and environmental conditions (Iglesias-Fernandez and Matilla, 2009). Complete reduction of time between testa and endosperm rupture can revert this model to the classical triphasic imbibition model (Bewley and Black, 1994).

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Conflict of interest

None.

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