Seed Science Research (2007) **17**, 165–174 DOI: 10.1017/S0960258507785872

Endogenous abscisic acid and precocious germination of developing soybean seeds

Carlos O. Gosparini[1], Hector A. Busilacchi[2], Paolo Vernieri[3] and Eligio N. Morandi[1]*

[1]Cátedras de Fisiología Vegetal and [2]Biología, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, Campo Experimental J. Villarino, CC14, S2125ZAA, Zavalla, Santa Fe, Argentina; [3]Dipartimento di Biologia delle Piante Agrarie, Universitá degli Studi di Pisa, Viale delle Piagge n° 23, 56124 Pisa, Italy

Abstract

The germination of developing seeds is very uncommon and is generally associated with deficiencies in abscisic acid (ABA) synthesis or sensitivity. This paper examines the quantitative relationship between the inhibition of precocious germination and endogenous ABA in the embryonic axis (ABAa) of hydrated soybean [*Glycine max* (L.) Merr.] seeds, isolated after the completion of histodifferentiation and before the beginning of dehydration, as well as the magnitude and evolution of axis sensitivity to endogenous ABA during that period. Developing seeds harvested at 25, 30, 35, 40 and 45 d after anthesis (DAA) were subjected to incubation or washing to induce changes in ABA content. ABA content was measured by radioimmunoassay, using a monoclonal antibody against free ABA. Germinability was measured as the time to 50% germination (t_{50}). Washing and incubation induced eight- and twofold increases, respectively, in the rate of ABAa decline compared with the *in planta* ABAa decline. The threshold ABAa for inhibition of precocious germination (ABAc) increased slightly from 25 to 40 DAA [1.15–1.66 μg ABA (g DW)$^{-1}$]. This contrasted with the substantial decline in ABAa [10.90–2.07 μg ABA (g DW)$^{-1}$] during the same period, and indicated that sensitivity to endogenous ABA of hydrated seeds was initially high and diminished slowly during development. The relationship between (ABAa–ABAc) and t_{50} was linear for immature seeds incubated before and after washing. Below the ABAc, there were no differences in the t_{50} of 25–45 DAA seeds. The ABAa contribution to the control of precocious soybean seed germination was evident, although other potentially interacting factors were also present.

Keywords: abscisic acid, *Glycine max*, precocious germination, seed development, seed maturation, soybean

Introduction

Abscisic acid (ABA) regulates key events during seed formation, such as the deposition of storage reserves, the acquisition of desiccation tolerance, the induction of primary dormancy and the prevention of precocious germination (Bewley and Black, 1994; Kermode, 2005). Germination of developing seeds is a very uncommon phenomenon, and it is generally associated with deficiencies in ABA synthesis or sensitivity (Black, 1991; Hilhorst, 1995; Karssen, 1995; Bewley, 1997). Support for this hypothesis comes from the isolation of mutants deficient in ABA content or responsiveness, such as maize *viviparous* (*vp*) (Robichaud *et al.*, 1980), tomato *sitiens* (*sit*) (Karssen, 1995), and *Arabidopsis ABA-deficient* (*aba*) and *ABA-insensitive* (*abi*) (Koornneef and Karssen, 1994; McCarty, 1995). Also, overexpression of genes for ABA biosynthesis increased seed ABA content and enhanced seed dormancy or delayed germination in *Nicotiana plumbaginifolia* (Frey *et al.*, 1999; Qin and Zeevaart, 2002), tomato (Thompson *et al.*, 2000) and *Arabidopsis* (Lindgren *et al.*, 2003). On the contrary, transgenic tobacco seeds expressing a seed-specific gene that produces an anti-ABA antibody precociously germinated when isolated (Phillips *et al.*, 1997). Despite this evidence, it is controversial that control of developing seed germinability universally rests with ABA (Bewley and Black, 1994).

In soybean [*Glycine max* (L.) Merr.] seeds, ABA content is low at the start of embryogenesis, increases rapidly, reaching a peak between 18 and 21 d after

*Correspondence:
Fax: 54-341-4970085
Email: emorandi@unr.edu.ar

anthesis (DAA), and then decreases slowly until physiological maturity (PM; Ackerson, 1984; Schussler *et al.*, 1984; Morandi *et al.*, 1990). At PM, ABA content in soybean seeds is very low, the connection with the mother plant is interrupted, and seeds start to dehydrate rapidly prior to harvest maturity. Around the time that the ABA peak is reached, the histodifferentiation phase is completed, and the embryo contains all the vegetative tissues of the new plant. From the end of histodifferentiation until almost PM, seed dry weight (DW) increases because the reserve accumulation programme is fully active (Egli, 1998). In addition, during the entire seed growth period, there is a net gain of water by the seed (Egli, 1998; Gosparini, 2002). However, developing soybean seeds do not germinate *in planta*, but if an immature soybean seed is isolated during the second half of its development (around 21 DAA), it can be induced to germinate if incubated in adequate conditions of temperature, humidity and oxygen pressure, without any additional requirement (Miles *et al.*, 1988; Morandi and Gosparini, 1991; Gosparini *et al.*, 1997). However, the rate of germination of isolated, immature fresh seeds is very low when compared with the rate of germination of a mature seeds. The rate of germination of isolated seeds increases with the increase in seed age from 25 to 45 DAA (Morandi and Gosparini, 1991; Gosparini *et al.*, 1997), indicating that constraints to germination are gradually released during the seed maturation period. This period is coincidental with the time at which the ABA content of seed tissues is declining (Ackerson, 1984; Morandi *et al.*, 1990). It seems logical to suppose that the effect of age on the rate of germination of excised immature soybean seeds is controlled by ABA. Ackerson (1984) was able to induce precocious germination of 21-day-old soybean seeds by depleting their embryonic ABA content by slow drying or washing treatments. He related the germination of 21 DAA seeds with ABA content in the entire embryo, without discrimination between cotyledons and embryonic axis. The identification of the target organ directly involved in a physiological response is crucial (Trewavas, 1991; Hilhorst, 1995; Bewley, 1997). Since germination of an immature soybean seed implies the resumption of its axis growth, the target organ for ABA action must be the embryonic axis. However, there is no information available for the relationship between the endogenous ABA content in the axis (ABAa) and the germinability of immature soybean seeds, isolated during the second half of development, as well as on the magnitude and evolution of the sensitivity of the axis to endogenous ABA during this period. In this study, we show that germinability of developing, non-dehydrated, soybean seeds is quantitatively associated with the amounts of endogenous ABA in the axes, and that the inhibition of precocious germination is very sensitive to endogenous ABA.

Materials and methods

Plant genotype and growing conditions

Soybean [*Glycine max* (L.) Merr.] cv. Williams 82 was grown in the greenhouse in 8 cm pots filled with humus-rich soil and perlite 3:1 (v/v). Seeds were treated with the fungicide, tiabendazol [2-(4-tiazolin)-bendimidazol], and inoculated with *Bradyrrizhobium japonicum* (Kirchner) Jordan before sowing. Pots were over-seeded, and seedlings at the unifoliate leaf stage were thinned to one uniform seedling per pot. The average temperatures during the day and night were 28 ± 2°C and 18 ± 2°C, respectively. The mean photosynthetic active photon flux density was 500 $\mu E\, m^{-2}\, s^{-1}$ (400–700 nm), measured with a LI-COR 185a radiometer and 190 s sensor (LI-COR Ltd, Lincoln, Nebraska, USA).

Fruit harvest and seed treatments

Uniform, synchronously growing fruits were harvested at 25, 30, 35, 40 and 45 d after anthesis (DAA) and superficially disinfected by immersion in 0.5% sodium hypochlorite solution for 10 min. Seeds were then separated from their fruits under a laminar flow hood. Isolated seeds were sterilized by immersion in a solution of 0.5% sodium hypochlorite for 1 min and washed twice in sterile water before incubation. The age of the developing seeds was considered equal to the DAA at which their respective fruits were harvested.

Forty immature, highly uniform seeds of each age were selected. Within each age, four seeds were randomly sampled and used for the measurement of ABA content at harvest (t_0). The remaining seeds were incubated in nine sterilized Petri dishes (four seeds per dish) on cotton and filter paper saturated with distilled water in the dark at 27 ± 1°C. One dish (four seeds) was randomly sampled after 12, 24, 48, 96 and 168 h for the measurement of ABA content during incubation. The number of germinated seeds was recorded daily and expressed as a percentage of the total (%G). The %G was measured previous to the sampling for ABA determinations. Thus, a total of 32, 28, 24, 24, 20, 20 and 20 seeds were used to calculate %G at 24, 48, 72, 96, 120, 144 and 168 h, respectively. A seed was considered germinated when its radicle protruded through the tegument. Time required for 50%G (t_{50}) was used to compare the effects of different seed ages and treatments on germination.

In another experiment, seeds isolated at 25, 30, 35 and 40 DAA were washed in distilled water for 0, 6, 12

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

and 24 h before incubation. Thirty seeds were used for each age and time of washing. Twenty seeds were washed together in 120 ml of distilled water and used for the germination assay. The other ten seeds were individually washed in test tubes containing 6 ml of distilled water and used for ABA analysis. Washing was performed in the dark at 27 ± 1°C with gentle shaking by aeration at low pressure. Washing treatments were performed at the same temperature as the germination assay. Because seeds started imbibition at the beginning of the washing, the washing time was added to the incubation time for the calculation of t_{50}.

ABA extraction

The sampled seeds were dissected (tegument, cotyledons and embryonic axis), frozen in liquid N_2 and lyophilized. The dry weight (DW) of each tissue was determined separately. Samples were kept at −70°C until ABA analysis. The ABA extraction was carried out in distilled water (Loveys and Van Dijk, 1988). In brief, each lyophilized sample was hydrated in 500 µl distilled water for 2 h, frozen in liquid N_2, thawed at room temperature and extracted overnight at 4°C in the dark. To discard the possibility of an incomplete ABA diffusion into the aqueous medium, the efficiency of aqueous extraction of intact cotyledons was compared with the extraction of homogenized cotyledons. Also, to discard an overestimation due to hydrolytic processes that liberate ABA from ABA conjugates, the measurement of free ABA from the 4°C extracts was compared with measurement of extracts previously boiled for 10 min to eliminate the enzymatic activity. There were no significant differences in ABA content of immature soybean cotyledons measured in aqueous extracts of intact tissue or in homogenized tissue extracts, or between extractions performed with and without boiling (data not shown).

Validation of radioimmunoassay

ABA quantification was performed on crude extracts by solid-phase radioimmunoassay (RIA) based on the use of the monoclonal antibody DBPA1, raised against S-(+)-ABA (Vernieri *et al.*, 1989a). All determinations were done in duplicate. The monoclonal antibody DBPA1 shows a high specificity for free S-(+)-ABA (Vernieri *et al.*, 1989a; Walker–Simmons *et al.*, 1991). Nevertheless, as it was the first time this antibody was used with soybean seed tissues, it was tested for the presence of competitive interference after high performance liquid chromatography (HPLC) fractionation of the crude aqueous extract (Vernieri *et al.*, 1989b). In brief, an HPLC instrument equipped with a UV absorbance detector at 254 nm (Laboratory Data Control, Riviera Beach, Florida, USA) was used. The column (15 cm × 0.635 cm outer diameter, packed with LiChrosorb RP18, 10 µm) was eluted at a flow rate of 1 ml min^{-1} using different ratios of methanol and water (with 0.05 M acetic acid) as follows: 30% methanol for 6 min; a linear gradient of 30–50% methanol for 20 min; 50% methanol for 6 min; a linear gradient of 50–100% methanol for 15 min. Two-ml fractions were collected, dried under vacuum, and resuspended in 75 mM phosphate-buffered saline (PBS, pH 7). Each fraction was assayed in triplicate by RIA. The DBPA1 antibody exhibited minimal cross-reaction for soybean embryo axis and cotyledon tissues, and none for tegument tissue (Fig. 1). Non-competitive interferences were evaluated by internal standardization experiments, adding an aliquot of crude aqueous extracts to increasing concentrations of ABA, and plotting measured ABA as function of ABA added. These experiments indicated the absence of non-competitive interferences for embryo axes, cotyledon and tegument tissues (data not shown).

Calculation of the rate of ABAa decline

The mean rate of *in vitro* endogenous embryonic axis abscisic acid (ABAa) decline (RAD) was calculated for the period prior to radicle protrusion. Thus, the RAD of incubated seeds was obtained during the first 96 h of incubation for 25 and 30 DAA and during the first 48 h of incubation for 35 and 40 DAA seeds, whereas RAD of washed seeds was obtained from the slope of the regression line of ABAa versus time during the 24 h of washing. The RAD during seed growth *in planta* was calculated as the difference in ABAa between two successive harvests (seed ages) divided by the days between harvests (5 d).

Results

ABA content in seed tissues during the second half of development

The changes of ABA content in embryonic axes, cotyledons and tegument tissues of immature soybean seeds from 25 to 45 DAA is shown in Fig. 2. ABA in a mature, dry seed (>60 DAA) was included as a reference. Data in Fig. 2 were pooled from several experiments and characterized the changes in ABA throughout the soybean seed maturation period, during which ABA declined in all seed tissues (Gosparini, 2002).

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

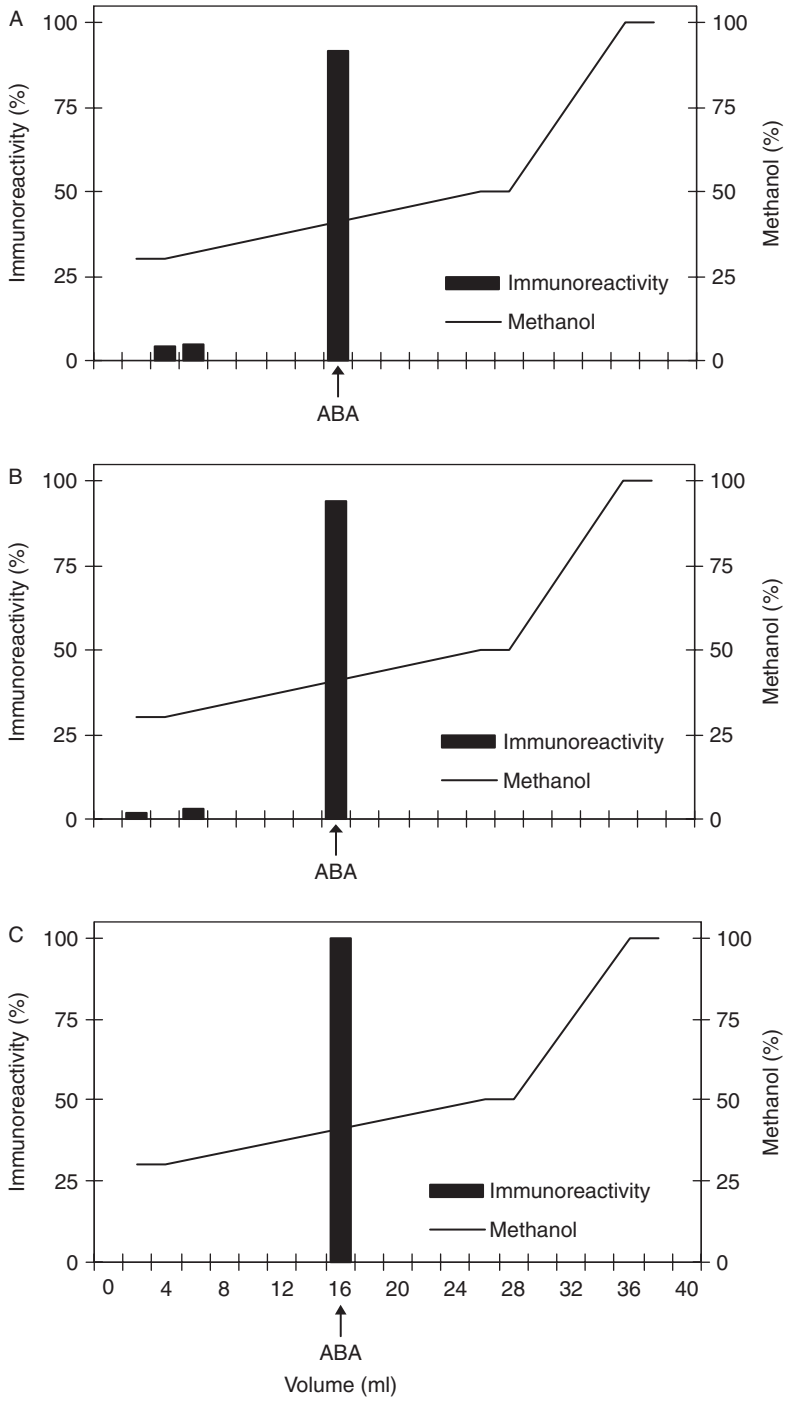

Figure 1. Elution of immunoreactivity to S-(+)-abscisic acid (ABA) by high performance liquid chromatography (HPLC) fractionation of soybean seed crude extracts from embryonic axes (A), cotyledons (B) and teguments (C).

Germination of incubated immature seeds

Germination started between 72 and 96 h of incubation for seeds of 25 and 30 DAA, between 48 and 72 h for seeds of 35 DAA, and between 24 and 48 h for seeds of 40 and 45 DAA (Fig. 3). The maximum percentage of germination was 50% for 25 and 30 DAA seeds, 80% for 35 and 40 DAA seeds and 90% for

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

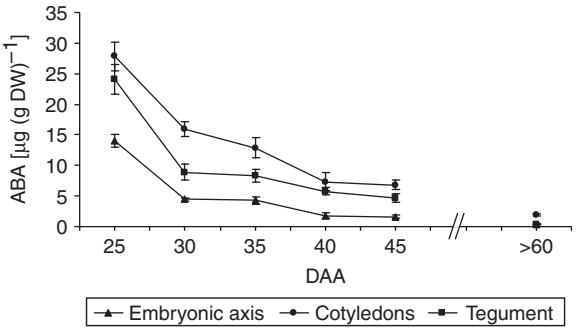

Figure 2. Abscisic acid (ABA) contents of embryonic axes, cotyledons and teguments of soybean seeds at 25, 30, 35, 40, 45 and > 60 d after anthesis (DAA). Points represent the mean ± SE of 19 replicates.

45 DAA seeds. The t_{50} was 144, 120, 84, 68 and 57 h, for 25, 30, 35, 40 and 45 DAA seeds, respectively (Fig. 3).

ABAa decline during incubation

The decline in ABAa of developing seeds as a function of incubation time is shown in Fig. 4. Initial ABAa was 10.90, 5.39, 2.52, 2.07 and 0.34 μg ABA (g DW)$^{-1}$, for 25, 30, 35, 40 and 45 DAA seeds, respectively (Fig. 4, t_0). The decline in ABAa occurred during the first 96 h of incubation in seeds of 25 and 30 DAA and during the first 48 h in seeds of 35 and 40 DAA. Seeds of 45 DAA displayed low and constant ABAa during the first 48 h (Fig. 4).

The ABAa decline in 25 to 40 DAA seeds occurred prior to radicle protrusion. The slight increase in ABAa measured after radicle protrusion in all seed ages was attributed to *de novo* synthesis associated with radicle growth, as observed previously by Iglesias and Babiano (1997).

Critical ABA levels for germination inhibition (ABAc)

Soybean seeds of 45 DAA were at physiological maturity (PM), and their ABAa was not different from the amounts of ABA found in the axis of mature dry (MD) seeds [0.34 and 0.33 μg ABA (g DW)$^{-1}$ for PM and MD seeds, respectively]. As germination of MD seeds is not inhibited by ABA, it was assumed that the ABAa present in 45 DAA seeds was no longer inhibiting germination. Thus, the t_{50} of 45 DAA seeds (57 h) was used as the reference time for germination in the absence of ABA inhibition for a non-dehydrated seed. The differences between the t_{50} corresponding to each seed age and the t_{50} of 45 DAA seeds were: 87, 63, 27 and 11 h, for 25, 30, 35 and 40 DAA seeds, respectively. These values corresponded to the additional incubation time (Δt) required by immature seeds (25–40 DAA) to reach 50% germination, when compared with the time required by PM seeds (45 DAA). ABAa contents at which germination was no longer inhibited by ABA (or ABAc) were obtained for each seed age, by replacing the corresponding values of Δt in the equations of ABAa decline during incubation (Fig. 4, inset). The ABAc values calculated

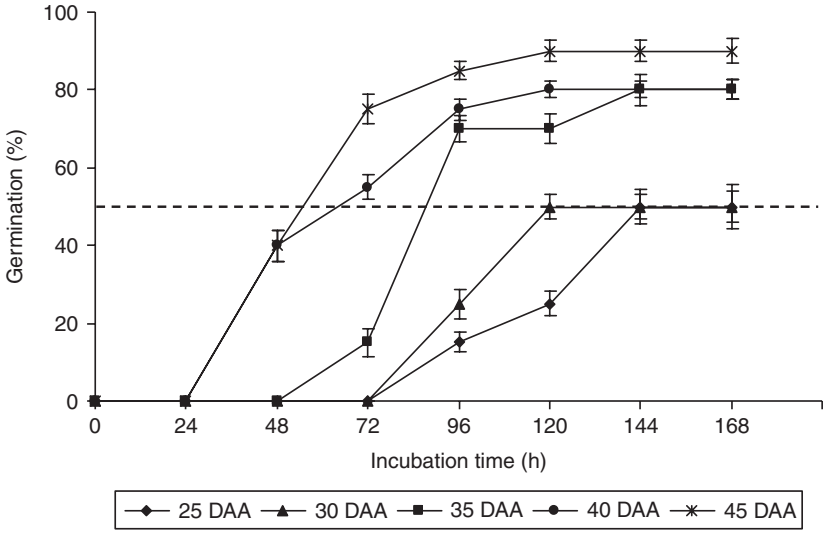

Figure 3. Time-course of germination of immature soybean seeds harvested at 25, 30, 35, 40 and 45 d after anthesis (DAA) at 27 ± 1°C. Points represent the mean ± SE of a maximum of 32 and a minimum of 20 seeds. The SE was within the data point if not shown. The dotted line corresponds to 50% germination.

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

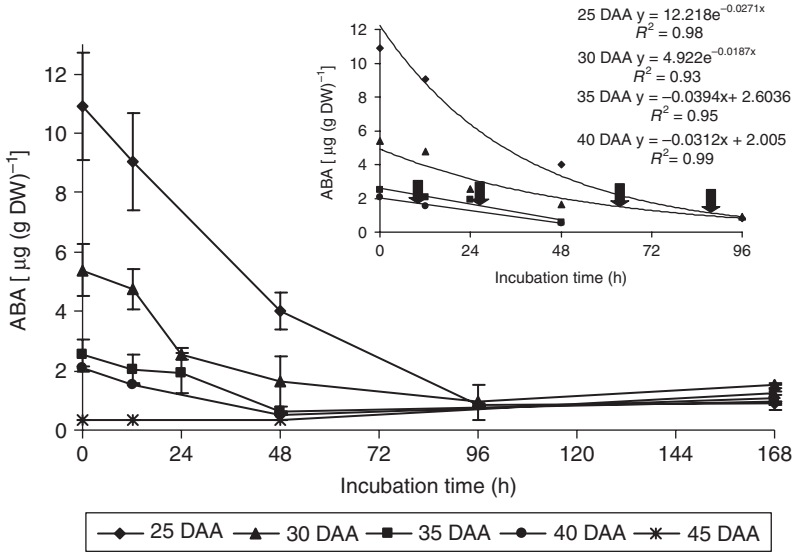

Figure 4. Patterns of endogenous abscisic acid in the embryonic axes (ABAa) during incubation of immature intact soybean seeds harvested at 25, 30, 35, 40 and 45 d after anthesis (DAA). Incubation conditions were the same as in Fig. 3. Points represent the mean ± SE of four replicates. The inset shows the curves that best fit the changes in ABAa before radicle protrusion. Arrows on inset curves indicate the additional times needed by 25–40 DAA seeds to reach 50% germination over the time required by seeds of 45 DAA (physiological maturity, $t_{50} = 57$ h).

in this way were: 1.15, 1.52, 1.54 and 1.66 µg ABA (g DW)$^{-1}$ for 25, 30, 35 and 40 DAA seeds, respectively. The delay in germination of an immature seed (25–40 DAA) was associated with the time required to reduce its ABAa to a value equal or lower than the ABAc. The relationship between t_{50} and (ABAa–ABAc) was represented by the equation:

$$t_{50} = 7.96(\text{ABAa} - \text{ABAc}) + 73.17,\ R^2 = 0.87 \quad (1)$$

Germination of immature washed seeds

The time course of germination of soybean seeds of 25, 30, 35 and 40 DAA, washed for 0, 6, 12 and 24 h is shown in Fig. 5. At t_0, 25 and 30 DAA seeds did not reach 50% germination during the germination assay, whereas the t_{50} of 35 and 40 DAA seeds was 153 and 98 h, respectively (Fig. 5A). After 6 h of washing, t_{50} was 162, 85, 96 and 62 h, for 25, 30, 35 and 40 DAA seeds, respectively (Fig. 5B). After 12 h of washing, t_{50} was reduced to 116, 65, 54 and 55 h, for 25, 30, 35 and 40 DAA seeds, respectively (Fig. 5C). After 24 h of washing, t_{50} was further reduced to 96, 62, 55 and 55 h, for 25, 30, 35 and 40 DAA seeds, respectively (Fig. 5D).

ABAa decline during washing

Washing induced a rapid decline in ABAa. For all seed developmental stages, ABAa declined with the duration of washing (Table 1). Except for 25 and 30 DAA seeds without washing (t_0), which did not reach the t_{50} by the time the germination assay was completed, the delay in t_{50} of the seeds with ABAa above ABAc was directly related to their ABAa (Table 1, values in bold). In contrast, when ABAa was below ABAc, there were small or no differences in the t_{50} of 30, 35 and 40 DAA seeds (Table 1, values in italics). Moreover, the mean t_{50} for 30 to 40 DAA seeds with ABAa below ABAc was 58 h, a value very close to the 57 h obtained for the t_{50} of an incubated, physiologically mature seed (Fig. 3).

Treatment efficiency in inducing a decline in ABAa

Seeds of the same developmental stage from different experiments showed variations in ABAa levels, probably due to differences in the environmental growing conditions of the mother plants (Kermode, 2005). In spite of this, the mean rate of ABAa decline (RAD), obtained for the period preceding radicle protrusion, was always directly related to the initial ABAa (ABAa at t_0) (Fig. 6). The treatment efficiency in reducing ABAa was represented by the slope of the regression line of the plot of ABAa at t_0 versus RAD under the assay conditions (Fig. 6). Comparing slopes under different conditions, it became clear that washing

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

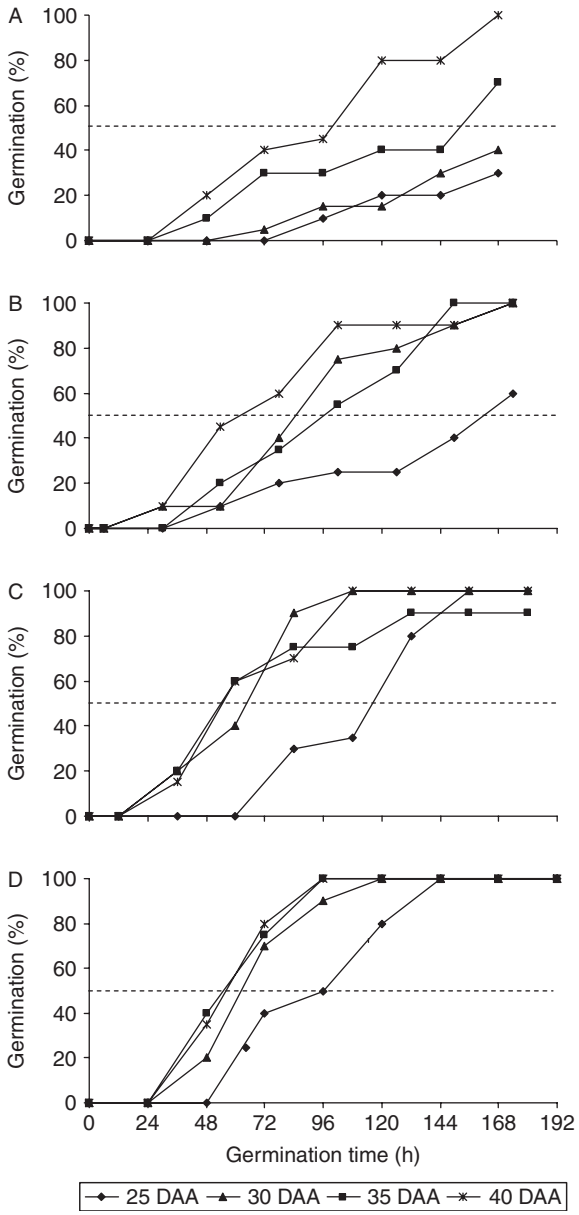

Figure 5. Time-course of germination of immature soybean seeds harvested at 25, 30, 35 and 40 d after anthesis (DAA), washed in distilled water for 0 (A), 6 (B), 12 (C) and 24 h (D) before incubation. Incubation conditions were the same as in Fig. 3. Points represent the mean of 20 seeds for each seed developmental stage. The dotted line corresponds to 50% germination.

and incubation induced ABAa declines 8.1 and 2.3 times faster, respectively, than the natural ABAa decline *in planta* (Fig. 6). Also, the ABAa decline was 3.5 times faster in washed than in incubated seeds (Fig. 6).

Table 1. Changes in the amounts of endogenous abscisic acid in the embryonic axes (ABAa) and time to 50% germination (t_{50}) of immature soybean seeds harvested at 25, 30, 35 and 40 d after anthesis (DAA) and first washed in distilled water for 0–24 h

DAA	Washing time (h)	ABAa [μg (g DW)$^{-1}$]	t_{50} (h)
25	0	**12.98**	–[a]
	6	**11.33**	**162**[b]
	12	**6.82**	**116**
	24	**2.68**	**96**
30	0	**7.40**	–[a]
	6	**2.97**	**85**
	12	*1.03*	*65*[c]
	24	*0.86*	*62*
35	0	**6.78**	**119**
	6	**3.45**	**96**
	12	*1.15*	*54*
	24	*1.19*	*55*
40	0	**4.87**	**98**
	6	*1.61*	*62*
	12	*0.88*	*55*
	24	*0.23*	*55*

[a] Seeds did not reach t_{50} by the time the germination assay was completed (168 h).
[b] ABAa and t_{50} in bold correspond to seed developmental stages and washing times in which ABAa > ABAc.
[c] ABAa and t_{50} in italics correspond to seed developmental stages and washing times in which ABAa ≤ ABAc.

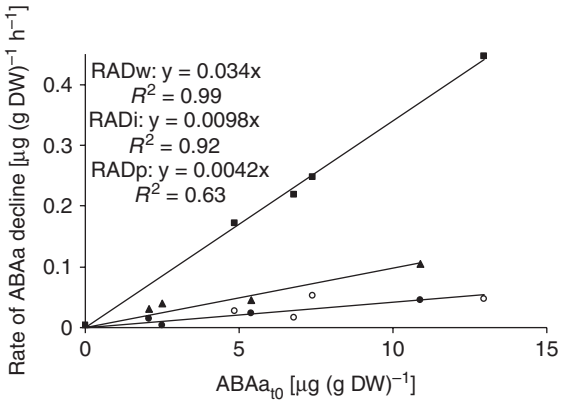

Figure 6. Mean rate of the decline (RAD) of endogenous abscisic acid in the embryonic axes (ABAa) before radicle protrusion, as a function of the initial ABAa for immature soybean seeds at 25, 30, 35 and 40 d after anthesis (DAA): washed, RADw (■) and incubated, RADi (▲). The mean RAD for seeds growing *in planta* (RADp), Experiment 1 (●) and Experiment 2 (○), was calculated between successive seed ages. See Materials and methods for details of the calculation procedure. Lines were forced to the origin.

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

Discussion

The monoclonal antibody DBPA1 against free S-(+)-ABA, used in a solid phase RIA, resulted in a very sensitive and specific quantification of endogenous ABA in soybean seed tissues. Our results demonstrated that the time an isolated, immature soybean seed requires to germinate was directly related to the ABAa at the moment of excision, and was inversely related to the rate of ABAa depletion during incubation or washing. In other words, germination will be arrested when ABAa remains above its critical level, ABAc. The relationship between (ABAa – ABAc) and t_{50} was linear for seeds of 25–40 DAA. This relationship is described by equation 1 and was applicable to both incubated and washed seeds. Incubated seeds of 25 and 30 DAA, however, did not germinate more than 50% after 168 h (Fig. 3). Similarly, seeds of the same developmental stages in the washing experiment did not reach 50% germination by 168 h when incubated only (t_0, Fig. 5A). Equation 1 implies that if ABAa were the only factor controlling germination, these seeds would be expected to have higher germination values after 168 h of incubation. These results suggest that in addition to ABA, other factors participate in the control of precocious germination. Those factors were more relevant in incubated seeds of 25 and 30 DAA.

Besides ABA, the water status and osmotic environment of the seed have been implicated in the inhibition of precocious germination (Bewley and Black, 1994; Hilhorst, 1995). Both osmoticum and ABA prevented precocious germination of developing alfalfa embryos (Xu *et al.*, 1990; Xu and Bewley, 1991). These authors also reported a gradual decline in ABA content and embryo sensitivity to sucrose during the second half of development. In developing soybean seeds, the sucrose concentration of the apoplastic interface (i.e. the free space between the inner face of the tegument and the outer face of the embryo) was calculated to be about 200 mM (Gifford and Thorne, 1985). Also, immature soybean seeds grown in a nutrient medium containing 200 mM sucrose did not germinate and continued dry matter accumulation (Egli, 1990). Therefore, it seems possible that apoplastic sucrose contributed to the additional delay in germination observed in incubated 25 and 30 DAA seeds, and perhaps to a lesser extent in seeds at later developmental stages. Several studies indicate that ABA and the osmotic environment interact, but their actions appear to be essentially independent (Hilhorst, 1995). Osmoticum prevented water uptake, whereas ABA prevented cell wall loosening in *Brassica napus* embryos (Schopfer and Plachy, 1985). One possible explanation for our results is that once the inhibitory effect of ABA is released (i.e. ABAa ≤ ABAc), the axis of an immature soybean seed requires extra time to overcome osmotic and/or tegument constraints to radicle protrusion.

Interestingly, equation 1 was able to predict the behaviour of washed seeds, including 25 and 30 DAA seeds, when ABAa was greater than ABAc (Table 1 and Fig. 7). The mean rate of water uptake was five times higher in washed than in incubated seeds (data not shown). Possibly, higher apoplastic dilution and/or axis turgor in washed, compared to incubated, seeds may account for the observed differences between treatments for 25 and 30 DAA seeds. In previous work, removing the tegument (which also eliminates the interfacial apoplast) accelerated the t_{50} of immature embryos of 30–45 DAA (Gosparini, 2002). Additional work is needed to quantify the relative contribution of the tegument and osmotic environment to the delay of germination in developing soybean seeds, as well as fluctuations of apoplastic sucrose during incubation or washing, and possible changes in embryo sensitivity to osmoticum during development.

Washing and incubation induced 8.1- and 2.3-fold increases, respectively, in the rates of ABAa decline, compared with the natural ABAa decline *in planta* (Fig. 6). Possible causes of this higher rate of ABA disappearance during incubation and washing could be due to: (1) isolated seeds stop receiving maternal ABA; (2) treatments stimulate ABA leakage to the medium; (3) increase in turgor due to water uptake

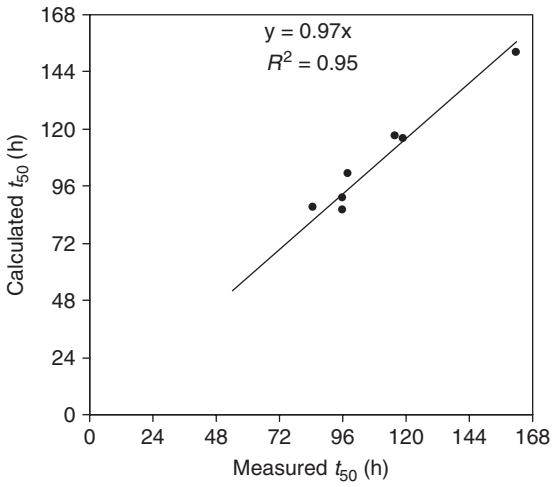

Figure 7. Relationship between the times to 50% germination (t_{50}) calculated by using Equation 1, and the t_{50} measured after first washing (0, 6, 12 and 24 h) immature soybean seeds, harvested at 25, 30, 35 and 40 d after anthesis (DAA). The values of the endogenous abscisic acid in the embryonic axes (ABAa) and t_{50} used were those corresponding to seed ages and washing times in which ABAa > ABAc (the threshold ABAa for inhibition of precocious germination) (Table 1, pairs of values in bold).

https://doi.org/10.1017/S0960258507785872 Published online by Cambridge University Press

accelerates ABA metabolism (degradation and/or conjugation); or (4) a combination of these. In addition, the faster ABAa decline during washing, compared to incubation, may be explained by a higher rate of water uptake in the former condition, which in turn results in an increase in seed tissue turgor and/or in ABA leakage into the washing solution. Because the ABA molecule is water soluble, seed ABA depletion could be due to ABA leakage. Also, preliminary results indicate that hydrated immature soybean seed tissues can metabolize ABA quite efficiently (Morandi *et al.*, 2002). More experimental work is necessary to elucidate the relative contribution of metabolism and leakage to ABA decline in highly hydrated immature seed tissues.

By using different washing times, we were able to generate a range of endogenous ABAa at all seed developmental stages. In general, the longer the time of washing, the lower the ABAa, and the shorter the t_{50} of immature seeds (Table 1). The tight relationship between the measured and calculated t_{50} for washed seeds (Fig. 7) indicated that equation 1 has predictive value for immature, highly hydrated, soybean seeds with ABAa above ABAc. On the other hand, when ABAa was below ABAc, differences in the t_{50} of washed seeds of 30, 35 and 40 DAA disappeared (Table 1). Moreover, their mean t_{50} (58 h) was very similar to the t_{50} of physiologically mature seeds (57 h). It is noteworthy that ABAc was very low and increased little during the period studied [from 1.15 to 1.66 μg ABA $(g\,DW)^{-1}$ for 25–40 DAA seeds]. The small change in ABAc contrasts with the substantial decline observed in initial ABAa during the same period [from 10.90 to 2.07 μg ABA $(g\,DW)^{-1}$ for 25–40 DAA seeds] (Fig. 4, t_0). Different seed tissues and processes have different sensitivities to ABA (reviewed by Kermode, 2005). Our results suggest that the inhibition of precocious germination of a hydrated, immature soybean seed was very sensitive to its endogenous ABAa. Trewavas (1991) suggested three basic requirements to avoid ambiguity in the measurement of growth substance sensitivity. First, the perturbations of hormone levels should be within the range of its natural endogenous concentrations. Secondly, experimental manipulation or tissue dissection should be limited. Thirdly, the contribution of the hormone to the control of a particular process should be evident, even in the presence of interacting factors. In our immature soybean seed system, all three proposed requirements were met: (1) the endogenous ABA fluctuated within biological levels (i.e. no exogenous ABA was added); (2) the seeds remained intact; and (3) the contribution of ABAa to the control of precocious germination was evident, although other potentially interacting factors (e.g. embryo osmotic environment, seed tegument) were present.

Acknowledgements

This research has been financed by Agencia Nacional de Promoción Científica y Tecnológica, Argentina, FONCyT, PIDs 0673 and 22995. E.N.M. is member of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas). The authors thank Dr Roberto Benech-Arnold for critical reading of the manuscript.

References

Ackerson, R.C. (1984) Abscisic acid and precocious germination in soybeans. *Journal of Experimental Botany* **35**, 414–421.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell* **9**, 1055–1066.

Bewley, J.D. and Black, M. (1994) *Seeds: Physiology of development and germination* (2nd edition). New York, Plenum Press.

Black, M. (1991) Involvement of ABA in the physiology of developing and mature seeds. pp. 99–124 *in* Davies, W.J.; Jones, H.G. (Eds) *Abscisic acid: Physiology and biochemistry*. Oxford, Bios Scientific Publishers Ltd.

Egli, D.B. (1990) Seed water relations and the regulation of the duration of seed growth in soybean. *Journal of Experimental Botany* **41**, 243–248.

Egli, D.B. (1998) *Seed biology and the yield of grain crops*. Wallingford, CABI Publishing.

Frey, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. (1999) Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. *Plant Molecular Biology* **39**, 1267–1274.

Gifford, R.M. and Thorne, J.H. (1985) Sucrose concentration at the apoplastic interface between seed coat and cotyledons of developing soybean seeds. *Plant Physiology* **77**, 863–868.

Gosparini, C.O. (2002) Regulación del desarrollo embrional en soja: Rol del ABA en el control de la germinación de semillas inmaduras de soja. Doctoral thesis, Facultad de Cs. Bioquímicas y Farmacéuticas. Universidad Nacional de Rosario, Argentina.

Gosparini, C.O., Morandi, E.N. and Cairo, C.A. (1997) Efecto de la edad, el lavado y la temperatura sobre la germinación de las semillas inmaduras, el crecimiento radicular y el tiempo hasta la floración, de la soja. *Revista de la Facultad de Agronomía, La Plata* **102**, 1–9.

Hilhorst, H.W.M. (1995) A critical update on seed dormancy. I. Primary dormancy. *Seed Science Research* **5**, 61–73.

Iglesias, R.G. and Babiano, M.J. (1997) Endogenous abscisic acid during the germination of chick-pea seeds. *Physiologia Plantarum* **100**, 500–504.

Karssen, C.M. (1995) Hormonal regulation of seed development, dormancy, and germination studied by genetic control. pp. 333–350 *in* Kigel, J.; Galili, G. (Eds) *Seed development and germination*. New York, Marcel Dekker.

Kermode, A.R. (2005) Role of abscisic acid in seed dormancy. *Journal of Plant Growth Regulation* **24**, 319–344.

Koornneef, M. and Karssen, C.M. (1994) Seed dormancy and germination. pp. 313–334 *in* Meyerowitz, E.M.; Somerville, C.R. (Eds) *Arabidopsis*. New York, Cold Spring Harbor Laboratory Press.

Lindgren, L.O., Stalberg, K.G. and Höglund, A.S. (2003) Seed-specific overexpression of an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll and abscisic acid. *Plant Physiology* **132**, 779–785.

Loveys, B.R. and Van Dijk, H.M. (1988) Improved extraction of abscisic acid from plant tissue. *Australian Journal of Plant Physiology* **15**, 421–427.

McCarty, D.R. (1995) Genetic control and integration of maturation and germination pathways in seed development. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 71–93.

Miles, D.F., TeKrony, D.M. and Egli, D.B. (1988) Changes in viability, germination, and respiration of freshly harvested soybean seed during development. *Crop Science* **28**, 700–704.

Morandi, E.N. and Gosparini, C.O. (1991) Modificación del balance hormonal en embriones de soja para inducir su germinación precoz. pp. 137–143 *in Actas Primera Reunión Nacional de Oleaginosos*. Rosario, Argentina.

Morandi, E.N., Schussler, J.R. and Brenner, M.L. (1990) Photoperiodically induced changes in seed growth rate of soybean as related to endogenous concentrations of ABA and sucrose in seed tissues. *Annals of Botany* **66**, 605–611.

Morandi, E.N., Gosparini, C.O., Busilacchi, H.A. and Vernieri, P. (2002) Seed coat blocks exogenous ABA inhibition of germination in developing soybean seeds. p. 87 *in Proceedings from the VII international workshop on seed biology,* May 2002, Salamanca, Spain.

Phillips, J., Artsaenko, O., Fiedler, U., Hortsmann, C., Mock, H.P., Muntz, K. and Conrad, U. (1997) Seed-specific immunomodulation of abscisic acid activity induces a developmental switch. *EMBO Journal* **16**, 4489–4496.

Qin, X. and Zeevaart, J.A.D. (2002) Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiology* **128**, 544–551.

Robichaud, C., Wong, J. and Sussex, I.M. (1980) Control of *in vitro* growth of viviparous embryo mutants of maize by abscisic acid. *Developmental Genetics* **1**, 325–330.

Schopfer, P. and Plachy, C. (1985) Control of seed germination by abscisic acid. III. Effect on embryo growth potential (minimum turgor pressure) and growth coefficient (cell wall extensibility) in *Brassica napus* L. *Plant Physiology* **77**, 676–686.

Schussler, J.R., Brenner, M.L. and Brun, W.A. (1984) Abscisic acid and its relationship to seed filling in soybeans. *Plant Physiology* **76**, 301–306.

Thompson, A.J., Jackson, A.C., Symonds, R.C., Mulholland, B.J., Dadswell, A.R., Blake, P.S., Burbidge, A. and Taylor, I.B. (2000) Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *Plant Journal* **23**, 363–374.

Trewavas, A. (1991) How do plant growth substances work? II. *Plant Cell and Environment* **14**, 1–12.

Vernieri, P., Perata, P., Armellini, D., Bugnoli, M., Presentini, R., Lorenzi, R., Ceccarelli, N., Alpi, A. and Tognoni, F. (1989a) Solid phase radioimmunoassay for the quantitation of abscisic acid in plant crude extracts using a new monoclonal antibody. *Journal of Plant Physiology* **134**, 441–446.

Vernieri, P., Perata, P., Lorenzi, R. and Ceccarelli, N. (1989b) Abscisic acid levels during early seed development in *Sechium edule* Sw. *Plant Physiology* **91**, 1351–1355.

Walker–Simmons, M.K., Reaney, M.J.T., Quarrie, S.A., Perata, P., Vernieri, P. and Abrams, S.R. (1991) Monoclonal antibody recognition of abscisic acid analogs. *Plant Physiology* **95**, 46–51.

Xu, N. and Bewley, J.D. (1991) Sensitivity to abscisic acid and osmoticum changes during embryogenesis of alfalfa (*Medicago sativa*). *Journal of Experimental Botany* **42**, 821–826.

Xu, N., Coulter, K.M. and Bewley, J.D. (1990) Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. *Planta* **182**, 382–390.

Received 18 October 2006
accepted after revision 8 May 2007