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EFFECT OF GA AND ABA ON GERMINATION BEHAVIOR OF BLACK RASPBERRY (*RUBUS COREANUS* MIQUEL) SEEDS

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Abstract

Black raspberry (*Rubus coreanus* Miquel) seed is covered with hard seed coat and enveloped in an endospermic layer. Seeds failed to germinate without scarification and stratification, suggesting that seeds exhibit profound both physical and physiological dormancy. Scarification with sulfuric acid for 15 minutes and stratification for 45 days produced best results. The application of GA was not effective in alleviating dormancy compared to GA-untreated seeds. *R. coreanus* seeds in contrast to other plant species, in which radicle emerges first during germination process, seed coat cleaved at the center or little more toward radicle end and whole seed emerged out (cotyledons plus radicle) as if the chick hatched out of egg. Normally seed germination is defined based on the radicle emergence but in case of *R. coreanus* due to lack of radicle emergence seed was considered germinated when half or more than half of the seed (radicle plus cotyledons and some time endospermic layer) emerged from seed coat. Furthermore, seed germination was completed in two steps, (1) cracking of seed coat and (2) breaking of endospermic layer. The rupturing of endospermic layer was critical in emergence of cotyledons or radicle. The role of ABA in breaking the seed coat and endospermic layer and effect of H_2SO_4 vs. manual scarification is discussed in this article.

Introduction

Rubus coreanus reproduces through seed and also regenerates vegetatively. *R. coreanus* seeds have a hard, thick, impermeable coat and dormant embryo that has dual dormancy. Seeds have the ability to become dormant a second time in response to environmental factors. Consequently, germination is often slow and low, creating substantial problem for breeders. There is a very long partly successful list of dormancy breaking treatments, which incorporate several stimulatory treatments indicating that the germination of *Rubus* spp. is difficult and rarely sufficient to promote full germination. Most raspberry seeds require a minimum warm stratification at 20 to 30°C for 90 days, followed by cold stratification at 2 to 5°C for an additional 90 days (Brinkman, 1974; Jennings, 1988). Cold or warm stratification alone is insufficient to induce germination in raspberry because seeds have hard coat impermeable to water, radical and gaseous exchange. Therefore, it is recommended to scarify seeds prior stratification. It is suggested to expose seeds to sulfuric acid solutions. The time of exposure ranges from 20 min to few hours. However, it is important to avoid seed damage from longer period of acid exposure (Brinkman, 1974; Jennings and Tulloch, 1964).

Although, the germination pattern of *R. coreanus* is not fully investigated, however, our investigation showed that *R. coreanus* has hard seed coat followed by covering of endospermic layer which is very much like other seeds bearing endospermic layer e.g. tobacco (*Nicotiana tabaccum*) seed (Manz *et al*., 2005). Tobacco seed has been established as a model for endospermic seed germination. It has been reported that tobacco seed follows a distinct pattern of germination that is rupturing of testa followed by rupturing of endospermic layer (Manz *et al*., 2005). It has been reported that rupturing of endosperm is critical in seed germination (Watkins and Cantliffe, 1983) and furthermore, abscisic acid (ABA) specifically inhibit endosperm rupturing (Manz *et al*., 2005). The role of ABA in seed dormancy is well documented (Gubler *et al*., 2005) and has been shown that dormancy in developing seed is dependent on ABA which is synthesized in the embryo and not on the maternal sources (Karrsen *et al*., 1983). Furthermore, dormancy release has been correlated with the ABA contents of the dormant seeds (Ali-Rachedi *et al*., 2004).

The current work was designed to investigate germination pattern of *R. coreanus* seeds including role of endosperm in seed germination and effect of ABA on the rupturing of seed coat and endospermic layer, and devising specific scarification and stratification treatments to break seed dormancy and achieve maximum germination.

Materials and Methods

R. coreanus seeds were collected from fruits stored at –20 °C for 1 year at Bramble Research Institute, Gochan, Korea. Seeds were scarified either manually by chipping the testa at the cotyledon end or by treating with H_2SO_4 for 15 minutes. Seeds were thoroughly washed with deionized water after treatment with H_2SO_4. A batch of seed was soaked or soaked and redried in distilled water (DW) for 1 and 8 h after scarification either manually or with H_2SO_4. Seeds were moist stratified either in DW or 100 ppm gibberellic acid (GA) solution at 4°C for 15, 30, 45 and 60 days. For surface sterilization seeds were washed with 2% sodium hypochlorite (NaOCl) for 5 minutes and thoroughly rinsed.

In order to observe the role of endospermic layer in seed germination and the effect of ABA on the rupturing of endospermic layer, stratified seeds were germinated in the presence of 100 ppm ABA before and after cracking of testa.

Seeds were germinated in 9 cm Petri dish with two layers of Whatmann No. 1 filter paper. Each treatment was repeated 3 times and 50 seeds were used per replication. Seeds were incubated at 25°C in constant light. Seed germination was recorded every day until no further germination was observed for many days. Germinated seeds were moved to 9 cm Petri dishes containing fine sand to observe root and shoot growth for 30 days. Data were subjected to ANOVA and LSD was calculated ($p>0.05$) by using Tukey's test.

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Results

***R. coreanus* seed hatching:** Normally in plant species radicle emerges first from the seed coat during germination process but in case of *R. coreanus*, seed coat cleaved at the center or little more toward radicle end and whole seed emerged out (cotyledons plus radicle) as if the chick comes out of egg. The radicle of some of seeds emerged first and then cotyledons appeared but the proportion of this kind of seeds never exceeded 10%, the maximum. The seed either germinated or not germinated is measured in terms of length (or appearance) of radicle but in case of current results due to the lack of radicle emergence seed was considered germinated when half or more than half of the seed (radicle plus cotyledons and some time endospermic layer) emerged from seed coat (Fig. 1A).

Two steps germination

Role of endospermic layer in radicle emergence: Seed germination was completed in two steps, (1) cracking of seed coat and (2) breaking of endospermic layer (Fig. 1). Although, both steps were important in seed germination but the breakage of endospermic layer was probably more critical in emergence of cotyledons or radicle. A thin layer of endospermic layer enveloped the cotyledons and radicle. The endospermic layer either ruptured at the (1) radicle site which later extend to the cotyledons site eventually releasing whole seed (Fig. 1a & b), (2) ruptured after seed coat cracked at the middle of seed, releasing the whole seed (Fig. 1c & d) or (3) ruptured at the radicle end, in case if radicle emerged first from seed coat (Fig. 1e).

Fig. 1. Seed of *Rubus coreanus* considered germinated (A - above top) when half or more than half of the seed (radicle plus cotyledons and some time endospermic layer) emerged from seed coat. Seed coat cracked at the center or little more toward radicle end of seed resulting emergence of whole seed (cotyledons plus radicle) as if the chick comes out of egg (insets a – d). In rare case radicle emerged first (inset e). c: cotyledons; e: endoplasmic layer; r: radicle and sc: seed coat.

Furthermore, stratified seeds were treated with 100 ppm ABA before and after cracking of seed coat and it was found that ABA inhibited endospermic layer from rupturing and ultimately inhibited the release of cotyledons and radicle that is failed seed germination (Fig. 2).

Seed germination

Manual Vs H_2SO_4 scarification: A small percentage of unscarified seed germinated. Sulfuric acid (H_2SO_4) scarified seeds gave better germination than manually scarified seeds (Table 1). Manually scarified seeds failed to germinate except a small percentage was germinated after 30 days of stratification and furthermore, only those seeds germinated whose endospermic layer was cut or ruptured during scarification (Fig. 3). Therefore, seeds mentioned germinated in this article are H_2SO_4 scarified unless stated otherwise.

Unstratification Vs stratification: Unstratified seeds failed to germination. However, germination increased with the stratification period. The germination reached to

51, 60, 73 and 75% after stratification for 15, 30, 45 and 60 days respectively at 4°C (Table 1). Seeds soaked or soaked and redried for 8 h prior stratification failed to germinate but soaked or soaked and redried for 1 h gave better germination. However, maximum germination was less than as achieved after 30 (60%) and 45 (73%) days of stratification without soaking and redrying.

Distilled water (DW) Vs Gibberellic Acid (GA): The seeds moist stratified with DW gave relatively higher germination than the seeds moist stratified with GA (100 ppm) that was contrary to the previous findings showing the GA enhances seed germination (Table 1).

Germination Rate: The germination rate of seeds increased with the increase of stratification period (Table 2). It was observed that more than 50% seeds germinated even during stratification after 45 days in case of seeds stratified in DW or GA. The seeds soaked for 1 h and redried were quick to germinate and even 50% seeds germinated after 30 days of stratification.

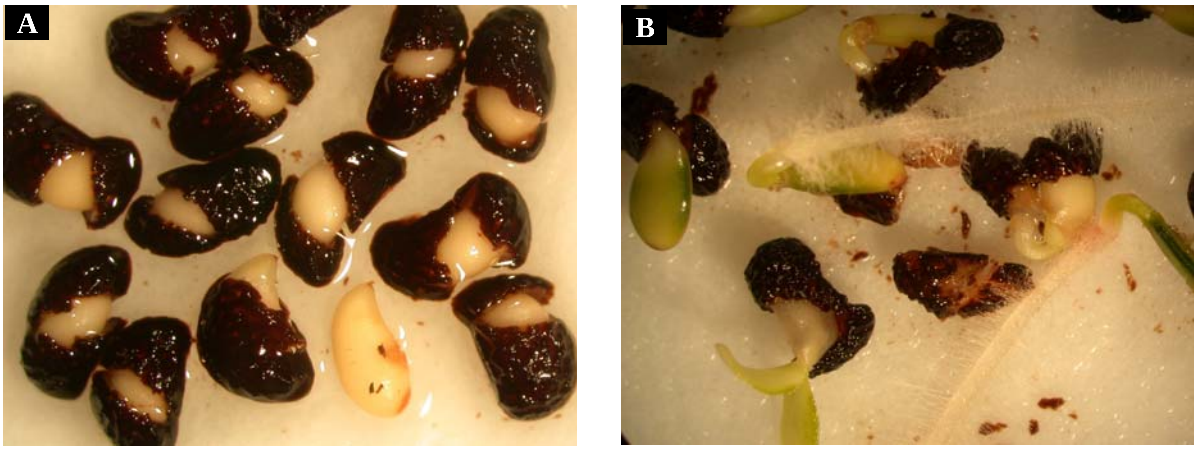

Fig. 2. Effect of ABA on seed germination of *Rubus coreanus*. (A) seeds treated with 100ppm ABA after cracking of seed coat. The endospermic layer did not break and radicle/cotyledons could not emerge while (B) embryonic layer of seeds without ABA ruptured and radicle/cotyledons emerged.

Table 1. The effect of exogenously applied gibberellic acid (GA) solution; moist chilling in distilled water (DW) or GA solution and wetting-drying on the germination of manually or H_2SO_4 scarified black raspberry (*Rubus Occidentalis*) seeds. Values are the means of the three replicates of 50 seeds.

Treatments		Germination (%)				
		Fresh seeds	Stratification time (days)			
			15	30	45	60
Control (untreated)		0	0	0	0	0
H_2SO_4 scarified	DW	10	-	-	-	-
	Exogenous 100 ppm GA solution	9	-	-	-	-
Manually scarified	DW	0	-	-	-	-
	Exogenous 100 ppm GA solution	0	-	-	-	-
H_2SO_4 scarified	Chilling in DW		51	60	73	75
	Chilling in 100 ppm GA solution		48	43	67	63
Manually scarified	Chilling in DW		0	14	0	0
	Chilling in 100 ppm GA solution		0	14	0	0
Soaking-redrying	Soaked (8H)		0	0	0	0
	Re-dried		0	0	0	0
Soaking-redrying	Soaked (1H)		43	54	35	29
	Re-dried		49	53	55	33
	LSD (p=0.05)		4.5			

Root and Shoot Growth

a. Manual Vs H_2SO_4 scarification: Not only very few manually scarified seeds germinated but also their subsequent root and shoot length was very small. Fig. 3b shows that even if the cotyledons emerged out of seed coat, the radicle remained trapped inside the coat for several days after germination. It is not clear how long seed would take to decompose, releasing the root of the seedling. On the other hand root and shoot of H_2SO_4 scarified seeds grew faster and better (Fig. 4).

b. Distilled water (DW) Vs Gibberellic Acid (GA): The root and shoot lengths of one-month-old seedlings were measured and it was found that root length of DW-scarified seeds was generally longer than GA-stratified seeds (Fig. 4A). However, shoot length remained same in both treatments except shoot length was significantly higher after 45 days of DW stratification (Fig. 4B).

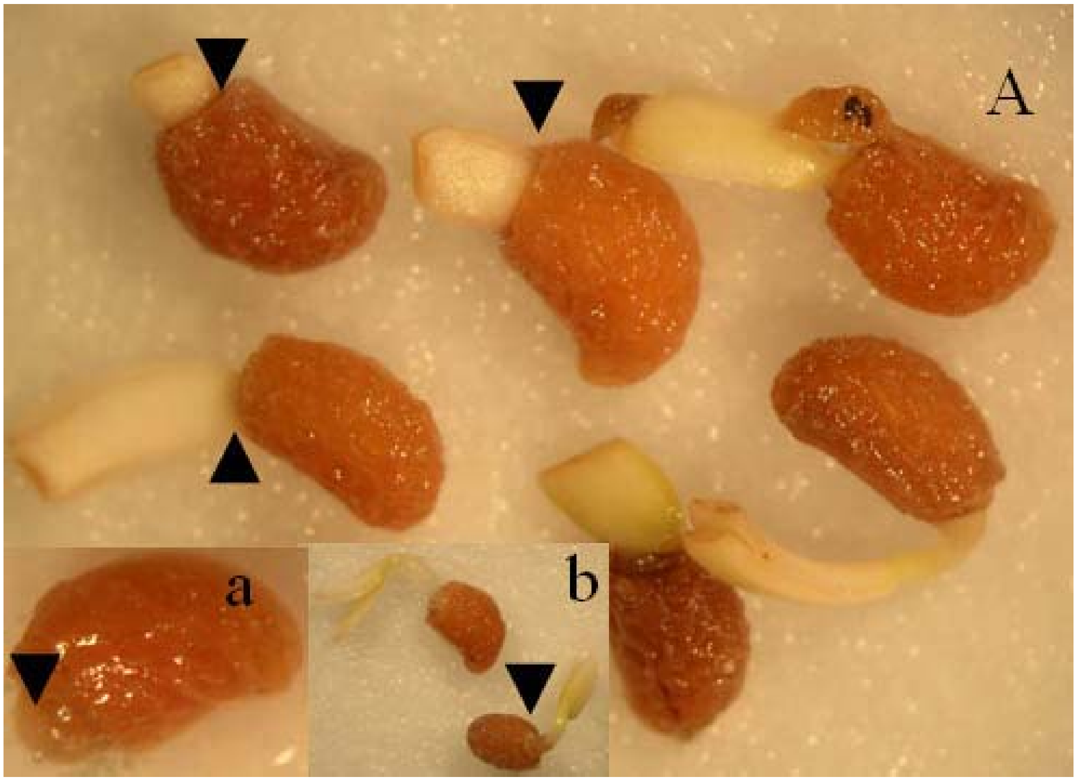

Fig. 3. Seed germination and seedling growth of manually scarified *R. coreanus* seeds. Seed with removed or damaged endosperm during scarification germinated (A) while others failed (a). Radicle remained trapped in the coat even after many days of germination (b). Therefore, root and shoot growth was less than compared to H_2SO_4 scarified seeds. Arrow-head indicates the point of scarification.

Table 2. The effect of exogenously applied gibberellic acid (GA) solution and the moist chilling in distilled water (DW) or GA solution on the germination rate ($1/t_{50}$) of manually or H_2SO_4 scarified *Rubus Occidentalis* seeds. Values are the means of the three replicates of 50 seeds.

Treatments		Germination (%)				
		Fresh seeds	Stratification time (days)			
			15	30	45	60
Control (untreated)		0	0	0	0	0
H_2SO_4 scarified	DW	0.29	-	-	-	-
	Exogenous 100 ppm GA solution	0.29	-	-	-	-
Manually scarified	DW	0	-	-	-	-
	Exogenous 100 ppm GA solution	0	-	-	-	-
H_2SO_4 scarified	Chilling in DW		0.4	0.67	*	*
	Chilling in 100 ppm GA solution		0.4	067	*	*
Manually scarified	Chilling in DW		0	0.4	0	0
	Chilling in 100 ppm GA solution		0	0.4	0	0
Soaking-redrying	Soaked (8H)		0	0	0	0
	Re-dried		0	0	0	0
Soaking-redrying	Soaked (1H)		0.67	*	*	*
	Re-dried		0.67	*	*	*
	LSD (p=0.05)		0.29			

* more than 50% seeds were already germinated during stratification (4°C) before incubation at 25°C for germination

Discussion

In general seed germination is initiated with the emergence/visualization of radicle and therefore seed germination is defined based on "the emergence of radicle or the size of the emerged radicle" (Bewley, 1997). However, in case of *R. coreanus* whole seed comes out of the seed coat rather emergence of radicle. The phenomenon of seed germination is much like chick hatching, the seed coat is cracked at the middle or towards the radicle side which gets wide and wider until the whole seed comes out. The traditional definition does not fit in to describe the seed germination of *R. coreanus*. Therefore, seed was considered germinated in current study when half or more than half of the seed emerged from seed coat (Fig. 1).

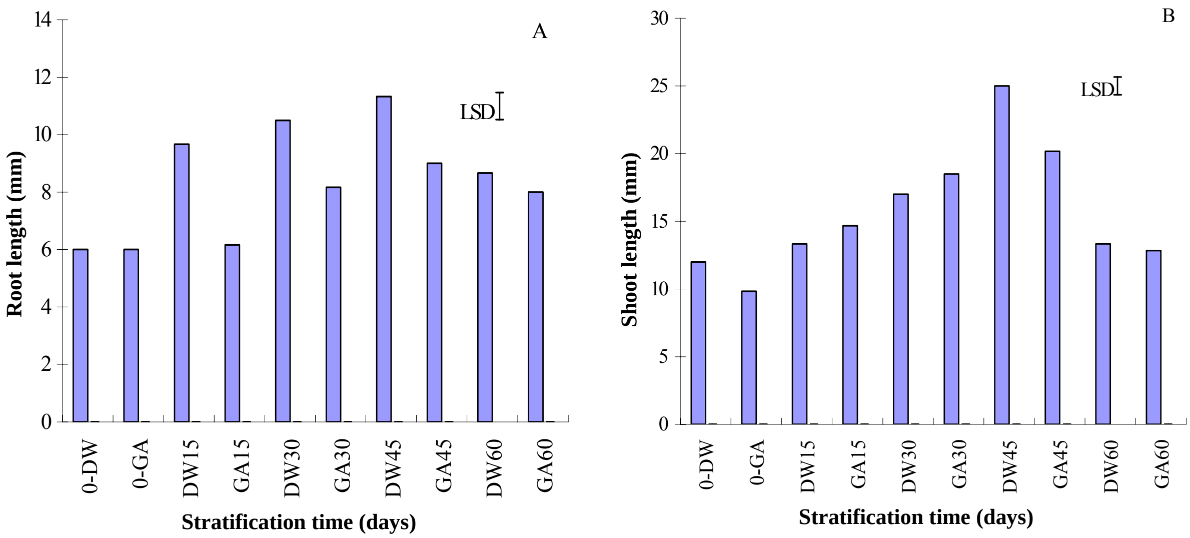

Fig. 4. Root (A) and shoot (B) growth of *R. coreanus* seedlings grown for 30 days from seeds stratified in distilled water (DW) or gibberellic acid (GA) for 0, 15, 30, 45 and 6o days. Root and shoot growth increased with the stratification period until 45 days but decreased with further stratification of seeds. The root growth (A) of DW stratified seedlings was higher than GA stratified seeds while shoot growth in both cases was same except DW stratified seeds had better shoot growth after 45 days of stratification.

It was furthermore observed that *R. coreanus* seed was covered with a thin endospermic layer. Seed germination initiated with the cracking of seed coat but completed by the rupturing of endospermic layer. It was found that seedling would not establish unless endospermic layer ruptured. It is believed that endosperm strength must decrease before germination could occur. Similar mechanism was observed in *Capsicum annum* (Watkins and Cantliffe, 1983) and *Nicotiana tabacum* (Manz *et al*., 2005) where seeds failed to germinate due to the failure of loosening and rupturing of endospermic layer. ABA is perhaps the best known inhibitory substance and is generally implicated in control of seed dormancy (Villiers, 1972). Seeds were treated with ABA prior and after seed coat cracking and it was found that endospermic layer failed to rupture and subsequently seed failed to develop into seedling (Fig. 2). The breakage of seed coat was not affected by the application of ABA. It is reported that ABA related dormancy in seed lies in the endospermic layer and could be alleviated by the reduction of ABA level. Manz *et al*. (2005) found that ABA in tobacco inhibited endosperm rupture and phase III water uptake, furthermore, ABA inhibited the induction of β-1,3-glucanase in the micropylar end (major water entry point in tobacco seed) just before its rupture but did not affect the rupture of testa.

Manual scarification (at cotyledons end) is considered one of the safest way of seed scarification, therefore, ensures maximum germination (Rehman *et al*., 1999). In current study it was found that manually scarified seeds failed to germinate with few exception in which endospermic layer was cut with razor during scarification (Fig. 3). Seed coat also failed to crack at the middle and furthermore, cotyledons were pushed towards the cut end of coat rather to the radicle side. These results suggest that probably the imbibitional pressure necessary to build in seed to crack seed coat in the middle is released or imbalanced by cutting coat at the cotyledon end. This was evident from the fact that seeds endosperm bulged towards cut end but could not exude or ruptured. Therefore, seed could not exert enough pressure to crack seed coat. These results further explain the seed hatching behavior of *R. coreanus* in which seed germination takes place by cracking seed at the middle and emerging whole seed.

R. coreanus seeds failed to germinate without scarification and stratification (Tables 1 & 2), which indicates that seeds have profound physical and physiological dormancy (Brinkman, 1974; Finch-Savage and Leubner-Metzger, 2006; Jennings and Tulloch, 1964). Therefore, seeds require dormancy alleviation treatment prior germination. It has been widely reported that *Rubus* seeds require scarification and warm or cold stratification, or warm and cold stratification in a cycle (Jennings, 1988). Furthermore, great number of literature reported a slow and low seed germination, a list of varied stratification period ranging minimum 2 months period at 4^oC and subsequent 2 months for germination of seeds at 25^oC (Brinkman, 1974; Jennings and Tulloch, 1964). In contrary to previous reports, we have found 51% of *R. coreanus* seeds germinated just after 15 days of stratification. Moreover, germination started after 2 days of incubation at 25^oC. The germination percentage increased to 75% after 60 days of stratification (Tables 1 & 2).

In general GA is considered important in releasing seed dormancy and improving seed germination (Iglesias and Babiano, 1997; Rehman *et al*., 2000a). However, in current study, the application of GA whether directly in germinating medium or seeds stratified in, did not enhance germination compared to GA–untreated seeds. The GA analysis of seeds showed (results not shown) higher concentration of GA present in the seeds, which indicates that additional application of GA might have toxic/negative effect on seed germination. The subsequent effect of GA-treated seeds on root and shoot growth was very much as its effect on seed germination compared to GA-untreated seeds. However, root growth was more affected than shoot growth. Over all, shoot and root growth was maximum from seeds stratified for 45 days which was very much same for seed germination.

Soaking and soaking-redrying of seeds for 1 h after scarification and prior stratification improved seed germination, however, germination percentage was not higher than seeds stratified without soaking and redrying. Furthermore, seed germination declined after 30 days of scarification. Seeds soaked or soaked and re-dried for 8 h failed to germinate (Table 1). The failure of germination or decreasing germination after 30 days of stratification in case of 1 h soaking or soaking-redrying, could be due to higher moisture contents, which had possibly restricted oxygen supply to the germinating seed and probably induced secondary dormancy (Rehman and Park, 2000b; Skinnes and Sorrells, 1990).

The germination rate increased with the stratification period (Table 2). The unstratified seeds were the slowest to germinate while seeds soaked or soaked and redried prior stratification were the fastest to germinate. It is suggested that stratification could have activated the metabolic and physiological activities including changes in hormones resulting in early germination (Khan, 1977).

In conclusion, *R. coreanus* seed has both physical and physiological dormancy dormancy. The best germination was achieved by stratifying seeds for 30-45 days prior scarifying with H_2SO_4 for 15 min. The germination was completed in two stages, (a) cracking of coat and (b) rupturing of endospermic layer. The rupturing of endospermic layer was critical in germination and was probably controlled by ABA. Furthermore, whole seed (radicle plus cotyledons) emerged from coat and/or endosperm like chick hatching from egg.

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