Encouraging of embryo development and seed germination in *Actaea racemosa* L. by gibberellic acid

I.M. Pinker, T. Degischer, A.-Chr. Rath and R. Schenk

Humboldt-Universität zu Berlin, Albrecht Daniel Thaer-Institute, Germany

Summary

Actaea racemosa L. (syn. *Cimicifuga racemosa* (L.) Nuttall) or black cohosh is a perennial plant of the buttercup family (Ranunculaceae) growing wild in deciduous forests of North America. Because mature seeds of *A. racemosa* have a rudimentary embryo and exhibit deep simple epicotyl morphophysiological dormancy, germination is a long-lasting process requiring warm-cold stratification. The study has been conducted in 2015–2016 and aimed to develop an efficient germination protocol. For imbibition, graded seeds (1.22–2.5 mm in length) were incubated in tap water or aqueous gibberellic acid solution for 12 hours. The imbibed seeds were kept in darkness at 23°C for 4 weeks (warm phase) followed by 4 weeks of incubation at 14°C or 7°C (cold phase) in darkness or light. Most embryos in mature seeds were developed to the heart stage. They reached after 4 weeks of warm phase the torpedo stage. During the following cold phase, the embryo development was more pronounced in seeds imbibed in gibberellic acid. This promoting effect of gibberellic acid showed a dose-effect response. Application of 2 g L⁻¹ gibberellic acid resulted in germination of around 60% seeds after 4 weeks of cold phase while 1 g L⁻¹ gibberellic acid caused germination of up to 20% seeds. Due to the application of 2 g L⁻¹ gibberellic acid to the imbibition solution the germination of most seeds was possible after 8 weeks warm-cold stratification. The observation of the embryo development proved the promoting effect of gibberellic acid, especially during the cold phase.

Keywords

Cimicifuga racemosa (L.) Nuttall, epicotyl dormancy, GA₃, imbibition, stratification temperature, warm-cold stratification

Introduction

Actaea racemosa L. (syn. *Cimicifuga racemosa* (L.) Nuttall) or black cohosh is a perennial plant of the buttercup family (Ranunculaceae) growing wild in deciduous forests of North America (Fischer et al., 2006). Since a long time *A. racemosa* is well known as medicinal plant used by the Native Americans to treat various diseases and also snakebites (Predny et al., 2006). Nowadays, products from its dried roots and rhizomes are offered especially against climacteric symptoms (ESCOP, 2011; Huang et al., 2015; Henneicke-von Zepelin, 2017). Clinical researches over decades collected data about efficacy and safety of those products (Henneicke-von Zepelin, 2017; Friederichsen et al., 2019). By pharmaceutical studies

Significance of this study

What is already known on this subject?

- Actaea* seeds exhibit deep simple epicotyl morphophysiological dormancy and need warm-cold stratification to germinate. Most authors used fluctuating temperatures during warm- and cold phase for some months each. Germination is long lasting (minimum 5 to 6 months). For the effect of gibberellic acid on germination there are only very few and conflicting reports. In these reports, gibberellic acid concentration used was not always indicated or rather low concentrations were applied, e.g., 2–4 mg L⁻¹. Gibberellic acid was so far applied continuously during warm- and cold phase, respectively.

What are the new findings?

- This is the first report showing the effect of a high concentration of gibberellic acid applied only during imbibition for 12 hours on embryo development during warm- and cold phase. The embryo development was accelerated in samples imbibed in gibberellic acid solution especially during the cold phase. Germination (emergence of radicle and plumule) started in these samples after 7 weeks already. The application of gibberellic acid to the imbibition solution supported the effect of warm-cold stratification regarding dormancy breaking and reduced the required stratification time considerably. Four weeks of warm phase (23°C) followed by 4 weeks cold phase (14°C or 7°C) were sufficient to get around 60% germination after 8 weeks already. The germination continued during the following 2 weeks and the percentage of germinated seeds increased.

What is the expected impact on horticulture?

- Actaea racemosa* is a valuable medicinal plant used in phytotherapy, especially against climacteric symptoms. Field production for *A. racemosa* should be established to substitute its harvest from the wild. However, young plant production is still the bottleneck for field production. With this protocol, young plant production can be accelerated considerably and moreover, young plants can be produced in time effectively in large amounts for field production.

action mechanisms of the active substances as triterpenoids and polyphenols are investigated more in detail. It seems, e.g., that some triterpene glycosides may suppress growth of breast cancer cells (Einbond et al., 2008). As plants for drug production were mostly harvested from the wild, concerns

about over-collection and diversity losses initiated attempts to field cultivation leading consequently to research activities from the 1980s on (Craker et al., 2003; Albrecht and McCarthy, 2007). Researchers as Popp et al. (2003), Fischer et al. (2006), Albrecht and McCarthy (2007), McCoy et al. (2007), and Kaur et al. (2013) stressed the necessity to develop cultivation systems to protect the wild populations. There were also reports on forest farming, but these results were not promising and depended largely from the environmental conditions (Small et al., 2014). Up to now, field cultivation of black cohosh is still hampered by the plant availability. The main problem for young plant production is the seed dormancy in black cohosh. Therefore, alternative clonal propagation techniques were applied as root stock division (Fischer et al., 2006; McCoy et al., 2007), *in vitro* multiplication (Lata et al., 2002), and somatic embryogenesis (Pinker and Schenk, 2018). Though, seed propagation would be the cheapest method guaranteeing also a certain genetic diversity in the cultivation area.

A. racemosa is characterized as other members of the *Actaea* genus (Fu et al., 1998; Li and Chen, 2002) by a rudimentary embryo in mature seeds measuring about 0.34 mm in length (Martin, 1946). According to Baskin and Baskin (2014) this species exhibits deep simple epicotyl morphophysiological dormancy, a type of morphophysiological dormancy (MPD). In nature, radicles of those seeds emerge in autumn while epicotyls or shoots require chilling to break its dormancy and they emerge in late winter or early spring (Baskin and Baskin, 1998). The critical temperatures for warm phase are mentioned to be $\geq 15^{\circ}\text{C}$ and for cold phase $0\text{--}10^{\circ}\text{C}$ in moist seeds (Nikolaeva, 1977; Hidayati et al., 2005). In seed technology warm-cold stratification is used for such seeds and application of gibberellic acid (GA_3) can sometimes help to break the dormancy (Baskin and Baskin, 2004).

Only few reports are available for stratification effects in *Actaea*. Baskin and Baskin (1985) applied fluctuating temperatures (daily maximum and minimum temperatures: $20.7^{\circ}\text{C}/8.6^{\circ}\text{C}$ for warm phase and $14.1^{\circ}\text{C}/4.5^{\circ}\text{C}$ for cold phase). After 8 months they found that more than 80% of seeds germinated. Popp et al. (2003) investigated various temperature regimes but worked with non-fluctuating temperatures. They applied 1–6 weeks' warm phase at 25°C followed by 8 weeks of cold phase at 12°C with a later change to 25°C again. Popp et al. (2003) published that in total 12 weeks were necessary for radicle emergence and concluded for the temperature regime that *C. racemosa* seeds require at least 4 weeks' warm phase followed by a cold phase. Albrecht and McCarthy (2007) applied 12 weeks fluctuating temperatures at $15/6^{\circ}\text{C}$ followed by 12 weeks at 5°C . They concluded that at least 8 weeks for cold phase are necessary to break the physiological dormancy. Kaur et al. (2013) studied 15 *Actaea* accessions and applied two methods to break the dormancy under *in vitro* conditions. In the two-step-approach for 2 weeks, 25°C were applied in darkness followed by alternating temperatures ($20^{\circ}\text{C}/8^{\circ}\text{C}$) for 12-h periods within a 16-h photoperiod for 12 months. The three-step-approach started also with 25°C for 2 weeks, but for the next 3–4 months 4°C were applied in darkness. The seeds with emerging radicle and shoot were transferred to 25°C into light (third step). With the three-step-approach higher germination percentages (up to 80%) were reached and germination was accelerated with radicle emergence within 4 months and shoot emergence after around 6 months.

Application of GA_3 may promote seed germination in

some types of MPD (Baskin and Baskin, 2004). In five species with deep simple epicotyl MPD there are reports that GA_3 substituted at least partly temperature effects during stratification by supporting the embryo growth (Baskin and Baskin, 2014). In *Fraxinus excelsior*, 1×10^{-3} M or 5×10^{-3} M GA_3 applied from the imbibition till the end of stratification at high ($17\text{--}24^{\circ}\text{C}$) or low ($4\text{--}6^{\circ}\text{C}$) temperatures accelerated the embryo growth (Wciślińska, 1977). In both temperature conditions the higher embryo size was reached with GA_3 application after 12 weeks. Warm-cold stratification without gibberellic acid treatment resulted also in embryo growth and 60% seed germinated after 40 weeks. By application of GA_3 in cold stratification, however, seed germinated after 21 weeks already. Wciślińska (1977) concluded that GA_3 accelerates the embryo growth by breaking the first kind of dormancy but is not responsible for breaking the physiological dormancy, for which cold stratification is required. In *Cardiocrinum cordatum* 0.1 g L^{-1} GA_3 application for 90 days substituted partly the effect of high temperatures regarding embryo development (Kondo et al., 2006). In *A. racemosa* there are only few and conflicting reports regarding the effect of GA_3 . Popp et al. (2003) reported that 4 mg L^{-1} GA_3 treatment accelerated somewhat the leaf development in *C. racemosa* while Kaur et al. (2013) did not find a promoting of GA_3 on *in vitro* germination whereas concentrations used were not indicated. Histo-differentiation of somatic embryos *in vitro* was encouraged by the application of GA_3 up to the torpedo stage, although, further development was rather supported by the application of 6-benzylaminopurine (Pinker et al., 2016). Based on the results reported for other species (*Fraxinus excelsior* and *Cardiocrinum cordatum*) it is hypothesized that application of high GA_3 concentrations before warm phase supports the embryo development during stratification in *Actaea* and accelerates hereby the germination.

Effects of GA_3 application and growing conditions (temperature and light) on embryo development, dormancy breaking, and seed germination were studied with the aim to force the germination process in *A. racemosa*.

Materials and methods

Seed and embryo quality in mature seeds

The mature follicles (fruits) were harvested in September 2015 from plants propagated through rhizome division and established in a research field in Berlin-Dahlem (Germany). Each follicle had 8–12 seeds (half-moon shaped, lens shaped, or round) (Figure 1a) arranged in two rows. The seed colour ranged from light to dark brown. Seeds were stored for 6 weeks in 45% air humidity at 18°C . After seed purification the seeds were graded through sieving. First, they were sieved with mesh size of 1.4 mm and then with mesh size of 2.0 mm. Remaining seeds were evaluated visually and dead or broken seeds were eliminated.

The mean thousand seed weight was determined in 8 graded seed samples of 100 seeds each according to the ISTA rules (ISTA, 2018). Length and width of air-dry seeds were determined by measuring 60 seeds of the graded seed samples. The length was measured along the flat seed side including the ridges. Seed width was recorded at the widest lateral dimension from flat side to the arched side.

The embryo developmental stage of 90 embryos isolated from mature imbibed seeds (1.8–2.2 mm long) was evaluated. Their longitudinal and lateral dimensions were measured under the dissection microscope (Vision Cobra, Vision Engineering).

FIGURE 1. a: Seed sample used for dissection (view in dissection microscope); b: Globular embryo with suspensor in mature seeds (view through transmission light microscope); c: Heart stage embryo with suspensor in mature seeds (view through transmission light microscope); d: Heart stage embryo (view in dissection microscope), length and width (L in mm).

Embryo evaluation methods

Embryo viability was evaluated with TZ-test according to ISTA (2018). The seeds were halved with a razor blade and a 1% aqueous solution of 2,3,5-triphenyltetrazoliumchlorid (TZ), pH 6.5, was dropped on the embryo. After one hour at 30°C the color of the embryos was observed and their dimensions measured under the dissection microscope (Vision Cobra, Vision Engineering).

For more detailed analyses transmission light microscopy was used. The embryo development after harvest and during warm and cold phase had been observed in seeds fixed in FAA (formalin: glacial acetic acid: 50% ethanol, 5:5:90, v/v/v) for 48 h. After fixation the specimens were dehydrated with a graded series of alcohol and then embedded in the plastic resin Technovit 7100® (Heraeus Kulzer GmbH & Co. KG, Wehrheim, Germany). Cross-sections (5 μm thick) were stained with Toluidine Blue O (0.05%) and a tincture of iodine/potassium iodide and then examined under a microscope (Olympus BH-2). Pictures of 30 embryos per treatment and week were taken with the Olympus UC30 camera and analyzed with cellSens Entry 1.6 software of Olympus.

Determination of water uptake during imbibition

To determine the water uptake time-course during imbibition, 40 samples with 3 g air-dry seeds (DW) each were filled into 50-mL glass bottles and incubated in 10 mL tap water or aqueous 2 g L⁻¹ gibberellic acid (GA₃, Roth) solution at 22°C on a rotation shaker (100 rpm) for 7 h. The first weight record on a precision balance (150/0.000 g Sartori-

us Laboratory) was done with 2 × 2 samples after 5 min incubation in tap water and 2 g L⁻¹ GA₃-solution, respectively. The seeds were incubated in tap water and GA₃-solution, respectively, and at hourly interval, for 7 h long, two seeds samples per treatment were weighted. Before weighting the incubated seed samples were collected in a sieve to drip the incubation solution. After that, the seeds were enwrapped in filter paper to suck the free water from seed surface and now the imbibed seed weight (IW) was determined. The water uptake was calculated as % water uptake = (IW - DW) * 100 / DW.

Evaluation of embryo development during stratification

Air-dry seed samples, 100 g each, were first pre-treated with a seed protectant solution (CELEST®, Syngenta) and then imbibed at 22°C for 12 h in 250 mL tap water (0 g L⁻¹ GA₃) or aqueous GA₃-solution (1 and 2 g L⁻¹) on a rotation shaker (100 rpm). After that the free solution was separated from seed samples by dripping through a sieve.

The wet, imbibed seeds (8 g per treatment) were incubated on wet filter paper (only tap water) in closed germination vessels for 4 weeks at 23°C in darkness (warm phase). After that, cold phase started and seeds were incubated at 14°C under a photoperiod of 12 h daily with 20 μmol m⁻² s⁻¹ photosynthetic active radiation, or at 14°C and 7°C, respectively, in darkness. The cold phase lasted for 4 weeks, which means, from week 5 to week 8. If necessary, during stratification the filter paper was watered with tap water. Details for the seed treatments are described in Table 1. Weekly 60 seeds per

TABLE 1. Variants of seed treatments during imbibition as well as warm and cold phases.

Phase	Treatment						Duration
Imbibition GA ₃ (g L ⁻¹)	0		1		2		12 hours
Warm (23°C)	Darkness		Darkness		Darkness		4 weeks
Cold (14°C)	Light	Darkness	Light	Darkness	Light	Darkness	4 weeks
Cold (7°C)*						Darkness	4 weeks

*: alternative to 14°C.

treatment were randomly taken off and 30 embryos of that sample examined under the dissection microscope (Vision Cobra, Vision Engineering).

Evaluation of seed germination

After 8 weeks, from each seed treatment a sample of 0.2 g (between 272 and 313 seeds per treatment) was taken and for all seeds of this sample germination was recorded. Seeds were recorded as germinated when radicle was visible and plumule was fully developed.

Statistical evaluation

All data were collected as described above and subjected to statistical analyses using IBM SPSS Statistics, v.22. For each sample mean value and standard deviation (SD) was calculated and differences were accepted as statistically significant when $p \leq 0.05$ according to Tukey's test for quantitative data and Chi square test (Pearson) for qualitative data.

Results

Embryos and mature seeds quality

The thousand seed weight was 2.72 ± 0.005 g for the graded seed lots. Air-dry seeds ranged after grading in length from 1.22 to 2.5 mm and from 0.94 to 1.5 mm in width. For seed dissection a subset of half-moon shaped seeds between 1.8 and 2.2 mm in length were used (Figure 1a). The rather

small, round shaped seeds and the larger seeds as well were unsuitable for dissection procedure.

After 6 weeks' storage, around 80% of embryos were stained with TZ and considered therefore as viable. Seeds smaller than 1 mm in length were empty. Some globular embryos (Figure 1b) were found in mature seeds in the histological observation (Figure 1b) but most embryos inside the dissected seeds were in the heart stage (Figures 1c and 1d). The embryo size in heart stage embryos of these seed lots was determined with 312 ± 0.05 μ m in length and 208 ± 0.03 μ m in width.

Determination of water uptake during imbibition

The seed samples were immersed in tap water and aqueous 2 g L⁻¹ GA₃-solution, respectively. Already after 5 min incubation, the seed testa changed its appearance and 2 h later, these changes were more pronounced. In Figure 2 the time course of water uptake is presented. The seeds weight increased due to water uptake by more than 50% already after 5 min immersion in the imbibition solutions indicated as hour 0. After one-hour imbibition the water uptake was between 80 and 90% of the initial seed weight. After 3 h the seed weight had doubled (around 100% water uptake). During the following 4 h the values were a little bit fluctuating but no further significant weight increase was determined. The values for water uptake were similar for seeds incubated in tap water or 2 g L⁻¹ GA₃ (Figure 2). The

FIGURE 2. Uptake of the imbibition solution (tap water or 2 g L⁻¹ GA₃ solution) calculated based on the weight increase in percent of seed samples with initially 3 g air-dry seed samples (0% = base line value). The values indicated at 0 hours are the values measured 5 min after immersion. Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$. (See also Supplemental Information, Figure S1).

TABLE 2. Seed length (mm) during imbibition of graded seed samples ($n = 60$).

	Seed length*
Air-dry	1.68 ± 0.19^c
5 min imbibition	1.81 ± 0.12^b
2 hours imbibition	1.94 ± 0.23^a

*: mean $\pm$ standard deviation.Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$.

microscopic observations after 2 h of imbibition showed an imbibed, aqueous endosperm already (Supplemental Information, Figure S1). As measurements of seed length proved, the seed size increased during the first 2-hours' imbibition significantly (Table 2).

Embryo development during stratification

Embryos in the mature seeds were in early developmental stages; in most cases, the heart stage was observed (Figures 1c and 1d). The embryo development continued and after 4 weeks, embryos reached the early torpedo stage (Figure 3a). Embryos of seeds imbibed in $2 \text{ g L}^{-1} \text{ GA}_3$ reached the late

FIGURE 3. Embryo development during warm and cold phase (view through dissection microscope). a: Early torpedo-stage embryo after 4 weeks' warm phase – water imbibition; b: Late torpedo-stage embryo after 4 weeks' warm phase – $2 \text{ g L}^{-1} \text{ GA}_3$ imbibition; c: Early torpedo-stage embryo after one week cold phase – water imbibition; d: Early cotyledonary-stage embryo after one week cold phase – $2 \text{ g L}^{-1} \text{ GA}_3$ imbibition; e: Early torpedo-stage embryo after 2 weeks' cold – imbibition; f: Cotyledonary-stage embryo after 2 weeks' cold phase – $2 \text{ g L}^{-1} \text{ GA}_3$ imbibition; g: Early torpedo-stage embryo after 3 weeks' cold phase – water imbibition; h: Germinating embryo after 3 weeks' cold phase – $2 \text{ g L}^{-1} \text{ GA}_3$ imbibition. L in mm.

TABLE 3. Effect of GA₃ concentration (g L⁻¹) in the imbibition solution on embryo length (μm), during warm phase (23°C, week 1–4) and cold phase (14°C, week 5–7) in darkness (n = 30).

Week	GA ₃		
	0	1	2
	Embryo length*		
0	295.00 ± 45.94 ^d	312.37 ± 39.55 ^d	330.27 ± 63.14 ^d
1	322.47 ± 39.34 ^d	330.80 ± 30.13 ^d	349.93 ± 52.88 ^d
2	317.77 ± 52.55 ^d	340.07 ± 56.59 ^d	414.03 ± 79.50 ^d
3	327.77 ± 51.36 ^d	373.87 ± 56.66 ^d	398.60 ± 75.05 ^d
4	393.63 ± 59.66 ^d	409.00 ± 47.22 ^d	473.60 ± 83.10 ^d
5	348.60 ± 44.82 ^d	448.23 ± 292.13 ^d	1,180.00 ± 919.18 ^c
6	380.03 ± 56.27 ^d	1,159.67 ± 823.34 ^c	1,720.07 ± 998.21 ^b
7	484.20 ± 278.46 ^d	1,471.83 ± 998.22 ^{bc}	2,485.03 ± 645.94 ^a

*: mean ± standard deviation.

Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$.

torpedo stage already (Figure 3b). During the warm phase the embryo size seemed to increase, however, showing no significance to statistical analysis (Table 3). The cold phase stimulated the embryo growth (Table 3) and histo-differentiation of the embryos in GA₃ treated seeds considerably (Figure 3d). The effect of GA₃ depended on concentration. In week 5, embryos treated with 2 g L⁻¹ GA₃ were around three times longer than embryos after imbibition (Table 3). Embryos of seed imbibed in 1 g L⁻¹ GA₃ were grown by around 40%, however, this was insignificant compared to water imbibed seeds which increased in length by around 20% (Table 3). The embryo developmental stage was also affected by GA₃ concentration. Many of embryos treated with 2 g L⁻¹ GA₃ were already in the early cotyledonary stage (Figure 3d) after 5 weeks of stratification. The embryos of seeds imbibed in tap water and 1 g L⁻¹ GA₃ were rather in the early torpedo stage (Figure 3c). In week 6, the embryos increased considerably in length in samples imbibed in GA₃ while those of water imbibed samples did not grow significantly (Table 3;

Figures 3e and 3f). In week 7 embryos of the 2 g L⁻¹ treatment continued to grow, exhibited finally a mean length of 2.5 mm (Table 3) and started to germinate (Figure 3h). The promoting effect of GA₃ treatment became now also obvious in the seed treated with 1 g L⁻¹ GA₃. They reach finally a mean length of around 1.5 mm (Table 3). In the seed treated with tap water the embryos developed but much slower than in the samples treated with GA₃ (Table 3; Figure 3).

Altering the growing conditions during cold phase (lower temperature or light instead darkness) had no effect on the embryo development. The largest embryos were found in week 7 after imbibition in 2 g L⁻¹ GA₃ regardless the growing conditions (Table 4). The embryo development was not significantly different in light or dark conditions (Table 4).

Germination

First seeds with radicle emergence (Figure 3h) could be observed after 7 weeks if they were imbibed in 2 g L⁻¹ GA₃. The promoting effect of treatment with gibberellic acid on

TABLE 4. Effect of GA₃ concentration (g L⁻¹) in the imbibition solution and growing conditions during cold phase (temperature and light or darkness) on embryo length (μm) in week 7 (n = 30).

GA ₃	Embryo length*		
	14°C, light	14°C, darkness	7°C, darkness
0	392.0 ± 62.8 ^c	484.2 ± 278.5 ^c	n.d.
1	1,686.1 ± 935.0 ^b	1,471.8 ± 998.2 ^b	n.d.
2	2,788.5 ± 600.0 ^a	2,485.03 ± 645.94 ^a	2,414.0 ± 588.6 ^a

*: mean ± standard deviation.

Different letters indicate significant differences according to Tukey's test, $p \leq 0.05$.

n.d.: not determined.

TABLE 5. The effect of GA₃ concentration (g L⁻¹) in the imbibition solution and growing conditions during cold phase (temperature and light or darkness) on seed germination (%) after 8 weeks' stratification (n > 270). (See also Supplemental Information, Figure S2).

GA ₃	Seed germination		
	14°C light	14°C darkness	7°C darkness
0	0 ^c	0 ^c	n.d.
1	0 ^c	19.6 ^b	n.d.
2	63.6 ^a	58.8 ^a	63.6 ^a

Different letters indicate significant differences according to Chi square test, Pearson, $p \leq 0.05$.

n.d.: not determined.

germination was clearly proved (Table 5). Seeds imbibed in water did not germinate at all after 8 weeks. Gibberellic acid treatment resulted in germination of around 20% (1 g L⁻¹ GA₃) to 60% (2 g L⁻¹ GA₃) after 8 weeks' stratification. During the following 2 weeks the germination process continued. Two weeks later up to 80% seeds of the 2 g L⁻¹ GA₃ treatment germinated.

For germination, the growing conditions – light or dark – were not important, however, seedlings developed faster and their cotyledons occurred earlier in light (Supplemental Information, Figure S2).

Discussion

Compared to literature data, seeds used in these experiments, especially that for dissection (1.8–2.2 mm long), were smaller than those used by Kaur et al. (2013) with 3 mm long seeds for seed dissection. Albrecht and McCarthy (2007) reported declining viability of seeds during storage reaching around 70% viability after 6 weeks storage. This is little less than the viability (80%) determined in this study. The embryo size in mature seeds was determined with around 312 µm in length confirming the value of 0.34 mm published earlier by Martin (1946). Most embryos were in the heart stage as also reported by Kaur et al. (2013).

Kaur et al. (2013) imbibed the *Actaea* seeds for *in vitro* germination experiments for 2 h while Baskin and Baskin (1985) did not mention an imbibition stage for seeds sown in soil in greenhouse. Based on the current results, 3 to 12 h in the imbibition solution seems to be recommended.

Regarding the effect of GA₃ on embryo development, the current results are in contrast to the results of Kaur et al. (2013) who mentioned that GA₃ treatment did not accelerate embryo development, however, they worked *in vitro* and the applied GA₃ concentration was not mentioned. The stimulating effect of GA₃ treatment in current experiments can be interpreted in line with the findings in other species regarding substitution of warm temperatures by GA₃ (Wciślińska, 1977; Kondo et al., 2006). Using this GA₃ effect, the total stratification time could be reduced considerably to 8 weeks compared, e.g., to 5 months reported by Kaur et al. (2013) and even to 12 weeks necessary for radicle outgrowth reported by Popp et al. (2003).

Considering the growing factors influencing the embryo development and germination the most important ones seems to be the temperature. The warm–cold stratification was successful at constant temperatures (23°C and 14°C, respectively) more or less those temperatures as Popp et al. (2003) applied. It seems to be clear that non-fluctuating temperatures may fulfill the requirements for dormancy breaking. But it should be added that 4 weeks' warm phase were only sufficient if GA₃ was applied with the imbibition solution. The observations of embryos clearly showed that during 4 weeks' warm phase the embryo development was rather slow. This seems to be in contrast to Baskin and Baskin (2004), who mentioned that warm temperatures are responsible for embryo growth. The effect of GA₃ application during imbibition became obvious first in the cold phase. In the literature different temperatures were indicated for cold phase. Baskin and Baskin (1985) worked successfully with 14.1°C/4.5°C. Kaur et al. (2013) used alternating temperatures (20°C/8°C) while Albrecht and McCarthy (2007) applied constantly 5°C in cold phase. In current experiment a temperature of 14°C worked very well and was used as standard. Maybe lower temperatures could be also used as shown here with the 7°C treatment.

Some authors exposed the seeds during stratification to light (Baskin and Baskin, 1985; Kaur et al., 2013). In the current experiments lighting resulted in the same embryo size as darkness. However, seedling development was better in light than in darkness and therefore this phase should be run in light.

The results confirm the hypothesis that high dosages of GA₃ applied before warm phase support embryo development in *Actaea*. Finally, a very efficient germination protocol was developed for *A. racemosa*. The role of gibberellic acid in dormancy breaking should be considered more in detail later on.

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Address of authors:

I.M. Pinker*, T. Degischer, A.-Chr. Rath and R. Schenk
Humboldt-Universität zu Berlin, Albrecht Daniel Thaer-Institute, Intensive Plant Food Systems, Lentzeallee 75, D-14195 Berlin, Germany

* Corresponding author;

E-mail: ina.pinker@cms.hu-berlin.de

Tel.: +49-30-209346297; Fax: +49-30-209346285

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL INFORMATION – FIGURE S1. a: Seed sample 5 min after immersion; b: Seed sample after 2 hours of imbibition; c: Seed after 2 hours' imbibition with indication of measuring points for length and width determination; d: Halved seed after 2 hours' imbibition with heart-stage embryo and aqueous endosperm.

SUPPLEMENTAL INFORMATION – FIGURE S2. a: Germinating seeds imbibed in 2 g L⁻¹ GA3 after 8 weeks' stratification (week 5–8 at 14°C in darkness); b: Seed with radicle protrusion after 8 weeks' stratification (week 5–8 at 14°C in darkness); c: Germinating seeds after 8 weeks' stratification (week 5–8 at 14°C in light); d: Germinating seed after 8 weeks' stratification (week 5–8 at 14°C in light).