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ORIGINAL PAPER

Continuous biosynthesis of abscisic acid (ABA) may be required for maintaining dormancy of isolated embryos and intact seeds of *Euonymus alatus*

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Abstract *Euonymus alatus* (Thunb.) Sieb. is a popular landscape plant in the United States due to its brilliant red fall foliage. It is also an important ornamental plant in many other areas of the world such as China, Japan and Europe. However, *E. alatus* is considered as a highly invasive plant species in the US. Mutation breeding can be used to create sterile, non-invasive cultivars. Seeds are the most commonly used explants for mutagen treatments, but *E. alatus* mature seeds possess prolonged dormancy and only a low percentage of them germinate even after 18 months of cold stratification. Here we report an immature embryo culture method for *E. alatus* 'Compactus' to circumvent the seed dormancy problem. Also, we have found that activated charcoal, gibberellic acid (GA_3) and 6-benzyladenine (BA) can reduce the dormancy of isolated embryos, which suggests that abscisic acid (ABA) might play a role in controlling seed dormancy. We have further demonstrated that exogenous ABA enhances dormancy of isolated *E. alatus* embryos while fluridone, an inhibitor for ABA biosynthesis, can effectively break their dormancy. These results, particularly the effect of fluridone, suggest that continuous ABA biosynthesis plays an important role in controlling the dormancy of *E. alatus* seeds.

Keywords Abscisic acid · Activated charcoal · Burning bush · Cold stratification · Fluridone · Germination · Gibberellic acid

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Introduction

Euonymus alatus (Thunb.) Sieb. (burning bush or winged euonymus) is an important landscape plant in the United States, China, Japan and Europe. It was introduced to US from Northeastern/Central China in 1860 (Chen et al. 2006) for horticultural purposes. With its ease of culture, tolerance to a wide range of soil conditions, orange ariled fruit and brilliant red autumn color, *E. alatus* has become a favorite of both landscapers and homeowners. The shrub is widely used as a hedge, border or specimen plant and it is a significant contributor to the $16 billion United States ornamental horticulture industry (US Department of Agriculture 2005). In Connecticut alone, sales of *E. alatus* 'Compactus' reach $5–10 million per year (Thammina et al. 2011). A mature *E. alatus* shrub produces large quantities of seeds. These seeds are transported and dispersed by birds, water or mechanical soil movement, and thus feral populations of the landscape shrub are established in a variety of habitats (Chen et al. 2008). A naturalized shrub competes with native flora for space, sun light, water and nutrients. As of 2006, *E. alatus* has been reported to be invasive in 21 states in the US (Ding et al. 2006). Hence, many states intend to

ban the sale, propagation and planting of this shrub (Thammina et al. 2011). However, due to the economic significance of *E. alatus* to the ornamental and landscape industries, completely banning its use would be undesirable (Li et al. 2004).

Transgenic plant technology and mutation breeding are effective in creating plants with desirable traits. Although, several gene technologies useful for producing sterile *E. alatus* have been developed (Chen et al. 2006, 2008; Li et al. 2004; Luo et al. 2006; Zheng et al. 2007), potential problem with transgene flow and also high costs associated with the deregulation process present obstacles for the use of transgenic technologies in horticultural crops (Luo et al. 2007; Moon et al. 2009). However, sterility is one of the most frequent phenotypes observed among the mutagen treated plants (Ahloowalia and Maluszynski 2001). Hence, this technique should be useful for generating sterile, non-invasive *E. alatus* cultivars. To produce sterile cultivars of *E. alatus*, seeds are the most convenient explants for mutagenesis. However, *E. alatus* seeds possess a prolonged dormancy that is a major hindrance for mutation breeding. Rudolf (1974) reported that three-month cold stratification would break seed dormancy in *E. alatus*. However, after following the protocol we observed that no seeds germinated after 3 months of cold stratification, while less than 1% of seeds germinated after 18 months of cold stratification at 4°C. In vitro embryo culture has been used as a suitable technique to obtain healthy plants from dormant seeds in a number of plant species when stratification and scarification treatments are ineffective (Raghavan 2003; Tamaki et al. 2011; Wen and Wang 2010).

Seed dormancy is a temporary failure of viable seed maintained under favorable physical conditions to complete germination (Baskin and Baskin 2004). Plant growth regulators have been documented to play an important role in the dormancy and germination of seeds in many plant species (Uma et al. 2011). Abscisic acid (ABA) is an important factor in inducing and maintaining seed dormancy (Kucera et al. 2005; Rai et al. 2011). Enhanced dormancy is observed in mutants and transgenic lines of tomato, tobacco and *Arabidopsis* that overaccumulate ABA (Okamoto et al. 2010). On the other hand, ABA deficient mutant seeds or seeds treated with fluridone (an ABA biosynthesis inhibitor) exhibit reduced seed dormancy and enhanced germination (McCarty 1995). Further, studies conducted with many plant species like *Pyrus malus* and *Fagus sylvatica* demonstrated the antagonistic effects of abscisic acid and gibberellic acid on the regulation of seed dormancy (Gendreau and Corbineau 2009; Kucera et al. 2005). Gibberellic acid (GA) plays an important role in dormancy release and in the promotion of germination by increasing the growth potential of embryo and GA-induced cell-wall hydrolases are necessary for endosperm weakening (Kucera et al. 2005; Siddiqui et al. 2011). It has also been reported that cytokinins can overcome the inhibitory effect of ABA in germination (Bewley and Fountain 1972; Dewar et al. 1998).

Here we report development of an efficient method for recovering plants from immature embryos of *E. alatus* 'Compactus' and also provide experimental evidence for a possible role of ABA in controlling embryo dormancy and therefore seed dormancy of *E. alatus*.

Materials and methods

Plant material

E. alatus 'Compactus' immature and mature fruits were collected in the first week of September and the third week of November, respectively, from 10-year-old plants located at Prides Corner Farms, Lebanon, CT, USA.

Cold stratification of mature seeds

Mature seeds were washed thoroughly, lighter seeds were rinsed off and the healthy ones were soaked in tap water for 24 h. After soaking, water was drained and the seeds were placed in plastic growth trays containing moistened sterilized sand. Trays containing the seed and sand mix were placed in a 4°C refrigerator and stratified in darkness for 3, 6, 12 or 18 months. Each replicate had 5,000 seeds and each treatment had three replicates. Trays were checked every week for molding and moisture contents. After cold stratification, the seeds were separated from sand and were sowed in growth trays containing Promix potting soil (Premier Horticulture Inc., PA, USA) and transferred to a greenhouse. Seeds stored at room temperature were used as a control treatment. All the trays were watered regularly and germination observations were taken after 30 days. Mean number of seeds germinated was based on three replicates for each treatment. Germination rate was calculated by dividing the number of seeds germinated by the total number of seeds used.

Culture media and conditions

All the culture media described here were comprised of either Murashige and Skoog (MS) medium salts (Murashige and Skoog 1962) or woody plant medium salts (WPM) (Lloyd and McCown 1980) with 100 mg l^{-1} *myo*-inositol, 2.0 mg l^{-1} glycine, 1.0 mg l^{-1} thiamine-HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine–HCl, 0.5 g l^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), 3% (w/v) sucrose and 0.7% (w/v) agar. Vitamin stocks were prepared with distilled water, while ABA, 6-benzyladenine (BA) and

gibberellic acid (GA_3) stocks were prepared with 75% (v/v) ethanol. Fluridone stock was prepared with 95% (v/v) ethanol. The pH of all culture media was adjusted to 5.8 with KOH or HCl before the addition of agar, and the media were autoclaved at 121°C for 20 min. All the cultures, except for those specifically mentioned, were maintained at 23 ± 2°C under 36 μmol m^{-2} s^{-1} light provided by cool-white fluorescent tube lamps with a 14 h photoperiod.

Initiation of aseptic cultures

Immature fruits were thoroughly washed under running tap water for 30 min and all further manipulations were carried out under aseptic conditions in a laminar air-flow hood. Immature fruits were surface-disinfected by immersion in 10% commercial bleach (containing 6% sodium hypochlorite) with 0.1% Tween 20 (Sigma, St. Louis, MO) for 30 min and rinsed three times with sterile distilled water. The fruits were then soaked in 75% (v/v) ethanol and swirled well for 2 min. After washing the fruits three times with sterile distilled water, they were dissected with a surgical blade to excise embryos. Immature embryos were cultured on media supplemented with full-strength MS or WPM salts and 0 or 1.5 g l^{-1} activated charcoal (Kang et al. 2009). Explants were transferred to fresh media every 2–3 weeks. Germination observations were taken every 2 weeks. An embryo was considered to be germinated when it had developed expanded cotyledons, an elongated hypocotyl and a radicle (Stage 5 of Fig. 1b). Germination rates were calculated based on the data collected on the 66th day of culture and were reported as an average over three replicates. Germinated embryos were cold treated to break dormancy and gradually acclimatized to the greenhouse for further growth as described by Thammina et al. (2011). To study the effects of plant growth regulators on the dormancy and germination, immature embryos were also cultured on the MS medium supplemented with 2.22 or 4.44 μM BA, 0.72 or 1.44 μM GA_3, 0.2 or 0.6 μM ABA and 20 μM fluridone. Immature embryos cultured on hormone-free MS medium were used as the control. Seventy immature embryos were cultured per replicate and each treatment had three replicates. All of the cultures were transferred to fresh media every 2–3 weeks. Germination rates were determined in every 2 weeks. Germination rates presented in this paper were recorded on the 15th and 30th days of culture for control, ABA and fluridone treatments, and on the 66th day of culture for control, BA and GA_3 treatments. Germination rates were calculated by dividing the number of immature embryos germinated by the total number of immature embryos cultured. Mean germination rates were based on three replicates for each treatment.

Mature seeds were surface-disinfected by immersion in commercial bleach. After disinfection, embryos were excised and cultured on MS media supplemented with 5, 10 or 20 μM fluridone and 0.5, 1.0, 1.5 or 3.0 g l^{-1} activated charcoal to study the effects of fluridone and activated charcoal on germination. Mature embryos were also cultured on basal MS medium as a control. Seventy mature embryos were cultured per replicate, and each treatment had three replicates. The culture conditions were same as those used for immature embryos. Embryo germination rates were determined every 2 weeks. Germination rates reported in this manuscript were recorded on the 15th day of culture for the fluridone treatments and on the 66th day of culture for the activated charcoal treatments. Germination rates were compared with control treatment data collected on the corresponding days.

Statistical analysis

Germination data were subjected to analysis of variance. Duncan's multiple range test was applied for mean separation, with alpha pre-determined at $P \leq 0.05$, using IBM SPSS Software (Version 19.0; IBM Corporation, Somers, NY).

Results

Effect of cold stratification on the germination of mature seeds

Germination was not observed in seeds that were stratified for 0 or 3 months. Germination rates were only 0.08, 0.24 and 0.76% for seeds stratified for 6, 12 and 18 months, respectively, after they were transferred to the greenhouse. There was no significant difference in the germination rates of 6- and 12-month stratified seeds compared to the control. We speculate that the low germination rates of mature seeds may be due to the presence of inhibitory substances in the embryo and/or endosperm.

Effects of culture media and activated charcoal on the germination and growth of immature and mature embryos

Due to the hard endosperm tissues in mature seeds of *E. alatus,* it is difficult to isolate embryos without damage. On the other hand, it is relatively easy to isolate embryos from immature seeds because endosperm and seed-coat tissues are soft in these seeds (Fig. 1a). Freshly isolated embryos were light green in color and 3–5 mm long (Fig. 1b, designated as Stage 1). Embryos started to enlarge on an appropriate culture medium (Stage 2; shown in Fig. 1b). Immature embryos turned dark green in color, and the two cotyledons were clearly distinguishable from each

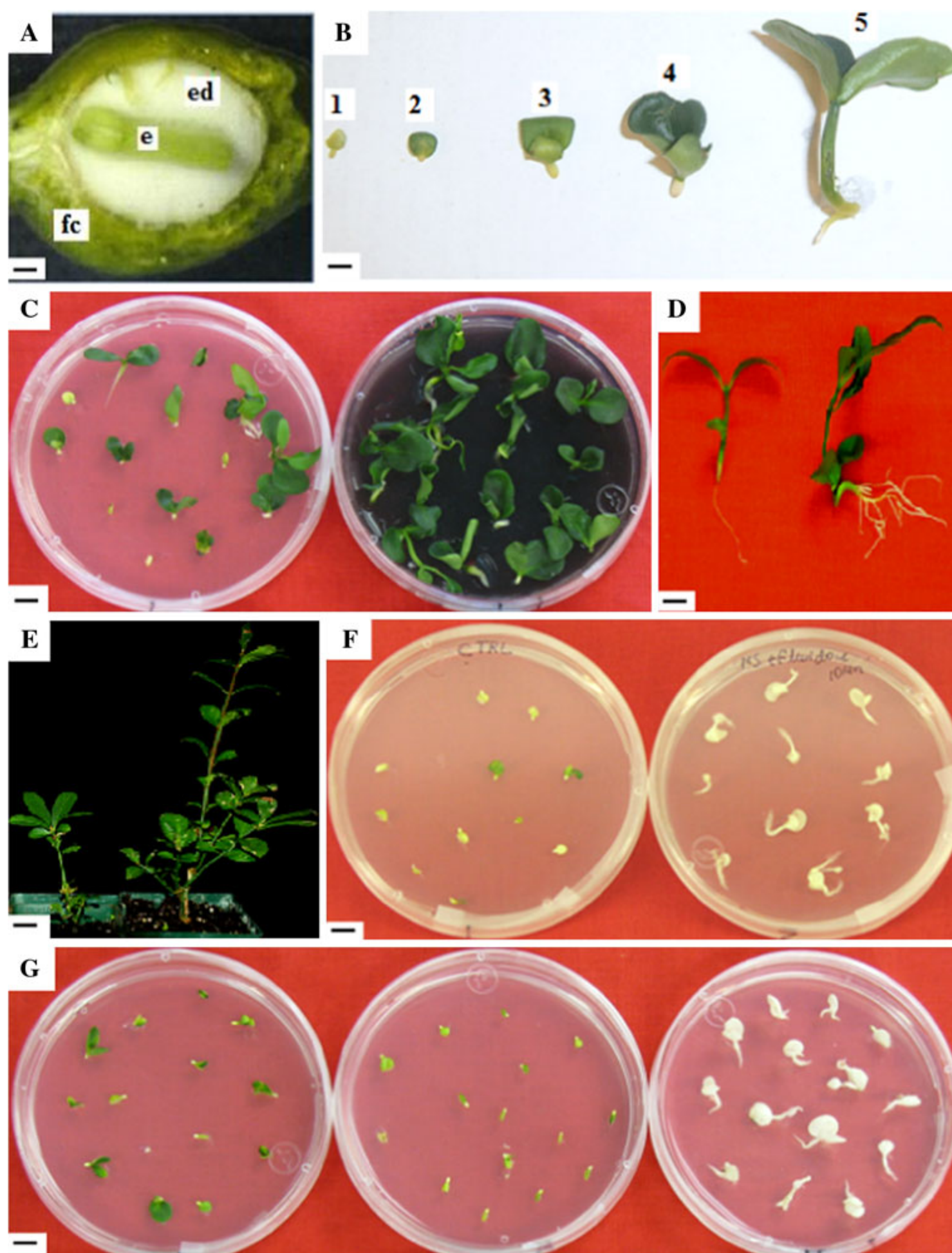

Fig. 1 Recovery of *Euonymus alatus* 'Compactus' plants from in vitro culture of immature embryos and the effect of ABA and fluridone on immature and mature embryo germination. **a** Longitudinal section of immature fruit: fc-fruit coat; ed-endosperm; e-embryo (*bar* 1 mm). **b** Different growth stages of immature embryos cultured on MS medium with 1.5 g l^{-1} activated charcoal: *Stage-1* freshly isolated immature embryo, *Stage-2* embryo cultured for 1 week, *Stage-3* embryo cultured for 2 weeks, *Stage-4* germinating embryo at 3 weeks of culture, *Stage-5* germinated embryo with expanded cotyledons, elongated hypocotyl and radicle after 5 weeks of culture (*bar* 3 mm). **c** Effect of activated charcoal on the germination of immature embryos after 30 days of culture: MS (*left*) and MS + 1.5 g l^{-1} activated charcoal (*right*) (*bar* 7 mm). **d** Plantlets obtained from immature embryos cultured on MS (*left*) and MS + 1.5 g l^{-1} activated charcoal (*right*) media after 66 days (*bar* 7 mm). **e** Nine- and fourteen-months-old plants derived from immature embryos cultured on MS + 1.5 g l^{-1} activated charcoal medium, growing in pots containing Promix soil in the greenhouse (*bar* 24 mm). **f** Effect of fluridone on the germination of mature embryos cultured for 15 days: MS (*left*) and MS + 10 μM fluridone (*right*) media (*bar* 7 mm). **g** Effect of ABA and fluridone on the germination of immature embryos cultured for 15 days: MS (*left*), MS + 0.2 μM ABA (*center*) and MS + 20 μM fluridone (*right*) media (*bar* 7 mm)

other by the 2nd week (Stage 3; Fig. 1b). After 3 weeks of culture, cotyledon expansion had been observed (Stage 4; Fig. 1b). By stage 4, embryos developed enlarged cotyledons, elongated hypocotyls and radicles by the 5th week of culture (Stage 5; Fig. 1b). When the growth and development of an isolated embryo reached Stage 5, we

considered the embryo as germinated. Using that standard, we counted the ones that reached Stage 5 on the 66th day of culture. We observed that 37.1% of immature embryos germinated and developed into seedlings on the MS medium, while the germination rate was only 16.2% on the WPM (Table 1). Because of that, MS medium was then used for all subsequent experiments.

Due to the low germination rate of isolated embryos, we included activated charcoal in the MS medium. As shown in Table 1 and Fig. 1c, d, the presence of activated charcoal significantly enhanced the germination rate of immature embryos, from 37.1% without activated charcoal to 76.7% with activated charcoal included. Activated charcoal also had a positive effect on the germination of mature embryos. After 66 days of culture on MS media supplemented with 0, 0.5, 1.0, 1.5 and 3.0 g l^{-1} activated charcoal, germination rates were 25.2, 46.7, 48.6, 64.3 and 67.1%, respectively (Table 1). The fact that activated charcoal reduces the embryo dormancy suggests that high levels of inhibitory substances, such as ABA, may be present in *E. alatus* embryos and thus induce dormancy.

Healthy seedlings derived from immature embryos were transferred to the soil and 95% of them established well and showed good growth in the greenhouse. Plants of different ages (9 and 14 months) derived from immature embryos cultured on MS + 1.5 g l^{-1} activated charcoal are shown in Fig. 1e.

Effects of plant growth regulators and fluridone on the germination and growth of immature and mature embryos

We have also observed that addition of GA_3 and BA into the MS medium significantly improved the germination rates of immature embryos. After 66 days of culture, embryo germination rates reached 53.3 and 62.9% with 0.72 and 1.44 μM GA_3, respectively. Similarly, 46.2 and 54.8% of embryos germinated on 2.22 and 4.44 μM BA, respectively. Only 37.1% of immature embryos germinated on the control medium (Table 2). Due to the antagonistic effects between ABA and GAs and between ABA and BA, our data suggest that high concentrations of ABA may be present in immature embryos.

To study a possible role of ABA in controlling the dormancy of embryos of *E. alatus*, we used both ABA and an ABA biosynthesis inhibitor to treat immature and mature embryos. We observed that germination of immature embryos was reduced to 3 and 0% when 0.2 and 0.6 μM ABA were included in the MS medium, respectively (Table 3). However, germination rates of immature embryos cultured on MS medium with 20 μM fluridone were drastically improved, with 73.8% of the embryos germinated by the 15th day and 89.1% by the 30th day,

Table 1 Effect of MS and WPM culture media and activated charcoal (AC) on the germination of *Euonymus alatus* 'Compactus' embryos

Type of embryos	Treatment	Germination rate (%)
Immature	MS	37.1 ± 2.2d
Immature	WPM	16.2 ± 1.3f
Immature	MS + 1.5 g l^{-1} AC	76.7 ± 3.1a
Immature	WPM + 1.5 g l^{-1} AC	21.4 ± 1.4ef
Mature	MS	25.2 ± 1.2e
Mature	MS + 0.5 g l^{-1} AC	46.7 ± 2.1c
Mature	MS + 1.0 g l^{-1} AC	48.6 ± 1.4c
Mature	MS + 1.5 g l^{-1} AC	64.3 ± 1.6b
Mature	MS + 3.0 g l^{-1} AC	67.1 ± 2.2b

Each value represents the mean ± SE of three replicates with 70 embryos per replicate. Data having the same letter in a column were not significantly different by Duncan's multiple range test ($P \leq 0.05$)

Table 2 Effect of BA and GA_3 on the germination of *Euonymus alatus* 'Compactus' immature embryos

Plant growth regulator (μM)		Germination rate (%)
BA	GA_3	
0	0	37.1 ± 2.2 d
–	0.72	53.3 ± 1.2 b
–	1.44	62.8 ± 1.4 a
2.22	–	46.2 ± 2.0 c
4.44	–	54.7 ± 0.9 b

Each value represents the mean ± SE of three replicates with 70 immature embryos per replicate. Data having the same letter in a column were not significantly different by Duncan's multiple range test ($P \leq 0.05$)

Table 3 Effect of ABA and fluridone on the germination of *Euonymus alatus* 'Compactus' embryos

Type of embryos	Concentration of ABA and fluridone (μM)		Germination rate (%)	
	ABA	Fluridone	15th day	30th day
Immature	0	0	0 ± 0 c	15.2 ± 0.5 b
Immature	0.2	–	0 ± 0 c	2.9 ± 0.8 c
Immature	0.6	–	0 ± 0 c	0 ± 0 c
Immature	–	20	73.8 ± 1.3 b	89.1 ± 1.7 a
Mature	0	0	0 ± 0 c	–
Mature	–	5	87.6 ± 2.0 a	–
Mature	–	10	93.3 ± 3.4 a	–
Mature	–	20	94.8 ± 2.3 a	–

Each value represents the mean ± SE of three replicates with 70 embryos per replicate. Data having the same letter in a column were not significantly different by Duncan's multiple range test ($P \leq 0.05$)

respectively (Table 3; Fig. 1g). Mature embryos also germinated on 5, 10 and 20 µM fluridone supplemented MS media by the 15th day of culture, while no germination was observed on the MS control medium (Fig. 1f). Approximately 87, 93 and 95% of mature embryos germinated on 5, 10 and 20 µM fluridone supplemented media, respectively (Table 3). Thus, fluridone significantly reduced the dormancy of both immature and mature embryos. These results have demonstrated that ABA plays an important role in the dormancy of both immature and mature embryos. Because fluridone blocks the synthesis of carotenoids, the ABA precursor, our results further suggest that ABA is continuously synthesized in both immature and mature embryos, and such a continuous ABA synthesis is important to maintain embryo dormancy. Since fluridone inhibits carotenoid synthesis, in the absence of protective carotenoids, chlorophyll breaks down in light and seedlings become white in color due to photo-oxidation (Fig. 1f, g). Most albino seedlings were restored after they were transferred onto fluridone-free MS medium (data not shown).

Discussion

Seed dormancy (often observed as "poor" seed germination) is an important characteristic of many invasive plant species (Miranda et al. 2011). Poor germination (prolonged seed dormancy) helps invasive plants to become more serious invaders because the dormancy ensures periodic germination and prevents seedlings from sprouting during unfavorable conditions (Dalling et al. 2011). This is obviously the case for *E. alatus* since prolonged seed dormancy has been observed. To circumvent seed dormancy problem of *E. alatus* we focused our research on immature embryo culture. In this study, we used both MS and WPM basal media to culture immature embryos of *E. alatus*, and observed higher percentages of seed germination with the use of the MS medium. Better germination and growth of immature embryos in *Boswellia serrata* and *Arachis hypogaea* has been reported on MS medium (Ghorpade et al. 2010; Qu et al. 1993). Nutritional requirements of immature embryos are complex and vary among different plant species (Hu and Zanettini 1995). We speculate that *E. alatus* immature embryos might require higher nitrogen content for germination and growth, which is available in abundance in the MS basal salt mixture. One liter of full-strength MS basal medium contains 1,650 mg of ammonium nitrate and 1,900 mg of potassium nitrate compared to the 400 mg of ammonium nitrate and 386 mg of calcium nitrate available in full-strength basal WPM salts.

Immature and mature embryos of *E. alatus* cultured on MS medium without activated charcoal germinated relatively poorly; but activated charcoal significantly improved their germination. The activated charcoal treated embryos then developed into healthy plantlets. With the addition of activated charcoal to the MS medium, an efficient germination protocol for immature embryos has been developed. As isolation of embryos from immature seeds of *E. alatus* is relatively easy and simple, our method provides a foundation for using embryos as explants for mutagenesis and therefore for the development of sterile and non-invasive *E. alatus* cultivars. Based on the data presented here, it is also obvious that addition of growth regulators such as gibberellins, cytokinins, or ABA biosynthesis inhibitors can further enhance germination rates of *E. alatus* embryos.

The fact that activated charcoal promoted immature and mature embryo germination and seedling growth led us to the conclusion that inhibitory substances are responsible for embryo dormancy of *E. alatus*. It has been previously demonstrated that ABA and phenols are the two important factors that maintain seed dormancy, and activated charcoal can effectively reduce the concentrations of these substances (Chen et al. 2007; Dhekney et al. 2011; Egerton-Warburton 1998; Thomas 2008). Polyphenol production in tissue culture experiments often results in explant and media browning, eventually leading to death (Kwapata et al. 2010). Nonetheless, we did not observe embryo or media discoloration even on activated charcoal free-MS control medium (Fig. 1c). Hence, phenols are unlikely to be responsible for the dormancy of *E. alatus* embryos.

ABA is a key growth regulator to control seed dormancy in higher plants. For instance, ABA has been reported to arrest embryo growth by inhibiting the extensibility of the embryonic cell wall (da Silva et al. 2004). It was observed that activated charcoal reduced anther ABA content in *Capsicum annuum* (Tıpırdamaz and Ellialtıoğlu 2002). Activated charcoal improved seed germination in *Rosa multiflora* by reducing ABA content in seeds (Yambe et al. 1992). Thus, ABA is likely a germination inhibiting factor for *E. alatus* embryos in our case, and the activated charcoal we used could have reduced ABA levels in embryos and therefore promoted their germination.

Indirect evidence to support the role of ABA in controlling *E. alatus* embryo dormancy is also obtained from the effects of GA_3 and BA. In our study, the use of GA_3 in the culture medium resulted in a 40–70% increase in the germination rates of immature embryos compared to the control. Gibberellins (GA_s) are known to play a significant role in the release of dormancy and promotion of germination in many plant species by overcoming inhibitory effects of ABA (Kucera et al. 2005; Mostafa et al. 2010). Grappin et al. (2000) and White and Rivin (2000) have reported that the inhibitory effect of 10 µM ABA on seed germination in *Nicotiana plumbaginifolia* and *Zea mays* respectively, was reversed by the addition of 100 µM GA_3. Dormancy of *Fagus sylvatica* seeds was also broken by

exogenous application of 100 μM GA_3 and expression of two specific genes induced by ABA was shown to be inhibited by GA_3 treatment (Nicolás et al. 1996).

We have also observed that BA in the culture medium improved the germination of *E. alatus* immature embryos. Cytokinins have been shown to be effective in overcoming ABA-inhibited germination of embryos in lettuce and sorghum (Bewley and Fountain 1972; Dewar et al. 1998). Germination of lettuce embryos was completely inhibited by 30 μM ABA. However, 100% germination was observed if 10 μM BA was added (Bewley and Fountain 1972). Our data from the GA_3 and BA experiments also support the hypothesis that ABA may be an important factor to control embryo dormancy of *E. alatus*.

More direct evidence supporting the role of ABA as an important factor in controlling dormancy of *E. alatus* embryos was provided in our experiments with ABA and an ABA biosynthesis inhibitor, fluridone. The fact that ABA promoted the dormancy of *E. alatus* embryos demonstrates that they are responsive to the ABA signal, and if these embryos contain an appropriate level of ABA, they will remain dormant. Fluridone, an inhibitor of carotenoid biosynthesis has been widely used to block ABA production in higher plants and to demonstrate the role of ABA in seed dormancy and germination, and other growth and developmental processes (Webb et al. 2009). Since fluridone effect on carotenoid biosynthesis appears to be specific, the inhibitor provides a powerful tool to study the roles of ABA in various physiological or developmental events, particularly seed dormancy (Kucera et al. 2005; Li and Walton 1987). Fluridone blocks phytoene desaturase, which is a critical enzyme in the formation of the ABA precursor carotene (Li and Walton 1990a, b; Walton and Li 1995; Webb et al. 2009). Our data shows that embryo dormancy is enhanced by ABA but drastically reduced by fluridone. These results provide more direct evidence that ABA plays an important role in the dormancy of isolated embryos of *E. alatus*. Further, because fluridone prevents continuous ABA synthesis, we believe that continuous ABA biosynthesis is needed to maintain the dormancy of immature and mature embryos. If ABA is responsible for the dormancy of isolated embryos of *E. alatus*, it should also be an important factor for the prolonged seed dormancy observed in intact *E. alatus* seeds.

In conclusion, we have shown that healthy *E. alatus* ‘Compactus’ plants can be recovered at a high rate, using immature embryo culture on full-strength MS medium with activated charcoal in about 4 months. We have further demonstrated that dormancy of immature and mature embryos can be significantly enhanced by ABA but drastically reduced by fluridone. We therefore suggest that continuous ABA biosynthesis is required for maintaining dormancy of *E. alatus* ‘Compactus’ seeds.

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