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The physiological basis for ethylene-induced dormancy release in three *Echinacea* species with special reference to the influence of the integumentary tapetum

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ABSTRACT

Seeds of *Echinacea* species have endogenous physiological dormancy. Dormancy release is induced by moist chilling stratification, but seeds treated with ethylene can show increased germination comparable to stratification. The primary aims of this work were to discover whether endogenous ethylene production was required for dormancy release and germination in *Echinacea* seeds and to investigate the physiological basis for stratification and ethylene-induced dormancy release. There were no significant differences in ethylene production in untreated versus stratified seeds. Seeds subjected to a dormancy release treatment showed reduced sensitivity to exogenous abscisic acid (ABA). Isolated embryos were completely released from dormancy when the outer envelope surrounding the embryo was removed. Isolated embryos with the envelope intact were also induced to germinate when treated with ACC (1-aminocyclopropane-1-carboxylic acid) to increase ethylene production. It was determined that the covering envelope was derived from enlargement of the endothelium layer surrounding the egg sac (integumentary tapetum) plus several crushed layers of the outer integument. Ethylene does not appear to be required for dormancy release and germination in *Echinacea* seeds. Two possible physiological mechanisms were discovered to explain stratification and ethylene-induced dormancy release. These included a change in seed sensitivity to ABA and changes in the tissues covering the embryo. The data suggests that ethylene-induced dormancy release is independent of stratification and possibly acts by inducing physiological events that are normally downstream of stratification.

1. Introduction

Echinacea comprises a small group of North American herbaceous perennials in the Asteraceae (Gleason and Cronquist, 1991). They are commonly used as garden plants, cut flowers, in ecological restorations, and as the source for the commercially important medicinal herb echinacea (Whitten, 2004). They are principally seed propagated although some of the species and interspecific hybrids are micropropagated (Lakshmanan et al., 2002). *Echinacea purpurea* (L.) Moench, known as purple coneflower, can be found growing throughout the United States and Canada. *Echinacea tennesseensis* (Beadle) Small is the Tennessee coneflower and is native to a small, restricted geographic area in the cedar glades of Tennessee, USA and is on the Federal endangered plant's list (Walck et al., 2002). It is available commercially as selected cultivars. *Echinacea simulata* McGregor, known commonly as the wavyleaf purple coneflower or glade coneflower, grows in the region of western Kentucky, southern Illinois, northeast Arkansas, and southeast Missouri.

Seeds of *Echinacea* species display endogenous physiological dormancy (Baskin and Baskin, 1998). Seed lots can contain a mixture of dormant and non-dormant seeds (Wartidiningsih et al., 1994). Therefore, germination in untreated *Echinacea* species can be erratic and can vary significantly even within seed lots of the same cultivar. Germination percentage and uniformity can be achieved with moist chilling stratification (Romero et al., 2005). Even seed lots with a high percentage of non-dormant seeds can show increased germination speed if seeds are exposed briefly to moist chilling (Wartidiningsih et al., 1994). Similarly, ethylene application to seeds of various *Echinacea* species has generally been an effective treatment to circumvent moist chilling stratification to increase germination percentage and speed (Feghahati and Reese, 1994; Macchia et al., 2001; Sari et al., 2001; Qu et al., 2004).

Ethylene production during germination is a typical observation for most seeds and there is often a burst of ethylene produced concomitant with radicle protrusion (Matilla, 2000). Studies using mutant or transgenic seeds impaired for ethylene production or perception show that ethylene is not a strict requirement for

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germination, but that these seeds can show a higher degree of dormancy compared to wild type seeds (Siriwitayawan et al., 2003; Matilla and Matilla-Vázquez, 2008). This may be due to an interaction between ethylene and ABA, as mutations in the ethylene signal transduction pathway alters seed sensitivity to ABA during germination (Beaudoin et al., 2000; Ghassemian et al., 2000).

In some species, the seed's ability to produce ethylene is associated with dormancy release (Kępczyński and Kępczyńska, 1997). This appears to be especially important in seeds experiencing thermodormancy as typified by lettuce (*Lactuca sativa* L.) and sunflower (*Helianthus annuus* L.). Lettuce and sunflower are fellow members of the *Asteraceae* and seeds (achenes) have a similar morphology to *Echinacea*. Therefore, the relationship between ethylene and dormancy in lettuce and sunflower could provide insight into *Echinacea* seed dormancy. Sunflower seeds are dormant when germinated at 15 °C (Corbineau et al., 1989). Ethylene application can release sunflower seeds from thermodormancy and ethylene inhibitors reduce or eliminate germination in non-dormant seeds suggesting a physiologically important role for ethylene in dormancy release in sunflower. Similarly, lettuce seeds imbibed at temperatures that induce thermodormancy (~35 °C) show a reduced ability for ACC synthesis as well as the conversion of ACC to ethylene (Khan and Prusinski, 1989; Huang and Khan, 1992). Also, the genes involved in ethylene production show down-regulation in thermodormant lettuce seeds (Argyris et al., 2008). Treatments that can alleviate thermodormancy (i.e. kinetin application) and those that prevent its induction (seed priming) have resulted increased capacity for ethylene production (Huang and Khan, 1992; Khan and Huang, 1988; Nascimento et al., 2004). Similarly, osmotically primed lettuce seeds were generally able to germinate at 35 °C, but the priming treatment was ineffective at preventing thermodormancy if the priming solution contained the ethylene action inhibitor silver thiosulfate (STS) (Nascimento et al., 2004).

Exposure of dormant *Echinacea* seeds to ethylene can induce germination, but the physiological significance of endogenous ethylene production related to germination and dormancy release has not been extensively studied. In a preliminary study, it appeared that ethylene may not be required to achieve dormancy release in *Echinacea* species induced by moist chilling stratification (Geneve and Wood, 2008). Therefore, the objectives of this research were to study the relationship between ethylene production and dormancy release in *Echinacea* and to determine if ethylene production or perception was an important mode of action for dormancy release in *Echinacea* seeds.

2. Materials and methods

2.1. Seed source

Three *Echinacea* species (*E. purpurea*, *E. tennesseensis*, and *E. simulata*) were used in this study. Seeds were produced at the South Farm experiment station of the University of Kentucky or obtained from Johnny Selected Seeds, Winslow, ME, USA (*E. purpurea* and *E. tennesseensis*), Everwilde Farms, Bloomer, WI, USA (*E. tennesseensis*), and Easyliving Wildflowers, Willow Springs, MO, USA (*E. simulata*). Initial seed germination within each species was similar across seed lots obtained from different sources; only one seed source was used per experiment.

2.2. Germination conditions

Achenes (hereafter referred to as seeds) or isolated embryos were germinated in Petri dishes (100 mm × 15 mm) containing 6 mL of treatment solution, two pieces of Grade 8001 germination paper (Stults Scientific Co., Mt. Holly Springs, PA, USA) and sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Germination occurred at a constant 25 °C in 8 h light (45 μmol s^{-1} m^2 from fluorescent lamps) and 16 h dark, and germination (radicle protrusion) was recorded daily for up to 12 days.

2.3. Moist chilling stratification conditions

Four replications of 25 seeds were put in Petri dishes with 6 mL of water, two pieces of germination paper, and sealed with parafilm. The seeds were then placed in a refrigerator at 5 °C for 0, 30, or 60 days, depending on the experiment. For germination, seeds were transferred to new Petri dishes containing 6 mL of test solution as previously described. For ethylene production measurements, there were 80 seeds per stratification Petri dish from which 20 seeds were subsequently transferred to 25 mL Erlenmeyer flasks. There were four replications of each flask.

2.4. Ethylene seed treatments

Twenty-five seeds were germinated in four replicated Petri dishes containing deionized water (control), 5 mM ACC (Sigma–Aldrich, St. Louis, MO, USA), or 1 mM ethephon (Sigma–Aldrich, St. Louis, MO, USA) in citrate buffer (pH 4.0). Ethephon-treated seeds were germinated in a separate incubator at 25 °C to prevent ethylene exposure to control treatments.

2.5. Ethylene production in stratified seeds

Twenty seeds were placed in four replicated 25 mL Erlenmeyer flasks containing 1 mL of test solution, one piece of Whatman #1 Qualitative filter paper (Maidstone, England), and rubber stoppers or parafilm was placed over the tops of the flasks. A 1 mL gas sample was taken every 24 h for 6 days and flasks were vented between samples. Ethylene was measured using a Buck Scientific Model 910 gas chromatograph with a flame ionization detector (FID), using a Supelco Custom Column 121799 packed with 80/100 Alumina F-1. The oven temperature was 100 °C, the nitrogen flow rate was 1 mL per minute, and the detector temperature was 150 °C. The standard used was 100 μL/L ethylene in helium.

2.6. Ethylene inhibitor treatments

Non-stratified or seeds stratified for 60 days at 5 °C were transferred to four replicate Petri dishes with 25 seeds containing water, 2 mM silver thiosulfate (STS) (Sigma–Aldrich, St. Louis, MO, USA), or a 2-day treatment with 1 μL/L MCP (EthylBloc powder, Biotechnologies for Horticulture, Burr Ridge, IL, USA) mixed in a pH 5 buffer. For seeds treated with MCP, 25 seeds were placed into four replicate Petri dishes (60 mm × 15 mm) with two pieces of germination paper and 2 mL of deionized water. Open dishes were placed inside a 3.9 L glass jar along with a 1.5 mL microcentrifuge tube containing the MCP powder and buffer solution. The tube containing the MCP was capped, shaken, and inserted inside the jar; the tube was then quickly uncapped, and the jar lid screwed on. The dishes were kept in the jar for 2 days, and upon removal the seeds were transferred to Petri dishes with 6 mL of deionized water.

In a second experiment, seeds were treated with 2 mM STS before, during, and following 30-day moist chilling stratification. Four replications of 25 seeds were placed into Petri dishes containing deionized water or 2 mM STS and were stratified for 30 days. Following stratification, seeds were rinsed to remove residual STS and transferred to new Petri dishes containing water or 2 mM STS for germination.

In a third experiment, seeds of *E. tennesseensis* were treated with 1 mM aminoethyoxyvinyl-glycine (AVG) (ReTain, Valent Biosciences, Libertyville, IL, USA) with and without stratification

and ethylene production and germination recorded for 6 days and final germination (12-day) being recorded as well. Seeds of *E. purpurea* were also treated with 1 mM AVG with and without stratification, and germination was recorded after 3 and 12 days.

2.7. ABA sensitivity

For the experiment investigating the effect of stratification on (±) cis-trans abscisic acid (ABA) sensitivity for germination, seeds were stratified for 0 or 60 days then transferred to new Petri dishes with 0, 10, or 50 μM ABA. To evaluate the impact of ethylene on ABA sensitivity for germination, seeds were placed directly in Petri dishes containing water, 5 mM ACC, 10 μM ABA (Sigma–Aldrich, St. Louis, MO, USA), 50 μM ABA, 5 mM ACC + 10 μM ABA, or 5 mM ACC + 50 μM ABA. There were 25 seeds in four replicate Petri dishes and germination was recorded daily for 12 days.

2.8. Embryo isolation and membrane removal

Embryos were removed for the pericarp and seed coat following imbibition for 24 h. The outer coverings were cut with a scalpel being careful not to damage the inner membrane. Ten isolated embryos with or without the envelope were placed in four replicate Petri dishes containing water or 5 mM ACC. In a second experiment, 10 *E. tennesseensis* embryos with or without the membrane were placed in four replicate Petri dishes or 20 embryos in four replicated 25 mL Erlenmeyer flasks with water or 5 mM ACC for 1 or 3 days and germination and ethylene production evaluated for 3 days.

2.9. Seed morphology

For sectioned seeds, tissue samples were fixed in formalin–acetic–alcohol (FAA) and vacuum-infiltrated for 24 h. Tissue was rinsed twice with 50% ethyl alcohol and dehydrated using a tertiary butyl alcohol dehydration series. Tissue was then embedded in paraffin blocks, attached to a rotary microtome (model 820, American Optical, Buffalo, N.Y.) and sectioned at 12–15 μm. Tissue sections were affixed to glass slides (8 cm × 3 cm) and stained with safranin-fast green.

2.10. Statistical analysis

The design of each experiment was completely randomized. Germination percentages were converted to $\arcsin\sqrt{x^2}$ before being analyzed statistically. Treatment means were compared by Tukey's test using SAS software (SAS Institute, Cary, NC).

3. Results

3.1. Germination in stratified ACC- and ethephon-treated seeds

For *E. purpurea*, stratification did not increase final germination, but affected germination speed as indicated by increased germination after 3 days (Table 1). In *E. tennesseensis* and *E. simulata*, stratification increased early and late germination. ACC-treated seeds showed increased germination in a similar pattern observed for stratification, except in *E. purpurea* where early germination was not increased to the same level as stratification (Table 1). Ethephon was not effective in improving germination in *E. purpurea* (Table 1). In *E. tennesseensis* and *E. simulata*, ethephon enhanced germination over untreated seeds, but only in *E. tennesseensis* was the treatment similar to stratification or ACC-treatment (Table 1).

Table 1
Germination percentages (3 and 12 days after imbibition) in *Echinacea* seeds following stratification for 60 days at 5 °C or germinated on a substrate containing water, 5 mM ACC or 1 mM ethephon.

Treatment	*E. purpurea*		*E. tennesseensis*		*E. simulata*	
	3-day	12-day	3-day	12-day	3-day	12-day
Water	3c[z]	62a	2c	52b	2c	26b
Stratified	83a	84a	66ab	86a	59a	78a
ACC	57b	86a	59a	82a	63a	82a
Ethephon	4c	37b	41b	81a	38b	83a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

3.2. Ethylene production in stratified and ACC-treated seeds

Ethylene production in *E. purpurea* and *E. tennesseensis* increased slowly during germination with the only significant increase occurring on day six (Fig. 1). Stratified *E. purpurea* seeds showed increased ethylene production during germination but *E. tennesseensis* and *E. simulata* showed similar ethylene production in stratified and non-stratified seeds (Fig. 1, Table 2). Including ACC in the germination substrate greatly enhanced ethylene production in all *Echinacea* species and this was further increased in stratified seeds of *E. tennesseensis* and *E. simulata* (Table 2).

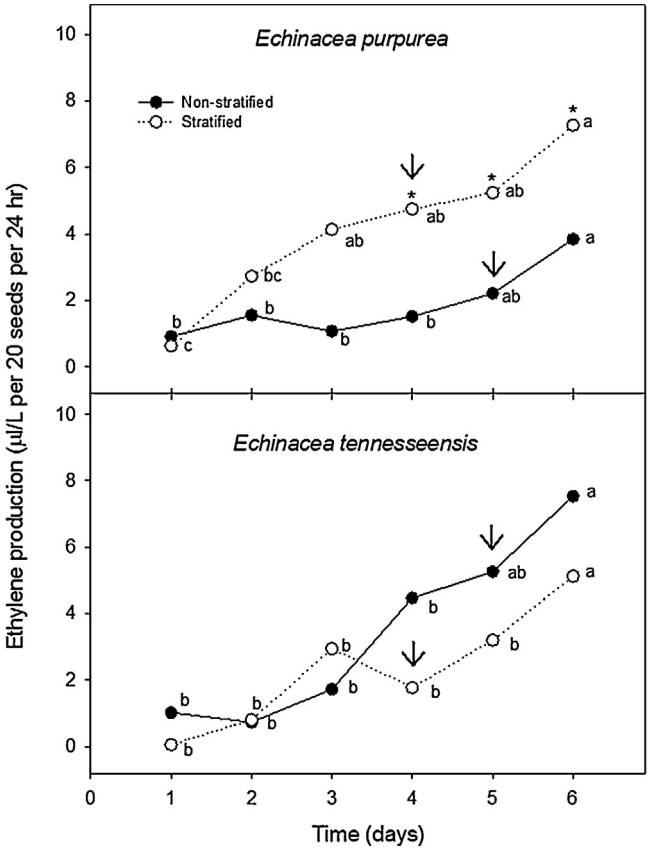

Fig. 1. Ethylene production (μL/L per 20 seeds per 24 h) in *Echinacea purpurea* and *E. tennesseensis* seeds with or without stratification for 30 days at 5 °C. Arrow indicates time to 50% germination. * Indicates a difference between stratified and non-stratified per day by single degree of freedom *F*-test ($P \leq 0.05$) and letters identify differences between days for either stratified or non-stratified seeds using 5% level by Tukey's HSD test.

Table 2
Ethylene production (μL/L per 20 seeds per 24 h) in *Echinacea* seeds with or without stratification for 60 days at 5 °C then germinated in the presence of water or 5 mM ACC.

Treatment		*E. purpurea*			*E. tennesseensis*			*E. simulata*		
Stratification time (days)	ACC	2-day	4-day	6-day	2-day	4-day	6-day	2-day	4-day	6-day
None	0	1.3b[z]	1.4b	2.3a	3.4c	5.6c	1.0c	1.7b	2.0b	2.7c
None	5	8.6b	44.3a	44.8a	13.2b	20.5b	18.1b	12.7b	85.7b	176.6b
60	0	7.0b	5.9b	8.6a	1.2c	8.6c	2.7c	5.7b	10.7b	7.2c
60	5	42.2a	38.6a	20.0a	116.8a	287.1a	307.2a	124.7a	347.1a	761.1a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

Table 3
Germination percentages (3 and 12 days after imbibition) in *Echinacea* seeds with or without stratification for 60 days at 5 °C then germinated in the presence of water or the ethylene inhibitors 2 mM STS or 1 μL/L MCP.

Treatment		*E. purpurea*		*E. tennesseensis*		*E. simulata*	
Stratification time (days)	Ethylene inhibitor	3-day	12-day	3-day	12-day	3-day	12-day
None	Untreated	5b[z]	57a	2c	47c	2c	26b
60	Untreated	45a	58a	66a	86a	59a	78a
None	STS	3b	58a	0c	57bc	1c	40b
60	STS	57a	72a	49a	77ab	58a	87a
None	MCP	1b	60a	0c	58bc	0c	21b
60	MCP	32a	57a	23b	76ab	23b	76a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

Table 4
Germination percentages (3 and 12 days after imbibition) in *Echinacea* seeds with or without stratification for 30 days at 5 °C in either water or 2 mM STS followed by germination on water or 2 mM STS.

Treatment		*E. purpurea*		*E. tennesseensis*		*E. simulata*	
Stratification substrate	Germination substrate	3-day	12-day	3-day	12-day	3-day	12-day
–	Water	13c[z]	79a	0d	53b	1c	22d
–	STS	11c	83a	0d	57b	1c	40c
Water	Water	50b	61a	35b	82a	59a	78b
Water	STS	62a	67a	16c	82a	57a	86a
STS	Water	68a	83a	51a	93a	57a	83a
STS	STS	61a	74a	38b	86a	53a	86a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

3.3. Germination in STS- and MCP-treated seeds

STS treatment had no effect on germination percentage in all *Echinacea* species regardless of stratification treatment (Table 3). MCP treatment did not affect final 12-day germination in any species, but there was a consistent reduction in 3-day germination in MCP-treated seeds of *E. tennesseensis* and *E. simulata* following stratification compared to the stratified control (Table 3).

3.4. Germination in STS-treated seeds prior to, during and after stratification

STS applied prior to, during or after stratification had no impact on *E. purpurea* germination (Table 4). For *E. tennesseensis*, STS did not affect final germination, but decreased germination speed (3-day germination) when it was included in the germination substrate. However, STS had no impact on 3-day germination when it was included during stratification (Table 4).

3.5. Impact of AVG on germination and ethylene production

In *E. tennesseensis*, AVG reduced early (3 and 6-day) germination in stratified seeds, but had no impact on final germination (Table 5). Ethylene production in *E. tennesseensis* seeds was reduced by exposure to AVG and seeds stratified in the presence of AVG and germinated on an AVG substrate had comparable ethylene levels to untreated controls (Table 5).

3.6. Sensitivity of stratified seeds to exogenous ABA

Final germination for non-stratified and stratified seeds was lower in all species at the higher ABA concentration (Fig. 2). ABA generally reduced germination speed in all species in both non-stratified and stratified seeds (Fig. 2). This effect was more pronounced at 50 μM compared to 10 μM ABA. Based on final germination and speed of germination, seeds that were stratified were no longer inhibited to the same extent as non-stratified seeds treated with the same ABA concentration suggesting that stratification reduced the sensitivity of seeds to ABA. This was most dramatic in seeds of *E. tennesseensis* where 50 μM ABA almost completely eliminates germination in non-stratified seeds (17%), while 50 μM ABA-treated stratified seeds germinated slowly but reaches a final germination percentage similar to seeds that received stratification alone (80%).

3.7. Sensitivity of ACC-treated seeds to ABA

ABA at 50 μM decreased final germination percentages in untreated (no ACC) seeds for *E. tennesseensis* and *E. simulata*, but not in ACC-treated seeds (Fig. 3). However, germination speed was reduced in ACC-treated seeds of both species at the higher ABA concentration compared to ACC alone or ACC plus 10 μM ABA. Including ACC in the medium reduced seeds sensitivity to ABA. As example, *E. tennesseensis* seeds treated only with 50 μM ABA germinated to 23%, while ACC-treated seeds exposed to 50 μM ABA germinated to 71%.

Table 5
Germination percentage (3, 6, and 12 days after imbibition) and ethylene production of *E. tennesseensis* seeds with or without stratification for 30 days at 5 °C in water or 1 mM AVG then transferred to water or 1 mM AVG.

Stratification substrate	Germination substrate	Germination percentage			Ethylene production (μL/L per 20 seeds per 24 h)	
		3-day	6-day	12-day	3-day	6-day
–	Water	0c[z]	12.5c	55.8c	6.5ab	9.0c
–	AVG	0c	10.8c	53.3c	2.3b	6.7c
Water	Water	45.8a	86.7a	86.7a	9.3a	31.1a
Water	AVG	40.8a	81.7a	81.7a	4.9b	17.7b
AVG	AVG	15.8b	68.3b	75.8a	2.4b	11.1c

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

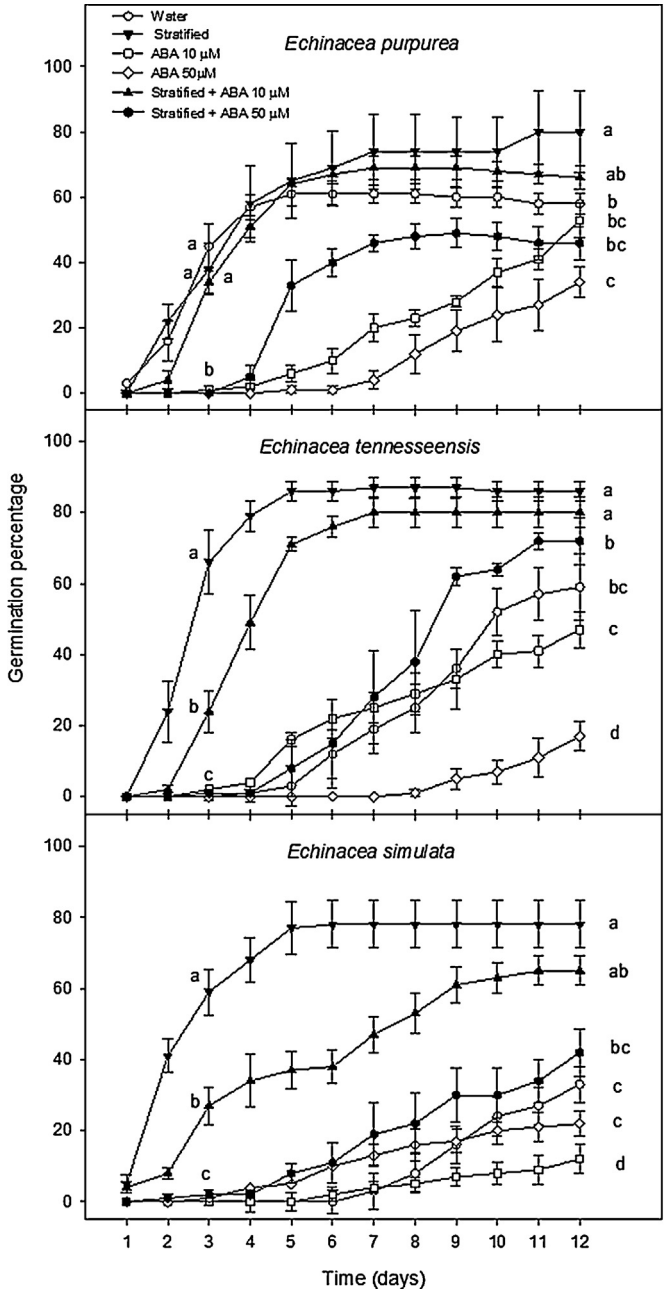

Fig. 2. Germination percentage in *Echinacea* seeds with or without stratification for 60 days at 5 °C and germinated with or without ABA in the substrate. Bars represent standard errors and final and 3-day means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

3.8. Seed (achene) morphology in E. tennesseensis

As in other members of the Asteraceae, the embryo sac in *Echinacea* develops from a tenuinucellate nucellus of just a few cell layers that degenerates early in development (Misra, 1965). Subsequently the ovule develops within a substantial unitegmic integument (Fig. 4). Early in embryo sac development, the inner layer of the integument divides anticlinally to form an outer layer of endothelium cells (Fig. 5) referred to as the integumentary tapetum (Kapil and Tiwari, 1978). These cells continue to expand as the ovule grows within the ovary. At the completion of seed development, the cell layers enclosing the embryo consist of endosperm, endothelium, crushed integument, and the seed coat (Fig. 6). The endosperm consists of two layers of cells covering the majority of the seed except for the multilayered micropylar endosperm

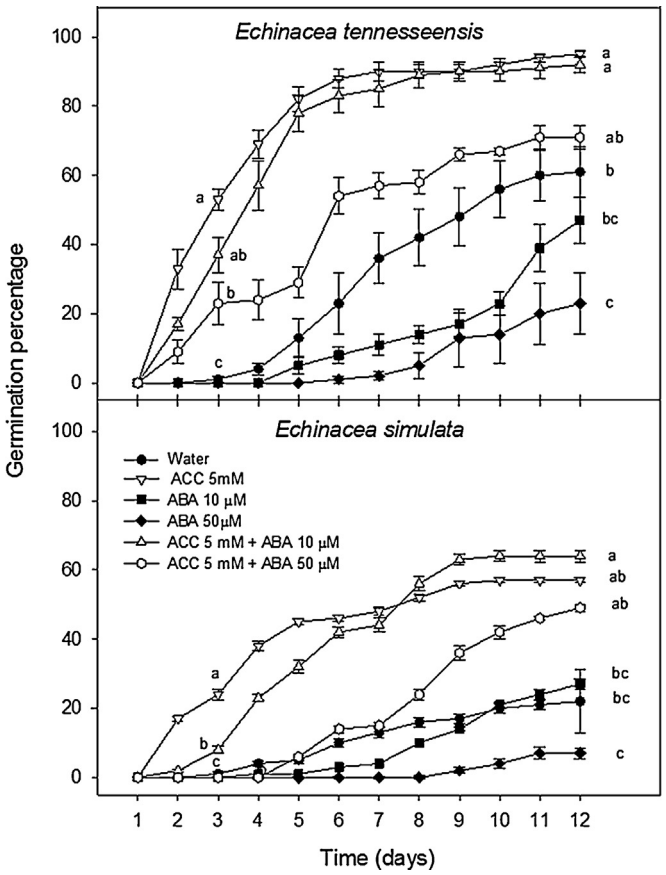

Fig. 3. Germination percentage in *Echinacea tennesseensis* and *E. simulata* seeds germinated with or without ACC and ABA in the substrate. Bars represent standard errors and final and 3-day means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

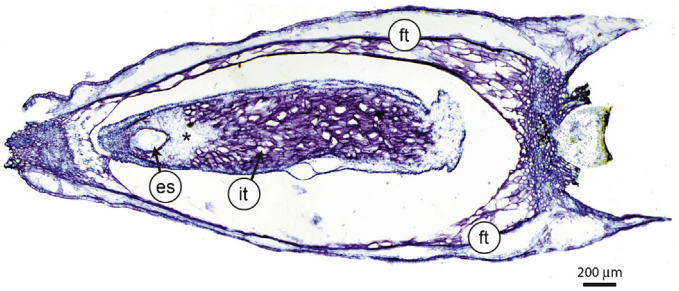

Fig. 4. Morphology of an *E. tennesseensis* achene following early egg sac development.

surrounding the radicle tip. In mature seeds, the endothelium and crushed integument form a distinct outer layer (envelope) that lies between the endosperm and seed coat and completely surrounds the embryo (Fig. 7).

3.9. Embryo isolation and envelope removal

Removal of the envelope from isolated embryos releases all embryos from dormancy (Table 6). With the envelope removed, all species showed embryo germination to 100% after 6 days compared to 98, 55, and 65% in embryos with the envelope intact for *E. purpurea*, *E. tennesseensis*, and *E. simulata*, respectively. There were also dramatic differences in germination after 3 days related to whether the envelope was removed or left intact. Similarly, embryos with the envelope intact treated with ACC resulted in nearly 100% germination for all species as well as an increase in germination speed (Table 6). Embryos with the envelope removed treated with an ethylene inhibitor (AVG) germinated at 100% (data not shown).

Ethylene evolution showed a similar pattern for intact seeds and isolated embryos with or without the envelope removed with ethylene production increasing with ACC treatment compared to untreated seeds or embryos (Table 7). Only a single day of ACC treatment was sufficient to increase germination in intact seeds and

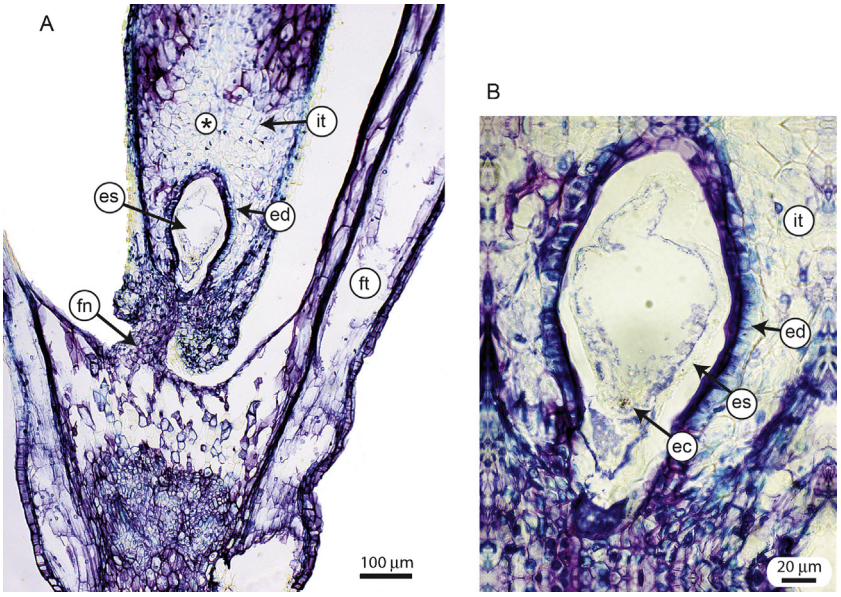

Fig. 5. (A) Morphology of the micropylar portion of a developing seed in *E. tennesseensis* during early egg sac development. (B) Developing embryo sac surrounded by a single-celled endothelium layer derived from the inner surface of the integument.

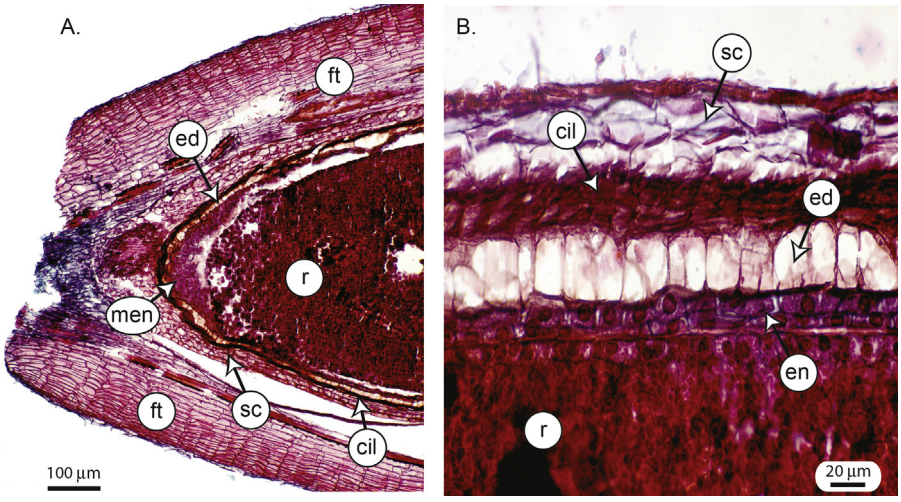

Fig. 6. (A) Morphology of the radicle portion of the achene just prior to maturation drying in *E. tennesseensis*. (B) Close up of the outer cell layers of the seed.

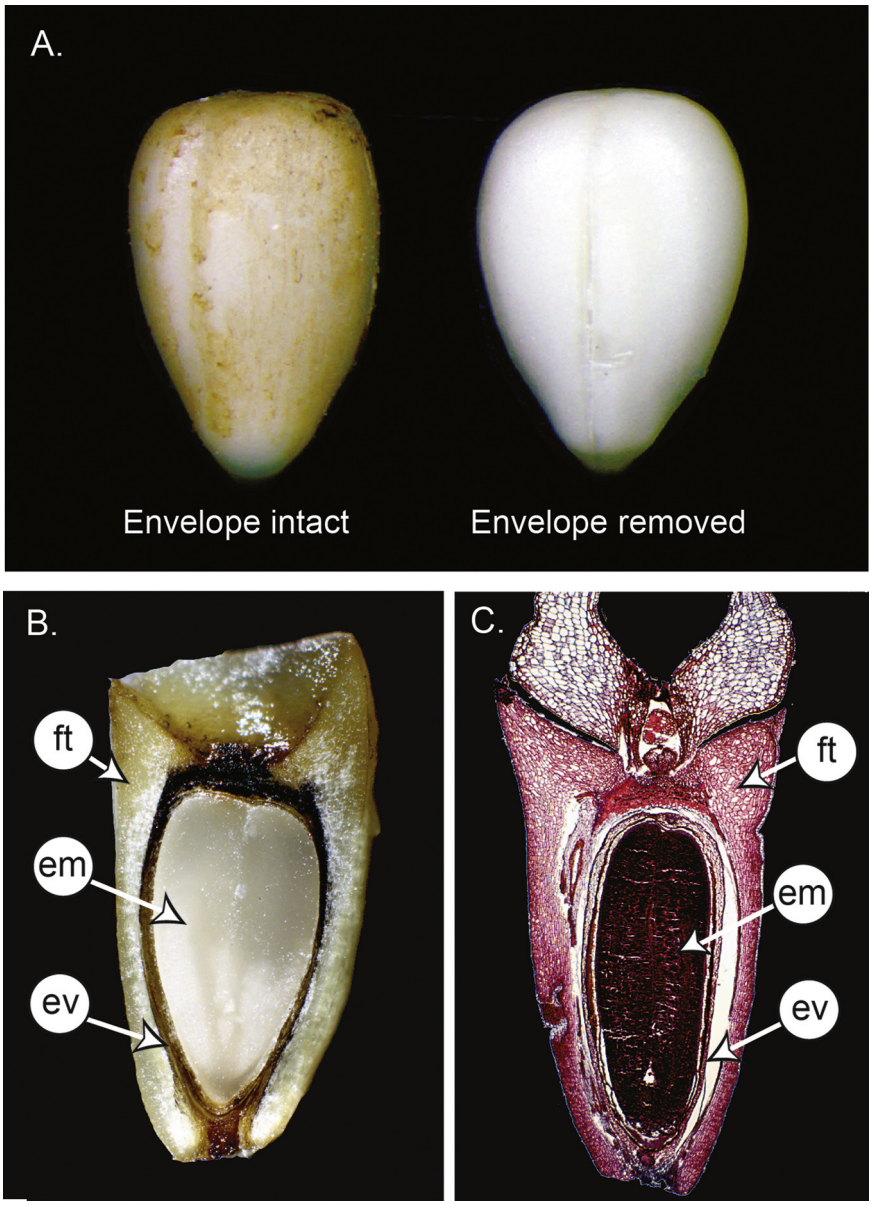

Fig. 7. Seed morphology in *E. tennesseensis*. (A) Embryo isolated from the pericarp and adhering seed coat showing the surrounding envelope in an imbibed seed. (B and C) Hand section and photomicrograph, respectively of a dry seed showing the morphology key parts of the seed (achene).

isolated embryos. Ethylene evolution was not different in embryos with or without the outer envelope when germinated in the presence of ACC for 3 days (Table 7).

4. Discussion

Echinacea species have seeds that display endogenous physiological dormancy, and moist chilling stratification can release seeds from dormancy (Tables 1 and 3). Seeds treated with ACC show a substantial increase in ethylene production (Table 2) and seeds treated with ACC or ethephon show that ethylene treatment can generally substitute for stratification to relieve dormancy (Table 1; Kochankov et al., 1998; Sari et al., 2001; Qu et al., 2004). *Echinacea* seeds produce ethylene during germination, but its levels are small and do not increase significantly until day 6 where over 50% of seeds have already germinated (Fig. 1). This is in contrast to many other

Table 6
Germination percentages (3 and 6 days after imbibition) in *Echinacea* embryos that were isolated from the pericarp with or without the outer envelope removed and treated with 1 mM ACC.

Treatment		*E. purpurea*		*E. tennesseensis*		*E. simulata*	
Envelope condition	Germination substrate	3-day	6-day	3-day	6-day	3-day	6-day
With envelope	Water	78b[z]	98a	20c	55b	15b	65b
With envelope	ACC	93a	100a	60b	93a	93a	93a
Envelope removed	Water	100a	100a	100a	100a	93a	100a
Envelope removed	ACC	100	100a	100a	100a	100a	100a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

Table 7
Germination percentage (3 days after imbibition) and ethylene production in *Echinacea tennesseensis* embryos that were isolated from the pericarp with or without the outer envelope removed and treated with 1 mM ACC.

Treatment		3-days after imbibition	
Membrane condition	Germination substrate	Germination percentage	Ethylene production (μL/L per 20 embryos per 24 h)
Intact seed	Water	5d[z]	2.4d
	ACC 1-day	65b	26.5c
	ACC 3-days	75b	70.2b
Isolated with envelope intact	Water	20c	3.7d
	ACC 1-day	70b	82.3b
	ACC 3-days	85ab	171.5a
Isolated with envelope removed	Water	85ab	4.8d
	ACC 1-day	90a	220.0a
	ACC 3-days	95a	235.2a

[z] Means followed by the same letter in a column were not different at the 5% level by Tukey's HSD test.

species where it is commonly observed that ethylene production increases with or just after radicle emergence, peaks soon after, and then declines (Matilla, 2000).

For *E. tennesseensis* and *E. simulata*, seeds receiving moist chilling stratification showed no increase in ethylene production compared to non-stratified seeds during germination (Table 2, Fig. 1). For *E. purpurea*, which showed less dormancy compared to *E. tennesseensis* and *E. simulata* (Table 1), there was a significant increase in ethylene production following stratification starting between 3 and 4 days after imbibition (Fig. 1). Following stratification, all species showed a dramatic increase in ethylene production when germinated in the presence of ACC (Table 2). This may be explained by a change in the permeability of seeds to ACC following stratification or in an increased production of ACC-oxidase, which would increase the seed's capacity to convert ACC to ethylene. Germination of isolated embryos of *E. tennesseensis* with or without its covering envelope suggests that the pericarp and envelope could slow ACC uptake, but seeds appear to be permeable to ACC (Table 7). Additionally, because stratified seeds germinate earlier and at higher percentages, radicle tissue could be converting additional ACC to ethylene resulting in higher ethylene production. Beechnut (*Fagus sylvatica* L.) is similar to *Echinacea* in that beechnuts have a hard pericarp covering the seed and are released from dormancy by either stratification or ethylene treatment (Calvo et al., 2004). In beechnuts, stratification causes a significant increase in both ethylene production and up-regulation in ACC-oxidase genes. It is therefore, plausible that ACC-oxidase could be up-regulated in *Echinacea* seeds causing the substantial increase in ACC-induced ethylene production in stratified seeds (Table 2) and suggests that stratified seeds not exposed to exogenous ACC fail to produce large amounts of ethylene because of limited endogenous ACC. One possible explanation for an increase in ACC-oxidase in *Echinacea* seeds following stratification not related to dormancy release could be as a mechanism allowing for a rapid rise in ethylene production as a defense response to potential biotic predation (Anderson et al., 2004). Ethylene can initiate a response cascade to stress and it was shown that wounding red rice (*Oryza sativa* f. *spontanea*) pericarps induced ethylene production that was not related to dormancy release (Gianinetti et al., 2007).

The small amounts of ethylene produced during germination and the lack of a substantial increase in ethylene production following dormancy release by stratification suggest that ethylene-induced dormancy release may not have physiological significance. For ethylene-sensitive species like thermodormant lettuce (Nascimento et al., 2004) and beechnut (Calvo et al., 2004), ethylene synthesis and action inhibitors were used to support a causal relationship for ethylene during dormancy release. For *Echinacea*, blocking ethylene production using AVG and ethylene perception using silver and MCP suggest that ethylene action was not required for germination or dormancy release via stratification (Tables 3–5). This held true for seeds treated during or following stratification. It is possible that a lack of response could be from limited inhibitor uptake, but ethylene production in AVG-treated *E. tennesseensis* seeds showed reduced ethylene production to or below untreated, dormant seeds without any impact on dormancy release (Table 5). These inhibitor studies indicate that ethylene may not play a major direct role in *Echinacea* dormancy release and that ethylene-induced dormancy release may operate through a stratification independent pathway or could induce downstream events that normally follow stratification.

Dormancy in *Echinacea* seeds seems to fit the proposed model for hormone interactions related to dormancy release in seeds (Finkelstein et al., 2008). The major hormones controlling seed dormancy maintenance and release are ABA and gibberellin (GA). ABA controls the establishment and maintenance for dormancy, while GA appears to control initiation and completion of germination. The ratio of ABA to GA induced signal transduction is as important as the active hormone levels for dormancy release (Finch-Savage and Leubner-Metzger, 2006; Kucera et al., 2005). Other hormones, especially ethylene, have a modifying impact on this relationship. In *Echinacea*, ABA inhibits germination (Fig. 2), GA promotes germination (Pill and Haynes, 1996; Kochankov et al., 1998) and ethylene promotes germination (Table 1; Kochankov et al., 1998; Sari et al., 2001; Qu et al., 2004). However, ethylene inhibitor studies from the current study bring into question the importance for ethylene action in natural dormancy release.

It has become well established that there is an antagonistic relationship between ABA and ethylene for a number of plant responses including seed dormancy and germination (Beaudoin et al., 2000; Ghassemian et al., 2000). Therefore, one common mechanism for stratification and ethylene-induced dormancy release in *Echinacea* could be through a change in ABA sensitivity. In all three *Echinacea* species, the negative effect of ABA on germination was reduced by stratification (Fig. 2) and likewise for *E. tennesseensis* and *E. simulata*, ACC-treated seeds showed less sensitivity to ABA (Fig. 3). These effects were seen for both germination speed and final germination percentages. These data suggest that both stratification and ethylene impact ABA control of dormancy independently in that the reduction in ABA sensitivity caused by stratification does not require ethylene production (Tables 3–5) and that the levels of ethylene required for significant dormancy release are extremely high relative to stratification-induced ethylene production (Table 2). Although not included in this study, ethylene has been shown to impact DELLA protein function (Achard et al., 2003). DELLA proteins are key negative regulators of GA effects during seed germination and therefore, ethylene could be acting on *Echinacea* seeds to reduce ABA effects while enhancing GA effects on germination.

Seed priming has been reported to be as effective as moist chilling stratification in promoting seed germination in various *Echinacea* species (Samfield et al., 1990; Wartidiningsih et al., 1994; Wees, 2004). Priming and stratification are similar as a pre-hydration treatment prior to germination but priming is performed at a higher temperature than is effective for chilling stratification (Geneve, 2003). Therefore, it is possible that priming may be operating on downstream events normally induced by stratification in a similar fashion to ethylene-induced dormancy release.

Seed coverings especially those tissues surrounding the radicle can be critical to maintaining seed dormancy (Finch-Savage and Leubner-Metzger, 2006). The seeds (achenes) of members of the Asteraceae have a covering pericarp with an inner layer of endosperm that is made up of 2–3 cells for the majority of the seed except in the area covering the radicle where there are multiple endospermic cell layers (Fig. 6). The pericarp offers very little resistance to germination as it is often open over the radicle end of the seed. For *Echinacea* seeds, removal of the pericarp and adhering seed coat does not impact germination (Table 7), but removal of the true seed tissue covering the embryo completely eliminates dormancy (Tables 6 and 7). In *Echinacea*, this covering is composed of endosperm, endothelium and crushed integument cell layers (Figs. 6 and 7). The micropylar endosperm cap surrounding the radicle is implicated in dormancy maintenance in a number of species where endosperm weakening is a prerequisite for germination (Finch-Savage and Leubner-Metzger, 2006).

Thermodormant lettuce seeds show changes in the micropylar endosperm cap that is correlated with dormancy release (Sung et al., 2008). This involved physical changes in the endosperm cells as well as changes in the protein bodies and vacuole size. Also, ethylene-induced thermodormancy release in lettuce seeds is associated with cell wall loosening enzymes (i.e. endo-β-mannanase) induction (Nascimento et al., 2004; Matilla and Matilla-Vázquez, 2008). In *Echinacea*, isolated embryos with an intact covering envelope were released from dormancy when treated with ACC (Tables 6 and 7). It is possible that ethylene is similarly inducing changes in the micropylar endosperm to weaken the tissue covering the radicle permitting germination in *Echinacea* seeds. Alternatively, ethylene has been shown to enhance seed vigor (Siriwitayawan et al., 2003) and may have a direct effect on embryo growth potential allowing the radicle to penetrate the seed coverings leading to germination without any changes in the surrounding covering tissue. Interestingly, Sung et al. (2008) showed that the properties of the micropylar endosperm in lettuce differed depending on the seed development environment. Lettuce seeds matured at higher temperatures produced cell walls in the micropylar endosperm that were more readily separated during high temperature germination compared to seeds matured at cooler temperatures. *Echinacea* seed germination and depth of dormancy can vary significantly within a single genus and even within differing seed lots of the same cultivar (Wartidiningsih et al., 1994). It is possible that the development environment for *Echinacea* seeds differentially impact the properties of the micropylar coverings possibly explaining the observed differences between seed lot germination and dormancy.

It was not possible to separate the endothelium and crushed integument cell layers from the endosperm when removing it from the embryo in *Echinacea*. Therefore, it is not currently possible to conclude whether the micropylar covering materials impose a physical restraint due to endosperm cells as previously discussed or whether the entire enclosing envelope might be maintaining dormancy due to its physical or permeability properties. There are some members of the cucumber family (Cucurbitaceae) that have a perisperm envelope that surrounds the embryo that is similar in appearance to the *Echinacea* seed envelope (Welbaum et al., 1995). This envelope shows reduced and selected permeability to ions due to lipid and callose content in the envelope (Welbaum et al., 1995; Ramakrishna and Amritphale, 2005). In dormant or non-dormant seeds germinated at low temperature, removal of embryos from the seed coat and perisperm-endosperm envelop is sufficient to permit germination (Edelstein et al., 1995). Prior to radicle emergence, cell wall enzymes work to make the envelope more ion permeable and to weaken the envelope around the radicle tip (Ramakrishna and Amritphale, 2005).

The enveloping seed coverings in *Echinacea* cannot be derived from nucellar tissue due to the limited amount and rapid depletion of tenuinucellate nucellar tissue characteristic to members of the Asteraceae (Kapil and Tiwari, 1978). Rather, it is derived from the endothelium cells that surround and grow with the developing embryo sac (Figs. 5 and 6). The endothelium cells act to digest adjacent integumentary tissue and transfer the nutrition into the embryo sac (Figs. 4 and 5; Kapil and Tiwari, 1978). The portion of the endothelium cells adjacent to the embryo sac outer wall have been shown to become thick walled and cutinized to exclude digestive enzymes from contacting the embryo sac (Kapil and Tiwari, 1978; Misra, 1965). A separate layer of crushed integument cells between the endothelium and seed coat has also been observed in other members of the Asteraceae (Misra, 1972). It is plausible that these tissue layers present a physical barrier or limit solute movement across the enclosing envelope that participate in dormancy maintenance in *Echinacea* in a similar way to the covering envelope in the Cucurbitaceae.

In conclusion, it does not appear that dormancy release via moist chilling stratification requires an intermediate step involving ethylene. Rather, ethylene-stimulated dormancy release appears to be working independent of stratification. A model for dormancy maintenance and release based on conclusions drawn from the current paper and inferences made from studies involving members of the Asteraceae (like lettuce and sunflower) suggests that the physical restraint exerted on the radicle by the micropylar cap is critical for maintaining dormancy and that dormancy breaking treatments act by weakening the cap or by inducing changes in the embryo that allow the radicle to generate sufficient force to penetrate the cap. It is suggested that reducing endogenous ABA titers or sensitivity to ABA are major events that precede dormancy release. Additionally, *Echinacea* seeds have a distinct enclosing envelope that could work in conjunction with micropylar endosperm to physically limit germination or to minimize efflux of solutes (i.e. ABA) from the seeds following imbibition in dormant seeds.

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