2013

# PHYSICAL DORMANCY IN SEEDS, WITH SPECIAL REFERENCE TO GERANIACEAE: MORPHO-ANATOMY, DEVELOPMENT, PHYSIOLOGY, BIOMECHANICS AND CLASSIFICATION OF WATER-GAP COMPLEXES

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# PHYSICAL DORMANCY IN SEEDS, WITH SPECIAL REFERENCE TO GERANIACEAE: MORPHO-ANATOMY, DEVELOPMENT, PHYSIOLOGY, BIOMECHANICS AND CLASSIFICATION OF WATER-GAP COMPLEXES

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DISSERTATION

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A dissertation submitted in partial fulfilment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences
at the University of Kentucky

By

Nalin Suranjith Gama Arachchige

Lexington, Kentucky

Director: Dr. Carol C. Baskin, Professor of Biology
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Lexington, Kentucky

2013

ABSTRACT OF DISSERTATION

# PHYSICAL DORMANCY IN SEEDS, WITH SPECIAL REFERENCE TO GERANIACEAE: MORPHO-ANATOMY, DEVELOPMENT, PHYSIOLOGY, BIOMECHANICS AND CLASSIFICATION OF WATER-GAP COMPLEXES

The primary aims of this dissertation were to (1) identify and characterize the water-gap complex in seeds of Geraniaceae, (2) investigate its role in physical dormancy (PY) break and (3) develop a new classification system for water-gap complexes in seeds of angiosperms. The winter annuals *Geranium carolinianum* and *G. dissectum* were selected as the main representative species for the study, and seeds of an additional 29 species from the Geraniaceae were used to compare the water-gap complex within the family. A new classification system for water-gap complexes in species with PY was developed by comparing the morpho-anatomical features of PY seeds and fruits of 16 families.

The water-gap complex of *G. carolinianum* was identified as a micropyle-hinged valve gap complex, and only a slight morpho-anatomical variation was observed within the family. Ontogenetic studies of the seed coat of *G. carolinianum* revealed that the water-gap region of Geraniaceae develops as an entity of the micropyle. The timing of seed germination with the onset of autumn can be explained by PY-breaking processes involving (a) two-temperature-dependent steps in *G. carolinianum,* and (b) one or two moisture-dependent step(s) along with the inability to germinate under high temperatures in *G. dissectum*. Step-I and step-II in PY-breaking of *G. carolinianum* are controlled by chemical and physical processes, respectively. This study indicates the feasibility of applying the developed thermal time model to predict or manipulate sensitivity induction in seeds with two-step PY-breaking processes. The model is the first and the most detailed one yet developed for sensitivity induction in PY-break. Based on the morpho-anatomical features, three basic water-gap complexes (types I, II and III) were identified in species with PY in 16 families. Depending on the number of openings involved in initial imbibition, the water-gap complexes were subdivided into simple and compound. The new classification system enables the understanding of relationships between water-gap complexes of taxonomically unrelated species with PY.

KEY WORDS, Geraniaceae, physical dormancy, thermal time, two-step model, water-gap complex

Nalin Gama Arachchige

April 10, 2013

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my major co-advisors Dr. Carol C Baskin and Dr. Jerry M Baskin for accepting me in their lab and providing me with inspiration, guidance and encouragement throughout my graduate studies at Lexington. I owe them for moulding my career, and being my guiding stars in the new path I took in Science. I am also grateful to Dr. Robert L Geneve, Dr. Denis B Egli. Dr. Scott Gleeson and Dr. Nicholas McLetchie for cordially serving in my PhD committee. I am particularly indebted to Dr. Robert L Geneve for his guidance with my seed anatomical studies, his valuable suggestions and allowing me to work in his laboratory. Also I would like to thank Dr. Bruce A Downie for serving as my external examiner in my PhD defence and for his valuable suggestions.

I very much grateful to Dr. Sharyn E. Perry for allowing me to use the microtome and tissue-sectioning equipment in her laboratory; Ms. Sharon T. Kester, for helping with sectioning and staining of tissues; Dr. Bruce A Downie for allowing me to use incubators in his laboratory and Mr. Larry Rice for the assistance with scanning electron microscopy.

I am extremely thankful to the following people and institutions for providing me seeds/fruits for my study. Mr. Alvin Yoshinaga, Dr. Ching-Te Chein, Dr. John Dickie, Dr. Robert Price, Dr. Elena Copete, Dr. Kun Liu, Dr. David Meritt, Dr. Cynthia S Jones, Dr. Mariam A Sahun, Dr. Chris Blazier, Dr. Ian Gillespie, Dr. Tracey Ruhlman, Dr. Doug Walker, Dr. Karen Sommerville, Dr. Richard Johnstone,

Dr. Peter Cuneo, Ms. Qinying Lan, Instituto de Botánica Darwinion in San Isidro, Argentina, Millenium Seed Bank Project, Royal Botanical Gardens, UK and Royal Botanic Gardens Sydney, Australia.

I would like to thank the visiting scholars and fellow graduate students, Dr. Elena Copete, Dr. Kun Liu, Dr. JJ Lu, Ms. Luan Zhihui, Ms. Jan Fry and Mr. James Shaffer for helping me with various tasks and being good friends.

I also want to offer my sincere thanks to Ms. Beverly Taulbee and other staff members of the Department of Biology for taking care of my administrative paperwork and their friendship during my stay in Lexington.

Finally, I would like to express my deep gratitude to my family and my wife (Lakshmie Rangama) for their support, encouragement, and unconditional love over the years.

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# CHAPTER 1

## Background and significance

## INTRODUCTION

### Germination-units and germination

Depending on the species, seeds or fruits (sometimes with accessory parts) can act as germination-units (Baskin and Baskin, 1998). Germination commences with the uptake of water by a quiescent germination-unit and terminates with the elongation of the embryonic axis, usually the penetration of radicle through the germination-unit (Bewley and Black, 1994; Bewley, 1997; Baskin and Baskin, 1998).

### The five-classes of seed dormancy

A dormant seed (or any other germination unit) is one that does not have the capacity to germinate within a specified period of time under any combination of normal physical environmental factors that otherwise is favourable for its germination, i.e. after the seed becomes nondormant (Baskin and Baskin, 1998, 2004).

Based on the seed dormancy classification scheme by Nikolaeva (1967[1969], 1977), Baskin and Baskin (1998, 2004, 2008) have proposed a detailed classification system for seed dormancy. Their classification system includes the five classes of dormancy: physiological dormancy (PD), morphological dormancy (MD), physical dormancy (PY), morphophysiological dormancy (MPD) and combinational dormancy (CD). Moreover, they have further subdivided dormancy classes into levels and types where appropriate (see table 1, Baskin and Baskin (2004).

Physiological dormancy (PD) is caused by low growth potential of embryos (Baskin and Baskin, 2004). The balance between the levels of Abscisic acid (ABA) and Gibberellic acid (GA) and their signaling pathways are important in induction, maintenance and release of PD. ABA acts as a positive regulator for PD induction and inhibits seed germination. Gibberellic acid (GA) counteracts inhibitory effects of ABA, releases seed dormancy and promotes seed germination (Kucera *et al*., 2005; Finklestein *et al*., 2008; Nambra *et al*., 2010; Graeber *et al*., 2012). Moreover, PD is influenced by several other factors such as ethylene, brassinosteroids, dormancy-specific genes, chromatin factors and non-enzymatic processes (Finklestein *et al*., 2008; Graeber *et al*., 2012).

Morphological dormancy (MD) occurs in species with small underdeveloped or undifferentiated embryos (without PD). MD is the delay of germination due to the requirement for a period of embryo growth within the seed after the mature seed has been dispersed (Baskin and Baskin, 1998). Baskin and Baskin proposed that a fresh seed has MD if the underdeveloped or undifferentiated embryo germinates within about 30 days at simulated autumn, spring or summer habitat temperatures (Baskin and Baskin, 2004).

Morphophysiological dormancy (MPD) occurs in seeds with small underdeveloped or undifferentiated embryos that also have PD (Baskin and Baskin, 1998). Unlike seeds with MD, seeds with MPD require more than 30 days to germinate under favourable conditions (Baskin and Baskin, 2004).

Physical dormancy (PY) is caused by a water impermeable palisade layer(s) in seed or fruit coats (Baskin and Baskin, 1998). This dormancy class will be discussed in detail below.

Combinational dormancy (CD or PY+PD) in seeds or fruits is caused by a water impermeable palisade layer(s), and additionally the embryo has some PD. Depending on the species, the loss of the PD component occurs before or after the loss of PY (Baskin and Baskin, 1998).

**Physical dormancy (PY) and its occurrence in the plant kingdom**

Physical dormancy (PY) can be defined as the incapability of a seed or fruit that has undergone maturation drying to imbibe water within a specified period of time due to a water impermeable palisade cell layer(s) in their seed or fruit coat, thus preventing germination.

PY is caused by a water-impermeable palisade cell layer(s) in seeds or fruit coats (Baskin *et al*., 2000) along with tightly sealed chalaza and micropyle openings (Gama-Arachchige *et al*., 2010). Seeds of some species with PY also have physiological dormancy (PD); hence, they are considered to have combinational dormancy (CD). Maturation drying i.e. loss of moisture during the final stages of seed or fruit development due to abscission of the maternal vascular supply, plays a major role in acquisition of PY (see table 2, Qu *et al*., 2010). During this stage, the moisture content of seeds/fruit decreases rapidly and once it reaches a certain level that varies from aprox. 5 to 20% among species, the seed or fruit can become water impermeable (Li *et al*., 1999*a*; Jayasuriya *et al*., 2007*b*; Qu *et al*., 2010).

## A special case of PY

Apart from seeds and fruits with PY, a special type of PY occurs in sporocarps of the aquatic fern, *Marsilea* (Bilderback, 1978). The hypodermal layer of the sporocarp of *Marsilea* differentiates into a palisade layer during development of the sporocarp, and after maturation drying this palisade layer becomes impermeable (Bilderback, 1978). Unlike seeds or fruits with PY, the sporocarp structures enclose megaspores and microspores (Nagalingum *et al*., 2006). However, similar to seeds and fruits, sporocarps are involved in reproduction.

## Phylogenetic distribution of PY and CD in the plant kingdom

PY and CD have been demonstrated or inferred to occur in species of 18 angiosperm plant families (Anacardiaceae, Biebersteiniaceae, Bixaceae, Cannaceae, Cistaceae, Convolvulaceae, Cucurbitaceae, Dipterocarpaceae, Fabaceae, Geraniaceae, Lauraceae, Malvaceae, Nelumbonaceae, Rhamnaceae, Sapindaceae, Sarcolaenaceae, Sphaerosepalaceae and Surianaceae) in 10 orders and are unknown in gymnosperms (Nandi, 1998; Baskin 2003; Horn, 2004; Baskin *et al*., 2000, 2006; Koutsovoulou *et al*., 2005; Angiosperm Phylogeny Group [APG III], 2009; Tsang, 2010) (Fig. 1.1). However, as discussed in the preceding paragraph PY also occurs in sporocarps of the fern family Marsileaceae (Bilderback, 1978). Another remarkable character regarding PY in the plant kingdom is that 14 of the 18 families with PY are in the eudicot fabid and malvid clades (Fig. 1.1).

## Water-gap region and PY-break

The breaking of PY involves disruption or dislodgement of 'water-gap' structures, which act as environmental 'signal detectors' for germination (Baskin *et al*., 2000). Therefore, the ability of water-gap structures to sense environmental conditions allows seeds with PY to become permeable under conditions favourable for germination and plant establishment (Taylor, 1996*a*, *b*; Jayasuriya *et al*., 2008*a*, 2009*c*). The water-gap region is a morpho-anatomically specialized area that differs from the rest of the seed or fruit coat. Location, anatomy, morphology and origin of water-gap regions can differ between and even within families (Baskin *et al*., 2000, Jayasuriya *et a*l., 2009*b*). Eleven different water-gap types have been characterized previously in six (excluding Geraniaceae) of the 18 angiosperm families with PY (Baskin and Baskin, 1998; Baskin *et al*., 2000; Baskin, 2003; Jayasuriya *et al*., 2007*a*, 2008*b*; Hu *et al*., 2008; Turner *et al*., 2009). The occurrence of more than one water-gap type is reported only in Convolvulaceae, Fabaceae and Malvaceae and it is possible that other families with PY may also have several water-gap types. However, water-gaps previously have not been characterized in Biebersteiniaceae, Cucubitaceae, Fabaceae (clade Cladrastis), Lauraceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae.

In the literature, the term 'water-gap' is interchangeably used to define both the opening formed during the PY-break and the whole specialized region of the seed or fruit coat (Jayasuriya *et al*., 2007*a*; Turner *et al*., 2009; Karaki *et al*., 2011; De Paula *et al*., 2012). However, to date no attempts have been made to define the different structures involved in early imbibition or to classify similar water-gap regions to study the relationship among unrelated taxa.

**Two step PY breaking process**

Taylor (1981) presented a temperature-dependent two-stage conceptual model for breaking of PY. In the first or preconditioning stage, the seeds are made sensitive to the second or PY-breaking stage (Taylor, 2005). This two-stage model is known to occur in seeds of several annual species of Fabaceae (Taylor, 1981; Taylor, 1996*a*, *b*; Taylor and Revell, 1999; Van Assche *et al*., 2003) and Convolvulaceae (Jayasuriya *et al*., 2008*a*, *b*; 2009c). The model consists of two distinct temperature and (or) moisture-dependent processes (Taylor, 2005; Van Assche and Vandelook, 2006; Jayasuriya *et al*., 2009*b*). However, the two-step process of PY-break is unknown in other families with PY or CD. Involvement of two stages in breaking of PY can be used to explain the PY-breaking behaviour and timing of germination under natural conditions (Taylor, 2005).

**Thermal time model for PY-break**

The concept of thermal time, i.e. exposure to a temperature above a threshold level for a particular time period, has been successfully applied in determining and comparing the rates of various physiological events in plants and poikilothermic invertebrates (Trudgill *et al*., 2005). This concept has been used in describing and quantifying physiological dormancy (PD)-break by after-ripening (Bradford, 2002; Batlla *et al*., 2009) and single step PY-break (McDonald, 2000). However, it has not been used to explain the stepwise PY-breaking processes.

**PY in Geraniaceae**

The family Geraniaceae belongs to the order Geraniales in the clade malvid (Fig. 1.1). The family is composed of the six genera *viz. California* (1 species), *Erodium* (70), *Geranium* (~420), *Hypseocharis, Monsonia* (39) and *Pelargonium* (~280) (Aedo *et al*, 1998b; Bakker *et al*., 2004; Fiz *et al*., 2008). *Geranium* consists of three subgenera (Erodoidea, Geranium and Robertum) (Aedo *et al*., 1998b) and *Erodium* of two subgenera (Erodium and Barbata) (Fiz *et al*., 2006). There are three clades (A, B and C) in *Pelargonium* (Bakker *et al*., 2004; Jones *et al*., 2009) and two sections (Monsonia and Olopetalum) in *Monsonia* (Aldasoro *et al*., 2001; Touloumenidou *et al*., 2007).

PY has been shown to occur in *California*, *Erodium, Geranium* and *Pelargonium* (Baskin and Baskin, 1974; Meisert, 2002; Gillespie and Andersen, 2005). Based on anatomical studies of the seed coat of *Hypseocharis remy* (Boesewinkel, 1988) and *Monsonia senagalensis* (Narayana and Arora, 1963), it can be inferred that PY also occurs in *Hypseocharis* and *Monsonia*. Thus, it appears that all six genera of Geraniaceae have PY or CD. However, the role of temperature in PY-breaking and development and morpho-anatomy of the water-gap region of the members of the family Geraniaceae are poorly understood. Therefore the current study is mainly focused on the PY of Geraniaceae.

## STUDY ORGANISMS

Since more than half of the species in family Geraniaceae belong to the genus *Geranium*, the two species *Geranium carolinianum* and *G. dissectum* were selected as the main representative species for this study. *G. carolinianum* and *G. dissectum* are

herbaceous winter annual species. *G. carolinianum* is native to eastern North America (Piper, 1906; Small, 1907; Aedo, 2000), while *G. dissectum* is native to Europe (Aedo *et al*., 1998*b*; Rhoads and Block, 2007) and is an introduced species in North America (Piper and Beattie, 1915). Both species are widely distributed weeds in North America and usually grow in disturbed habitats such as road-sides, old fields, waste places, gardens and fallow and cultivated fields (McCready and Cooperrider, 1984; Abbas *et al*., 1995; Wilson and Clark, 2001). Moreover, both species are reported to be naturalized weeds in many parts of the world including Australia, China, Great Britain, Japan, Italy and South America, (Mueller, 1885; Dunn, 1905; Macbride, 1949; Peng, 1978; Aedo *et al*., 1998*b*, 2005; Benvenuti *et al*, 2001; Xu and Aedo, 2008; Nishida and Yamashita, 2009).

Additionally, 29 species in Geraniaceae and 15 species in Bixaceae, Cistaceae, Cucurbitaceae, Fabaceae, Malvaceae, Nelumbonaceae, Rhamnaceae, Sapindaceae and Surianaceae were used to compare the morpho-anatomy of the water-gap region.

## RESEARCH QUESTIONS

Even though Geraniaceae previously has been shown to contain species with PY or CD, several questions arise that require answering in order to fully understand the role of the water-gap region in this family.

1. What is (are) the water-gap(s) in PY seeds of Geraniaceae?
2. What is the ontogenetic origin of the water-gap region?
3. What are the requirements for PY-break?
4. How many steps are involved in the PY-breaking process?

5. Is it possible to quantify the temperature requirement for PY-break?

6. What is the mechanism of opening of the water-gap?

7. What is the morpho-anatomical relationship of the water-gap region in Geraniaceae to those of other PY families?

## OBJECTIVES

The main aims of this dissertation research were to study the role of the water-gap region in the PY-breaking process in seeds of Geraniaceae and to compare the water-gap region of the Geraniaceae with that of the other 17 angiosperm families known to contain species with PY. Thus, the objectives of each chapter in this dissertation research were as follows:

1. Identify and morpho-anatomically characterize the water gap in PY seeds of Geraniaceae (chapter 2);

2. Compare the simultaneous development of the water gap region, seed coat, chalaza and micropyle (chapter 3);

3. Determine the number of steps involved in the PY-breaking processes and identify the temperature and moisture regimes that activate the dormancy-breaking process at each stage (chapter 4);

4. Investigate the role of temperature in driving the two steps of PY-breaking (chapter 5);

5. Establish a thermal time (degree-weeks) model to explain sensitivity induction quantitatively (chapter 5);

6. Propose a mechanism to explain PY-breaking, focusing on the water-gap region (chapter 5);

7. Morpho-anatomically characterize the water-gap regions in the families Biberstaineaceae, Cucubitaceae, Fabaceae (clade: Cladrastis), Lauraceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae (chapter 6); and

8. Compare the morpho-anatomical features of water-gap regions of all the PY families and classify them into basic groups (chapter 6).

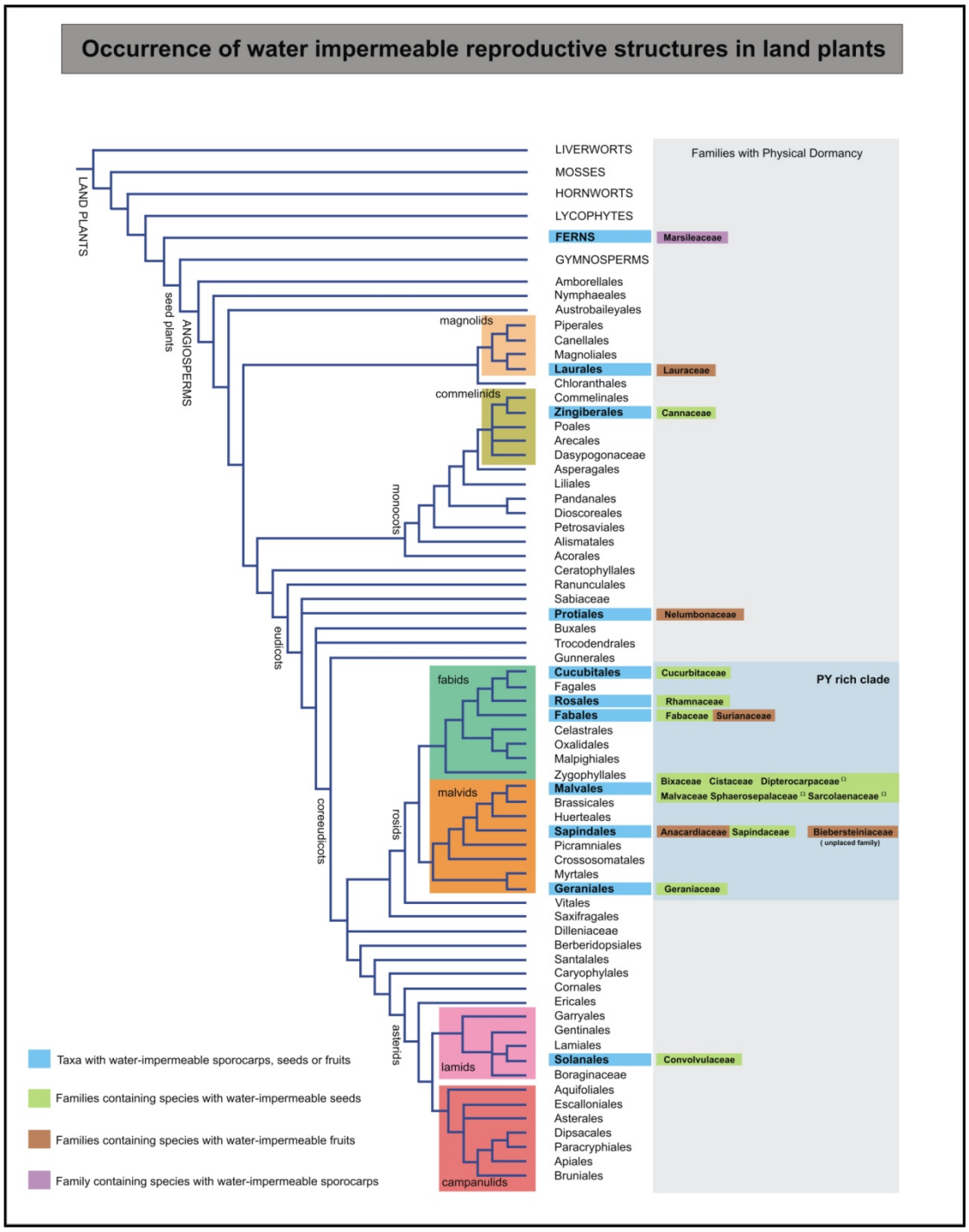



Figure 1.1. Occurrence of water impermeable structures in land plants. Phylogenetic tree of land plants is modified from APG-III (2009) and Nickrent *et al* (2000). Not all the species in families marked as PY contain water impermeable sporocarps, fruits or seeds. Abbreviations: Ω, PY of Dipterocarpaceae, Sphaerosepalaceae and Sarcolaenaceae are based on seed coat anatomy by Nandi (1998) and Horn (2004).

# CHAPTER 2

## Identification and characterization of the water-gap in physically dormant seeds of Geraniaceae, with special reference to *Geranium carolinianum* L.

## INTRODUCTION

Physical dormancy (PY) is caused by one or more water-impermeable layers of palisade cells in the seed (or fruit) coat (Baskin and Baskin, 1998; Baskin *et al*., 2000) along with a closed chalaza and micropyle. PY is known to occur only in angiosperms and thus is unknown in gymnosperms (Baskin and Baskin, 1998). One magnolids, one monocot and 16 eudicot families have been demonstrated or inferred to contain species that have PY (Nandi, 1998; Horn, 2004; Baskin *et al*., 2000; Baskin 2003; Koutsovoulou *et al*., 2005; Baskin *et al*., 2006; APG III, 2009; Tsang, 2010). Of the 16 eudicots, occurrence of PY in Dipterocarpaceae, Sarcolaenaceae and Sphaerosepalaceae has been based only on seed-coat anatomy (Nandi, 1998; Horn, 2004). Some families that contain species with PY also have species with physiological dormancy (PD), combinational dormancy (PY+PD) or non-dormancy (Baskin *et al*., 2000).

Seeds with PY cannot imbibe water even under favourable environmental conditions due to a water-impermeable layer(s) of cells. Specialized structures are involved in occlusion of the water-gaps (see table 3.5, Baskin and Baskin, 1998; table 1, Baskin *et al*., 2000). The breaking of PY involves disruption or dislodgement of 'water-gap' structures, which act as environmental 'signal detectors' for germination (Baskin *et al*., 2000). Once the closed water-gap opens, a seed can imbibe water rapidly and

germinate under a wide range of conditions (Baskin and Baskin, 1998; Baskin *et al*., 2000).

Water-gap anatomy, morphology, origin and location differ among families as well as within the same family (Baskin *et al*., 2000). Moreover, anatomy and morphology of the seed (or fruit) coat in the water-gap region differ from those of the rest of the seed (or fruit) coat. Eleven different water-gap types have been characterized in six (excluding Geraniaceae) of the 18 angiosperm families with PY (Baskin and Baskin, 1998; Baskin *et al*., 2000; Baskin, 2003; Jayasuriya *et al*., 2007a, 2008b; Hu *et al*., 2008; Turner *et al*., 2009).

Among the six genera of Geraniaceae, viz. *California*, *Erodium*, *Geranium*, *Hypseocharis*, *Monsonia* and *Pelargonium* (Fiz *et al*., 2008), PY has been shown to occur in *Erodium*, *Geranium* and *Pelargonium* (Baskin and Baskin, 1974; Meisert, 2002). The recently recognized monotypic genus *California* (Aldasoro *et al*., 2002; Fiz *et al*., 2006) can also be considered as exhibiting PY, since *Erodium macrophyllum* (identified as *California macrophyllum* by Aldasoro *et al*., 2002) was reported to have PY (Gillespie and Andersen, 2005). Based on seed-coat anatomical studies on *Hypseocharis remy* (Boesewinkel, 1988) and *Monsonia senagalensis* (Narayana and Arora, 1963), it can be inferred that PY also occurs in *Hypseocharis* and *Monsonia*. Thus, it appears that all six genera of Geraniaceae have PY or (PY+PD). Further, we are not aware of a species in any of these six genera with PD, morphological dormancy (MD), morphophysiological dormancy (MPD) or with non-dormant seeds.

The water-impermeable cell layer in the seed coat of Geraniaceae is a continuous layer of palisade cells (except in the chalazal region) that is located below the outer polygonal and middle parenchyma layer(s) of the outer integument (Kay and Lees, 1907; Boesewinkel and Been, 1979; Schulz *et al*., 1991; Meisert *et al*., 1999). The palisade cells are strongly lignified and contain tannin depositions and calcium oxalate crystals (Schulz *et al*., 1991; Aedo *et al*., 1998*a*; Meisert *et al*., 2001). The gap between palisade cells of the chalaza is filled with a chalazal plug (= suberized stopper, *sensu* Boesewinkel and Been, 1979) that maintains seed-coat impermeability. When the palisade layer is damaged, e.g. by mechanical scarification, acid scarification, etc., seeds lose their impermeability and imbibe water (Nell *et al*., 1981, Schulz *et al*., 1991).

Baskin and Baskin (1974) suggested that the hilum is the water-gap in *Geranium carolinianum*, but they did not document the presence of a water-gap. Nell *et al.* (1981) compared the morphology of the hilum region of four cultivars of *Pelargonium hortorum* using scanning electron micrographs and concluded that there is no relationship between occlusion of the hilar fissure and seed germination. Meisert *et al.* (1999) suggested that the chalazal opening is the main pathway of water entry into water-permeable seeds of *P. molliconium*. However, they described neither the initial water entrance into innately permeable seeds nor the water-gap of impermeable seeds after they become non-dormant.

Using scanning electron micrographs, Schulz *et al.* (1991) compared the effect of acid scarification of the seed coat of water-permeable and water-impermeable varieties of *P. zonale*. These authors concluded that neither the light line in the palisade layer nor

the palisade layer itself is responsible for water impermeability, but they did not study the effect of acid scarification on opening of the water-gap. Gillespie and Andersen (2005) tested the effect of different treatments on seed germination of *E. macrophyllum* (= *C. macrophyllum*). Based on the high germination percentage after manual scarification, they suggested that germination of *E. macrophyllum* may be enhanced by physical abrasion of the seed coat when seeds drill themselves into the soil.

The primary study species *Geranium carolinianum* (Carolina geranium) is a herbaceous winter annual that belongs to the family Geraniaceae, subgenus *Geranium*, section *Geranium* (which consists of 339 species; Aedo *et al*., 1998*b*). Two varieties, *G. carolinianum* var. *carolinianum* and var. *confertiflorum*, have been identified in Kentucky, USA (Jones, 2005). This species is native to eastern North America but is widely distributed throughout the North American continent (Piper, 1906; Small, 1907; Aedo, 2000). *Geranium carolinianum* has long been considered a weed that grows in disturbed habitats such as roadsides, waste places, gardens, old fields, turfs and fallow and cultivated fields in the United States (Kay and Lees, 1913; Britton, 1918; Spencer, 1976; Haragan, 1991). Moreover, it has been reported to be a naturalized weed in China, Japan, northern Europe, South America and Taiwan (Peng, 1978; Xu and Aedo, 2008; Nishida and Yamashita, 2009).

Seed dormancy of *G. carolinianum* is caused by a water-impermeable seed coat. Freshly matured seeds also have shallow PD (i.e. small amount of embryo dormancy); thus, the seeds have (PY+PD) (Baskin and Baskin, 1974). In the Baskin and Baskin (1974) study, embryos became non-dormant during a short after-ripening period

before the seeds became permeable; thus, seeds of *G. carolinianum* primarily have PY. In nature, seeds are dispersed in early summer and come out of PY by late summer due to dry and hot weather conditions and germinate in autumn when soil moisture becomes readily available.

Previous studies on Geraniaceae have considered water entry into innately permeable seeds only. Since breaking of seed dormancy is an ecologically significant event in the life history of a species, it is important to study the initial site of water entry into PY seeds after the breaking of dormancy. Therefore, the current study focused mainly on identifying and morphologically and anatomically characterizing the water-gap in PY seeds of Geraniaceae, with special reference to *G. carolinianum.* In addition, morphological changes in the seed coat during seed burial and after heat treatment were also studied.

## MATERIALS AND METHODS

### *Seed collection and preparation*

Stems of *Geranium carolinianum* L. bearing mature fruits were collected from plants growing on a railroad right-of-way in Rosemont, Lexington, KY, USA, in May 2009. They were covered with a net and allowed to dry for 3 d inside a non-heated greenhouse. Seeds released naturally were used in this study. An additional 30 species of Geraniaceae members were selected to compare water-gap regions (Table 2.1). Mature seeds of species of *California*, *Erodium*, *Hypseocharis*, *Geranium*, *Monsonia* and *Pelargonium* were obtained from commercial seed companies and personal seed collections made by colleagues in Australia, China, France, Spain, United Kingdom

and United States of America (Table 2.2). Subsequently, seeds were stored at room temperature (approx. 23 °C and 50–60 % RH, dry storage) until used.

*Characteristics of the two seed types of* G. carolinianum

*Geranium carolinianum* was observed to produce dark-brown and light-brown seeds. Length, width and mass of seeds of each colour type were measured. For measurement of length and width, 30 seeds from each colour type were randomly selected and measured using a dissecting microscope with a calibrated micrometer eyepiece. For measurement of mass, ten replicates of ten seeds each of the two colour morphology types were weighed to the nearest 0.0001 g.

To compare the anatomy of the micropylar and chalazal regions and, of the seed coat away from these two regions, 20-µm vibratome sections (longitudinal and transverse) of both dark-brown and light-brown seeds were taken using a VIBRATOME® 1500 sectioning system. Sections were observed under a light microscope (Olympus BX40) equipped with a digital camera (Olympus DP25) and micrographs taken and compared. During the rest of the study, either a dissecting microscope (ZEISS STEMI SVII) or the Olympus BX40 light microscope was used along with the Olympus DP25 digital camera for obtaining micrographs.

Ten replicates (each with ten seeds) of fresh mechanically scarified and non-scarified seeds of both dark-brown and light-brown colour types were germinated on moist sand at 20/10 °C (12 h/12 h) under a 14 h/10 h daily light/dark period. Photon irradiance during the light phase was approx. 40 µmol $m^{-2}$ $s^{-1}$, 400–700 nm, and the light source was cool white fluorescent tubes. The number of germinated seeds was

counted daily for 10 d (but only final germination is shown in Results). Hereafter, fresh seeds were selected for study irrespective of their colour.

*Dormancy breaking in seeds of* G. carolinianum

Fresh seeds of *G. carolinianum* were incubated on wet filter papers in Petri dishes for 3 d and any innately permeable seeds discarded. Subsequently, fully filled, undamaged seeds were selected from non-imbibed seeds. This procedure was used whenever impermeable seeds were selected. Based on the results of preliminary studies, the selected seeds were made permeable by subjecting them to dry heat at 80 °C for 7 d.

*Morphological changes during dormancy breaking*

Using the dissecting microscope equipped with a digital camera, micrographs of *G. carolinianum* seeds were taken before and after heat treatment to compare morphological changes that occur during breaking of dormancy.

*Germination*

Two hundred impermeable seeds of *G. carolinianum* were selected, and the outermost permeable cell layers (above the palisade layer) were removed carefully from 100 of them with the aid of a toothpick and a dissecting microscope. The other 100 seeds were left intact. The same procedure was repeated with permeable (heat-treated) seeds. Another set of 100 seeds was scarified mechanically with a razor blade at places on the seed coat away from micropylar and chalazal ends without damaging the embryo and was used as a control. All seeds were germinated as described above.

*Imbibition*

An imbibition test was carried out to compare water uptake in impermeable, permeable (heat-treated) and mechanically scarified seeds of *G. carolinianum*. Fifty seeds each of each group of seeds were used for the test. Each category was separated into ten replicates of five seeds each and weighed to the nearest 0.0001 g. Seeds of each replicate sample were placed on wet filter paper in Petri dishes. The seeds were blotted and weighed at 30-min intervals for 10 h; percentage mass increase (fresh mass basis) for each interval was calculated.

*Dye tracking*

Dye-tracking experiments were carried out to locate the site of water movement through the seed coat of permeable seeds (heat-treated) during imbibition. Concentrated solutions of both acid fuchsin and methylene blue dyes were used. After the dormancy-breaking treatment, 100 seeds of *G. carolinianum* were dipped in a concentrated acid fuchsin solution. Four seeds each were removed initially after 5 min and 15 min and then at 15-min intervals for 4 h and blotted with tissue papers. Seeds were cut longitudinally into two halves across the micropylar and chalazal regions, and the cut surfaces were observed under external artificial illumination using the light microscope. The pathway of the dye (marked in pink) was observed and micrographs taken. The same procedure was repeated with impermeable seeds.

Forty heat-treated seeds of *G. carolinianum* from which the outer water-permeable cell layers had been removed (for better visualization of the water-gap) were immersed in a concentrated methylene blue dye solution. Four seeds each were removed initially after 10 min, and then at 30-min intervals for 4 h. The surface of the

micropylar and chalazal regions (with and without removing the palisade layer) and cut-surfaces of seeds cut longitudinally into two halves through the chalazal and micropylar regions were observed and micrographs taken as described above. The pathway of methylene blue (marked in blue) was observed.

*Blocking experiment*

The outermost permeable cell layers in the micropylar and chalazal regions of heat-treated seeds were removed carefully from approx. 500 seeds of *G. carolinianum* with a toothpick. The micropylar region was blocked with Super Glue® (methyl 2-cyanoacrylate) in 100 seeds with a sharpened toothpick. One set of 100 seeds was blocked similarly at the chalazal region only and another 100 at both chalazal and micropylar regions. Ten replicates of ten seeds for each treatment were placed on wet sand in Petri dishes and incubated for 3 d under the same conditions used in germination experiments. The number of imbibed seeds (larger in size and lighter in colour) was counted at intervals of 24 h.

*Light microscopy*

Microtome sections.

Microtome and hand sections were taken to study the anatomy of the water-gap. Four fully matured seeds of *G. carolinianum* were scarified mechanically to facilitate wax infiltration and fixed in FAA solution for 7 d. Then, they were dehydrated in a series of tertiary-butanol (TBA) and embedded in paraffin wax. Subsequently, 12-µm longitudinal sections of the seeds were cut using a microtome (LEICA RM 2135). Sections were stained with 1 % safranin solution. The micropylar and chalazal regions

and the seed coat away from micropyle and chalaza were observed and photomicrographed.

Free-hand sections.

The outer permeable cell layers of three permeable *G. carolinianum* seeds were removed. Sections were cut periclinally through the micropylar region, including the presumed water-gap and palisade cells away from presumed water-gap. Sections were mounted on glass slides and micrographs taken.

*Scanning electron microscopy*

Seeds of 31 sprcies of Geraniaceae including *G. carolinianum* were made permeable by drying at 80 °C for 1 week. Three impermeable and three permeable (heat-treated) seeds, each with and without outer cell layers, were used to compare the morphological changes during the breaking of dormancy. To observe the morphological changes during early imbibition, six permeable seeds each with and without outer cell layers were immersed in water, and three seeds of each were removed from the water after 10 and 20 min of imbibition and blotted dry. The same procedure was followed with impermeable seeds. Two dislodged blisters were used to study the morphology of the lower surface of palisade cells of the presumed water-gap.

To compare the water-gaps of all 31 species, permeable (heat treated) seeds of each species from which the outer cell layers had been removed were immersed in water for 20 min and then blotted dry. All of the samples were mounted on scanning electron microscopy specimen stubs using double-sided carbon tapes. Then, the

samples were sputter-coated with gold-palladium (15 nm), scanned with an S-3200 Hitachi scanning electron microscope at an acceleration voltage of 5.0 kV and micrographs taken and compared.

*Effect of morphological changes of seed coat during burial on seed germination*

To identify the morphological changes in the seed coat *of G.carolinianum in vivo*, seeds that had been buried at a depth of 2 cm in soil for 4 months (June to October) in an open area on the campus of University of Kentucky were exhumed, observed under a dissecting microscope and micrographs taken. Fifty seeds each with and without a change in colour near the micropylar region were germinated as described above (five replicates of ten seeds each for both types).

*Statistical analysis*

Percentage germination and imbibition data were normalized by arcsine-transformation prior to the analysis. Seed length, width and mass data of the two *G. carolinianum* seed colour types and germination percentages data of the burial experiment were compared using an independent two-sample *t*-test ($P < 0.05$). All other germination and imbibition percentage data were analysed by one-way ANOVA, and Duncan's multiple range test was used to determine significant differences between each treatment ($P < 0.05$). All analyses were carried out using SAS® ver. 9.2 software.

## RESULTS

*Characteristics of the two seed types of* G. carolinianum

The light-brown and dark-brown seeds differ significantly in width but not in mass and length (Table 2.3).

The two seed types did not differ in anatomy of the chalazal or micropylar regions or seed coat away from micropylar and chalazal regions. There was more thickening in radial walls of the outermost polygonal cell layer of the outer integument in light-brown seeds than in dark-brown seeds (images not shown).

After mechanical scarification, all *G. carolinianum* seeds imbibed water within 24 h, but only a few seeds germinated at 7 d. Within the next 3 d, germination increased rapidly to 89.0 ± 5.3 % and 96.0 ± 1.7 % in scarified light-brown and dark-brown seeds, respectively. Non-scarified light-brown and dark-brown seeds germinated to only 3.0 ± 2.1 % and 2.0 ± 2.6 %, respectively, after 10 d of incubation. Germination differed significantly between scarified and non-scarified seeds, but not between scarified or between non-scarified seeds of the two seed colour types ($P < 0.05$).

*Dormancy breaking in seeds of* G.carolinianum

After the dry heat treatment (80 °C for 1 week), a brownish-orange circular area appeared near the micropylar region in all treated seeds of *G. carolinianum* (Fig. 2.1C, D). The colour of this area in fresh dormant seeds was similar to that of the seed coat (Fig. 2.1A, B).

*Imbibition*

Imbibition in manually scarified seeds of *G. carolinianum* was faster than it was in heat-treated seeds. Manually scarified seeds and heat-treated seeds reached their

maximum mass in 5.5 h and 6.5 h, respectively. After 10 h, the mass of manually scarified and heat-treated seeds had increased by 88.7 ± 1.1 % and 94.5 ± 1.9 %, respectively, whereas that of non-scarified seeds had increased by only 0.3 ± 0.2 % ($P < 0.05$; Fig. 2.2).

*Germination*

After 10 d of incubation on wet sand at 20/10 °C, heat-treated (outer cell layer intact), heat-treated (outer cell layers removed) and scarified seeds of *G. carolinianum* germinated to 100 %. Germination of impermeable (outer cell layers intact) and impermeable (outer cell layers removed) was 0.0 % and 2.0 ± 0.0 %, respectively ($P < 0.05$).

*Dye tracking*

Within 5 min, the outermost layers [outer layer and middle layer(s) of the outer integument] of both impermeable and permeable *G. carolinianum* seeds were stained pink (Fig. 2.3C, I). After 5 min, the palisade cells of the 'hinged valve' had stained pink in permeable seeds (Fig. 2.3B) and blue in the chalazal opening (Fig. 2.4B). The first appearance of dye below the palisade layer was observed after 10 min in the subpalisade cells of the micropylar region below the 'hinged valve' (Fig. 2.4E), and this stained area had increased in size after 30 min, indicating water movement between palisade and subpalisade layers (Fig. 2.4F). Thereafter, movement of the stain was slower than that of water, but the cells swelled due to imbibition. After 1.5 h, swelling of the embryo occurred first under the micropylar region towards the chalaza (Fig. 2.3D, E). At the same time, no staining or swelling occurred in the chalazal region (Fig. 2.3D, H). After 2 h, a pink colour was observed halfway through

the subpalisade directly below the micropylar region and after 2.5 h in the subpalisade cells adjacent to the micropyle towards the chalaza (Fig. 2.3F and G). The nucellus and the whole micropylar region were stained after 4 h of incubation in dye, while the whole embryo had imbibed (Fig. 2.3G). Even after 24 h, the embryo remained unstained (figure not shown).

*Blocking of presumed water-gap*

After 72 h of incubation, the percentage of imbibition in *G. carolinianum* seeds with the chalaza blocked (98 ± 1.1 %) was significantly higher than that in seeds with the micropylar region blocked (14.0 ± 3.1 %) or with the micropylar region + chalaza blocked (18.0 ± 3.3 %; $P < 0.05$). No significant difference was observed between seeds in which the micropylar region or chalaza + micropylar region were blocked ($P < 0.05$; Fig. 2.5).

*Light microscopy*

The seed coat of *G. carolinianum* consists of two integuments. The outer integument contains an outermost polygonal cell layer with thickened radial walls, middle parenchyma cell layer(s) and a palisade layer. The inner integument consists of a subpalisade layer, a middle compressed layer and an innermost large cell layer. The nucellus is much compressed (not shown).

The palisade layer forms a continuous water-impermeable layer except in the chalazal region. Near the chalazal region of mature seeds, the chalazal plug originates from the nucleus and fills the gap between palisade cells. The palisade cells are polygonal (transverse section), and they can have from four to twelve sides. Normally, when

compared with cells with four to six sides, cells with more than six sides are darker, larger and contain larger cell depositions at the base of the cell. These are called tanniferous cells (Fig. 2.6E, F). The light line in the tanniferous cells is slightly more raised than it is in normal palisade cells.

The anatomy of the seed coat in the micropylar region differs from that of the seed coat away from the micropyle. Near the micropylar region, the middle parenchyma cell layer of the outer integument is several layers thick, and the palisade cells are radially elongated. These elongated cells are lighter in colour than the palisade cells away from the micropylar region and have a wider light line (Fig. 2.6B, C). Elongated palisade cells form a dome-shaped structure by bending sideways, and the top of the dome is formed by parenchyma cells (Fig. 2.6B). Subpalisade cells in the micropylar region are multilayered, and the cells are elongated radially. Consequently, these subpalisade cells appear to be taller than other subpalisade cells away from the micropyle (Fig. 2.6B).

Palisade cells of the ‘hinged valve’ comprise elongated and bent palisade cells of the micropyle and non-elongated palisade cells of the micropylar region. These palisade cells differ from palisade cells away from the water-gap region due to the absence of tanniferous cells in this region. Near the micropyle, the width of palisade cells is less than that of normal palisade cells, and their size increases towards the radicle end of the seed. The inner periclinal cell wall of the palisade cells near the micropyle is flat and becomes convex towards the radicle. Also, cell lumens are visible above the light line of the palisade cells near the micropyle (Fig. 2.6G).

*Electron microscopy*

No apparent morphological differences were observed between dormant and non-dormant (heat-treated) seeds of *G. carolinianum* with or without outer cell layers (Fig. 2.7A and C). When permeable seeds started to imbibe water, a 'clam shell'-shaped blister formed near the microplylar region towards the radicle end of the seed (Fig. 2.7B and D). The blister was formed by swelling of palisade cells in this region, and it remained hinged to elongated palisade cells of the micropyle (Fig. 2.7C). Apparently, the outer two cell layers also play a role in preventing the blister from detaching. After 20 min, the blister was detached from the imbibed seeds (without outer cell layers) revealing an opening (Fig. 2.7G). The cell size of the palisade and subpalisade layers of the water-gap increased gradually – from the micropyle to the radicle end. Also, it was observed that the inner periclinal walls of palisade cells of the water-gap were flat near the micropyle and became convex towards the radicle end (Fig. 2.7E, F). Accordingly, outer periclinal walls of subpalisade cells of the water-gap are flat and concave (Fig. 2.7G).

No distinct water-gap region was observed for seeds in *Hypseocharis pimpinellifolia* (Fig. 2.8A). The water-gap of other study species of *California*, *Erodium*, *Geranium*, *Monsonia* and *Pelargonium* were located at the micropylar region (Fig. 2.8B-AD). However, water-gap morphology can vary with the species. In most species of *Geranium* and in all the *Pelargonium* species a circular opening was formed near the micropyle, similar to *G. carolinianum* (Fig. 2.8C-N; U-AD). However, in seeds of G. *bohemicum* a horse-shoe shaped opening was formed near the micropyle (Fig. 2.8F). In seeds of *C. macrophylla*, *E. angustifolia* and all the *Erodium* species, the whole micropylar region, including the micropyle, was removed during blister formation

(Fig. 2.8B; O-S; T). Moreover, in *E. ciconium* and *E. cicutarium*, elongated openings formed (Fig. 2.8P,Q).

*Effect of morphological changes of seed coat during burial on seed germination*

During burial, the outer permeable cell layers were damaged or lost. A change of colour near the micropylar region was observed in some *G. carolinianum* seeds (Fig. 2.9B) similar to that in seeds after heat treatment. Seeds with a change in colour germinated to a significantly higher percentage (100 %) than those without a colour change (20 ± 3.16 %; $P < 0.05$).

## DISCUSSION

The two seed-coat colours in *G. carolinianum* can be attributed to different degrees of radial wall thickening in the outermost polygonal parenchyma cell layer of the outer integument. This cell layer along with the middle cell layer(s) is responsible for the reticulate appearance on the surface of Geraniaceae seeds (Boesewinkel and Been, 1979; Schulz *et al*., 1991; Aedo *et al*., 1998*a*). Seeds with additional cell wall thickening in the outermost polygonal cell layer are lighter in colour, while those with less thickening are darker in colour. Due to the higher degree of thickening, the seed width of light-coloured seeds is significantly higher than that of darker seeds. However, the two seed types do not differ significantly in length, mass or seed-coat anatomy.

Even though all of the fresh scarified *G. carolinianum* seeds of both seed types imbibed water within 24 h, the percentage germination remained very low within the

first 7 d. However, it reached a mean of 92 % germination by the end of 10 d. This slow rate of germination indicates the presence of some non-deep PD in the embryo. Thus, the seeds have (PY+PD). PD of intact seeds was lost within 2 months, and then scarified seeds germinated rapidly. Loss of PD in Geraniaceae during dry storage has been reported by Baskin and Baskin (1974) and Van Assche and Vandelook (2006).

According to Meisert (2002), some physically dormant species of Geraniaceae have an innately permeable seed fraction. This fraction is <3 % in *G. carolinianum*. The permeable seed fraction of *G. carolinianum* seems to comprise three categories; viz. immature seeds, seeds with open chalaza and seeds with cracked seed coats. Seeds with PY acquire impermeability during maturation drying in the last stages of seed development (Egley, 1976; Egley *et al*., 1983; Baskin and Baskin, 1998; Li *et al*., 1999*a*, Jayasuriya *et al*., 2007b; Chapter 3). If seeds are shed prematurely before development of PY, they may not become dormant due to lack of depositions such as lignin, wax, phenolic compounds, etc., that make them impermeable (Werker, 1997). The width of the chalazal opening plays a role in impermeable and permeable seed fractions in Geraniaceae. Water-permeable seeds form a wider opening than impermeable seeds in the chalazal slit (Meisert *et al*., 1999). Cracks in the seed coat may also be responsible for the occurrence of an innately permeable seed fraction (Ma *et al*., 2004). Cracks in both permeable and impermeable seeds of *G. carolinianum* were observed in scanning electron micrographs. If the cracks have reached the subpalisade layer, seeds may have become permeable.

The outermost cell layers of the outer integument are not responsible for seed-coat dormancy due to the lack of mechanical barriers and presence of stomata. However,

as observed by Boesewinkel and Been (1979) in seeds of *Geranium pratense*, wax deposited on cell walls may act as a partial barrier to water. Apparently, when buried in soil these cell layers can be removed easily due to abrasion by soil particles and microbial and chemical breakdown. In the present study, removal of these outer cell layers in impermeable seeds did not break PY. Also, in dye-tracking experiments these cell layers were stained by dye in both permeable and impermeable seeds, indicating their permeability. Therefore, as reported in other species of Geraniaceae, PY of *G. carolinianum* is maintained by an impermeable palisade layer. The palisade layer is yellowish brown in vibratome sections, due to lignin and tannin depositions, while the other entire cell layers in the seed coat are colourless.

Impermeable seeds of *G. carolinianum* subjected to dry heat (80 °C) for 1 week became permeable and could be identified by their brownish orange colour in the area of the water-gap. This colour change is due to detachment of the palisade layer from the subpalisade layer in the water-gap. We suggest that when the two layers separate, calcium oxalate crystals are shattered and cause the incident light to scatter, resulting in lightening of colour. The colour change was also observed in fresh seeds when pressure was applied to the micropylar area. Formation of a blister of a lighter colour in the chalazal region after application of a small force was reported in *Sida spinosa* (Egley and Paul, 1981). Therefore, the colour change in the water-gap of *G. carolinianum* during heat treatment may not be due to a chemical change. Heat-treated seeds with a colour change germinated to 100 %, while none of the non-treated seeds germinated. Thus, the colour change in the water-gap region after heat treatment is related to breaking of dormancy in *G. carolinianum* seeds.

After 4 months of burial in soil, the germination of *G. carolinianum* seeds with a colour change in the water-gap area was significantly higher (100 %) than that of seeds without visible colour change (20 ± 3.2 %). These results are similar to those of heat-treated and non-treated seeds. The colour change in the water-gap begins at the micropyle and gradually spreads toward the radicle end of the seed. The initial stages of colour change are not visible even under a dissecting microscope. This possibly explains why a considerable amount of germination in seeds without a visible colour change was obtained.

The micropyle, lumens (located above the light line) and cracks in 'hinged valve' palisade cells near the micropyle may play an important role in imbibition. When the palisade cells of the 'hinged valve' imbibe water, they immediately start to swell, forming a blister in the micropylar region. When the blister erupts, it exposes an opening (water-gap) near the micropyle towards the radicle end of the seed. In heat-treated seeds, the outer permeable cell layers prevent dislodgement of the blister, but in buried seeds the blister may be dislodged due to previous erosion of these outer cell layers.

Dye-tracking showed that water enters through the water-gap and not through the chalaza, since the subpalisade layer was stained first below the water-gap and not in the chalazal region. Moreover, the embryo imbibed first near the water-gap after 1.5 h, while it remained non-imbibed near the chalaza. Swelling of palisade and subpalisade cells and of the embryo started first near the micropyle and then extended towards the chalaza. This pattern of swelling confirms that movement of water after opening of the water-gap is radial and is directed towards the chalaza. Once cells near

the chalaza are swollen, more water can be imbibed through the chalazal opening. Movement of the dye lagged behind that of water. This may be due to the larger size of dye molecules and lack of affinity of the dye for cell walls, thus slowing the movement of the dye. Moreover, the embryo was not stained by acid fuchsin or by methylene blue even after 24 h of incubation. These results agree with those obtained for *Dodonaea petiolaris* (Turner *et al*., 2009).

The idea that initial water entry occurs first through the water-gap near the micropyle and not through the chalazal opening was also supported by blocking experiments. Within the first 24 h of incubation, the percentage imbibition of seeds with the micropylar region sealed was significantly lower than that of those with the chalaza sealed. Even after 72 h, the difference remained the same. Therefore, water should first enter through the water-gap to initiate imbibition. The uptake of some water even after blocking the water-gap region may be due to imperfect binding of the glue. As previously reported by Egley and Paul (1981) and Turner *et al.* (2009), incomplete blocking is a common problem associated with sealants in blocking experiments. The role of the chalazal opening in initial water uptake can be disregarded since there was no significant difference in percentage imbibition of seeds with the chalaza blocked and with both the chalaza and micropylar regions blocked. Blocking of the water-gap only (i.e. without also blocking the micropyle) was not successful due to their closeness to each other. Therefore, the role of the micropyle in initial water uptake is still unresolved.

Meisert *et al.* (1999) documented the chalazal slit as the site of water entry into innately permeable seeds of Geraniaceae. In that study, the authors compared the

chalazal anatomy of water-permeable and impermeable seeds. The permeable seed fraction of *Pelargonium mollicomum* and seeds of the permeable species, *Erodium manescavii*, form a wide chalazal opening that maintains permeability, while the impermeable seed fraction of *P. mollicomum* and seeds of the PY species *P. candicans* form a narrow opening through which water does not pass. Meisert *et al.* (1999) used $OsO_4$ to visualize the entry of water into innately permeable *P. mollicomum* seeds and concluded that the chalazal opening was the main site of water entry into the seed. However, they documented neither the location of initial movement of water through innately permeable seeds nor the water-gap of impermeable seeds after breaking PY in Geraniaceae. The present study clearly shows that the initial water uptake in PY seeds after breaking of dormancy takes place through the water-gap near the micropyle and not via the chalazal opening.

Baskin and Baskin (1974) suggested that the hilum functions as a 'hygroscopic valve' that is involved in initial water entry into the seed, but based on the reports of Boesewinkel and Been (1979), they subsequently reported the chalaza as the water-gap in Geraniaceae (Baskin and Baskin, 1998; Baskin *et al*., 2000). Nell *et al.* (1981) compared the morphology of the hilum region of four cultivars of *Pelargonium hortorum* using scanning electron microscope photographs and concluded that there is no relationship between occlusion of the hilar fissure and seed germination. The hilar slit acts as the water-gap in certain species like *Cercis canadensis* (Jones and Geneve, 1995; Geneve, 2009), *Cuscuta australis* (Jayasuriya *et al*., 2008b) and *Sophora alopecuroides* (Hu *et al*., 2008). However, unlike the hilar fissure in these three species, the hilar fissure in Geraniaceae is located in the outer permeable layers of the seed coat and thus cannot act as a water-gap.

Gillespie and Andersen (2005) suggested that physical abrasions may play a role in the breaking of PY in *E. macrophyllum* (= *C. macrophyllum*). This would occur when seeds are drilled into soil by coiling and uncoiling of their awns in response to changing relative humidity levels. However, Baskin and Baskin (2000) argue that it is rather unrealistic to consider that abrasive action, which is an ever-present probability in the physical environment, has a significant effect on breaking dormancy in nature. Such a mechanism would not allow for control of timing of dormancy break, which is a critical event in the adaptation of plants to their environment.

Schulz *et al.* (1991) compared scanning electron microscope photographs of the seed coat of water-permeable and impermeable varieties of *Pelargonium zonale* before and after scarification with conc. $H_2SO_4$. They concluded that the palisade layer no longer acts as a water-impermeable barrier after the seeds are acid scarified. However, they did not show the effect of $H_2SO_4$ on either the chalazal or micropylar region, and therefore the effect of acid scarification on opening of the water-gap is not clear.

Germination of physically dormant seeds occurs only after a plug or lid that closes the discontinuity ('water-gap') in the water-impermeable layer(s) is dislodged or disrupted, thereby creating an opening for entrance of water to the embryo (Baskin *et al*., 2000). Eleven kinds of water-gaps in six angiosperm families (excluding Geraniaceae) have been characterized previously: (1) a bulge gap adjacent to the micropyle in Convolvulaceae (Jayasuriya *et al*., 2007a); (2) a carpellary micropyle in Anacardiaceae (Li *et al*., 1999*b*); (3) a chalazal blister gap in Malvaceae–Malveae (La Croix and Staniforth, 1964; Egley and Paul, 1981; Serrato-Valenti *et al*., 1992); (4) a

chalazal opening in Malvaceae–Gossypieae (Christiansen and Moore, 1959; Simpson *et al*., 1940); (5) a chalazal slit in Malvaceae-Hibisceae (Serrato-Valenti *et al*., 1992; Poljakoff-Mayber *et al*., 1994); (6) a gap adjacent to the hilum in Sapindaceae (Turner *et al*., 2009); (7) a hilar slit in Convolvulaceae (Jayasuriya *et al*., 2008b); (8) a hilar slit in Fabaceae–Caesalpinoideae (Jones and Geneve 1995; Geneve,2009) and Papilinoideae (Hu *et al*., 2008); (9) a lens gap in Fabaceae–Caesalpinoideae (Lersten *et al*., 1992) and Mimosoideae (Dell, 1980; Hanna, 1984; Morrison *et al*., 1998); (10) Lens slit in Fabaceae-Papilinoideae (Morrison *et al*., 1998) and (11) a raphal scar in Cannaceae (Grootjen and Bouman, 1988; Graven *et al*., 1997; Mass-Van De Kamer and Mass, 2008).

Even though the location of the water-gap of *G. carolinianum* seeds shows some similarity to that of *Ipomoea lacunosa* (Jayasuriya *et al*., 2007a), they differ in that two openings are formed in *I. lacunosa*. Moreover, blister formation in *G. carolinianum* is similar to that of *Abutilon theophrasti* (La Croix and Staniforth, 1964), *Albizia lophantha* (Dell, 1980), *Sida spinosa* (Egley and Paul, 1981, 1982; Egley *et al*., 1986), *Acacia kempeana* (Hanna, 1984), *Canna tuerckheimii* (Grootjen and Bouman, 1988), *I. lacunosa* (Jayasuriya *et al*., 2007a) and *Dodonaea petiolaris* (Turner *et al*., 2009). However, the water-gap in *G. carolianum* differs from all other characterized blister-forming water-gap types in anatomy, morphology or location. Therefore, the 'hinged valve gap' adjacent to the micropyle in Geraniaceae is the twelfth water-gap type to be characterized.

Based on scanning electron micrographs, the morphology of the water-gaps in members of Geraniaceae are similar in shape and location with slight variations

within the family. In *Geranium* and *Pelargonium*, one half of the micropyle is retained when the blister is dislodged from the seed coat, whereas, in *California, Erodium* and *Monsonia*, the whole micropylar region, including the micropyle, is removed when the blister is dislodged from the seed coat. Thus the water-gaps of the latter three genera slightly differ from that in other members of the family. Moreover, due to lack of seeds, the water-gap of *Hypseocharis* could not be identified. Thus further studies should be carried out to identify the water-gap in this genus.

Opening of the water-gap is essential for germination of *G. carolinianum*. During the initial stages of imbibition in permeable seeds (after PY is broken), eruption of the special structure, 'hinged valve', adjacent to the micropyle opens the water-gap in Geraniaceae. In *G. carolinianum*, morphology of the water-gap of seeds made permeable by exposing them to natural conditions was observed to be similar to that of heat-treated seeds. Therefore, as predicted by Baskin and Baskin (1974), dry hot weather conditions in summer can be an environmental factor affecting the dormancy breaking of *G. carolinianum* under natural conditions. The present study reveals that the 'hinged valve' gap which is adjacent to the micropyle, functions as the water-gap in seeds of *G. carolinianum* after PY is broken.

Table 2.1. Classification of study species of Geraniaceae

| | Genera | | Study species |
|---|---|---|---|
| 1. | *Hypseocharis* | | *H. pimpinellifolia* |
| 2. | *California* | | *C. macrophylla* |
| 3. | *Geranium* | | |
| | subgenera | | |
| | | Erodoidea | No species were studied |
| | | Geranium | *G. carolinianum, G. dissectum, G. sessiliflorum, G. tuberosum, G. bohemicum, G. grandistipulatum* |
| | | Robertum | *G. polyanthes, G. ocellatum, G. molle, G. pusillum, G. pyrenaicum, G. lucidum, G. madrense* |
| 4. | *Erodium* | | |
| | subgenera | | |
| | | Erodium | *E. taxanum* |
| | | Barbata | *E. ciconium, E. cicutarium, E. manescavii, E. moschatum* |
| 5. | *Monsonia* | | |
| | Section | | |
| | | Monsonia | No species were studied |
| | | Olopetalum | *M. angustifolia* |
| 6. | *Pelargonium* | | |
| | Clades | | |
| | | A | *P. alternans, P. capitatum, P. crithmifolium, P. nanum, P. vitifolium* |
| | | B | No species were studied |
| | | C | *P. dolomiticum, P. mirrhifolium, P. alchemilloides, P. quinquelobatum, P. tongaense* |

The classification of genera into clades, sections and subgenera based on Aedo *et al* (1998b); Fiz *et al* (2006); Aldasoro *et al* (2001); Touloumenidou *et al* (2007); Bakker *et al* (2004); Jones *et al* (2009).

Table 2.2. Seed sources of the additional study species of Geraniaceae

| Species | Seed source |
|---|---|
| *Hypseocharis pimpinellifolia* | Instituto de Botánica Darwinion, AR (March, 2012) |
| *California macrophylla* | University of Texas, Austin, TX, USA (March, 2012) |
| *Geranium dissectum* | University of Kentucky, Lexington, KY, USA (June, 2009) |
| *Geranium sessiliflorum* | B and T World Seeds Company, Paguingan, FR (March, 2012) |
| *Geranium tuberosum* | B and T World Seeds Company, Paguingan, FR (July-,2011) |
| *Geranium bohemicum* | Plant World Seeds, Devon, UK (March, 2012) |
| *Geranium grandistipulatum* | Plant World Seeds, Devon, UK (March, 2012) |
| *Geranium polyanthes* | Plant World Seeds, Devon, UK (March, 2012) |
| *Geranium ocellatum* | Plant World Seeds, Devon, UK (March, 2012) |
| *Geranium molle* | University Kentucky, Lexington, KY, USA (June, 2009) |
| *Geranium pusillum* | University Kentucky, Lexington, KY, USA (June, 2009) |
| *Geranium pyrenaicum* | Millennium Seed Bank collection, Kew Botanical Garden, Kew, UK (April, 2012) |
| *Geranium lucidum* | Millennium Seed Bank collection, Kew Botanical Garden, Kew, UK (April, 2012) |
| *Geranium madrense* | Plant World Seeds, Devon, UK (March, 2012) |
| *Erodium taxanum* | University of Texas, Austin, TX, USA (February, 2012) |
| *Erodium ciconium* | La Gineta, Albacete, Castile-La Mancha, SP (June, 2011) |
| *Erodium cicutarium* | Spindletop Farm , Lexington, KY, USA (June, 2009) |
| *Erodium manescavii* | Plant World Seeds, Devon, UK (March, 2012) |
| *Erodium moschatum* | Plant World Seeds, Devon, UK (March, 2012) |
| *Monsonia angustifolia* | B and T World Seeds Company, Paguingan, FR (March, 2012) |
| *Pelargonium alternans* | University of Texas, Austin, TX, USA (February, 2012) |

Table 2.2 (continued)

| | |
|---|---|
| *Pelargonium crithmifolium* | University of Connecticut, Storrs, CT, USA (February, 2012) |
| *Pelargonium nanum* | University of Texas, Austin, TX, USA (March, 2012) |
| *Pelargonium vitifolium* | University of Connecticut, Storrs, CT, USA (February, 2012) |
| *Pelargonium capitatum* | West Perth, WA, AUS (October, 2009) |
| *Pelargonium dolomiticum* | University of Texas, Austin, TX, USA (February, 2012) |
| *Pelargonium mirrhifolium* | University of Texas, Austin, TX, USA (February, 2012) |
| *Pelargonium alchemilloides* | University of Texas, Austin, TX, USA (February, 2012) |
| *Pelargonium quinquelobatum* | University of Texas, Austin, TX, USA (February, 2012) |
| *Pelargonium tongaense* | University of Connecticut, Storrs, CT, USA (February, 2012) |

Table 2.3. Mass, length and width of the two seed types of *G. carolinianum*

| Seed type | Seed mass (mg) | Seed length (mm) | Seed width (mm) |
|---|---|---|---|
| Light brown | 2.44 ± 0.03 | 1.92 ± 0.02 | 1.26 ± 0.01* |
| Dark brown | 2.35 ± 0.03 | 1.96 ± 0.02 | 1.22 ± 0.01 |

Values are means ± s.e.

* Indicates significant difference at $P < 0.05$, with an independent two-sample t-test

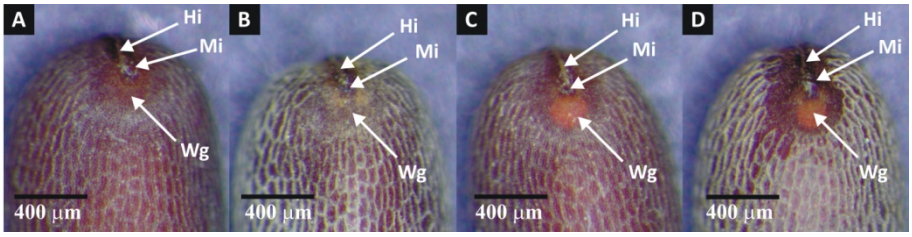



Figure 2.1. The micropylar region of *Geranium carolinianum*: (A) dark-brown and (B) light-brown impermeable seeds with no colour change in the area adjacent to the micropyle; (C) dark-brown and (D) light-brown permeable seeds with colour change in the area adjacent to the micropyle after dry heat treatment at 80 °C for 1 week. Abbreviations: Hi, hilum; Wg, water-gap; Mi, micropyle.

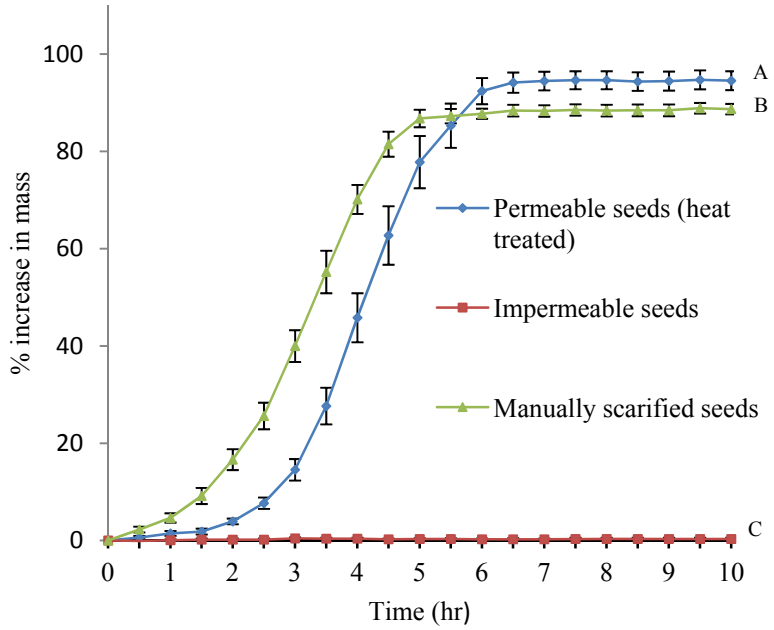



Figure 2.2. Percentage mass increase (mean ± s.e.) in permeable (heat-treated), manually scarified and impermeable seeds of *G. carolinianum* during 10 h of incubation at ambient room temperature. Different letters indicate significant differences between treatments ($P < 0.05$).

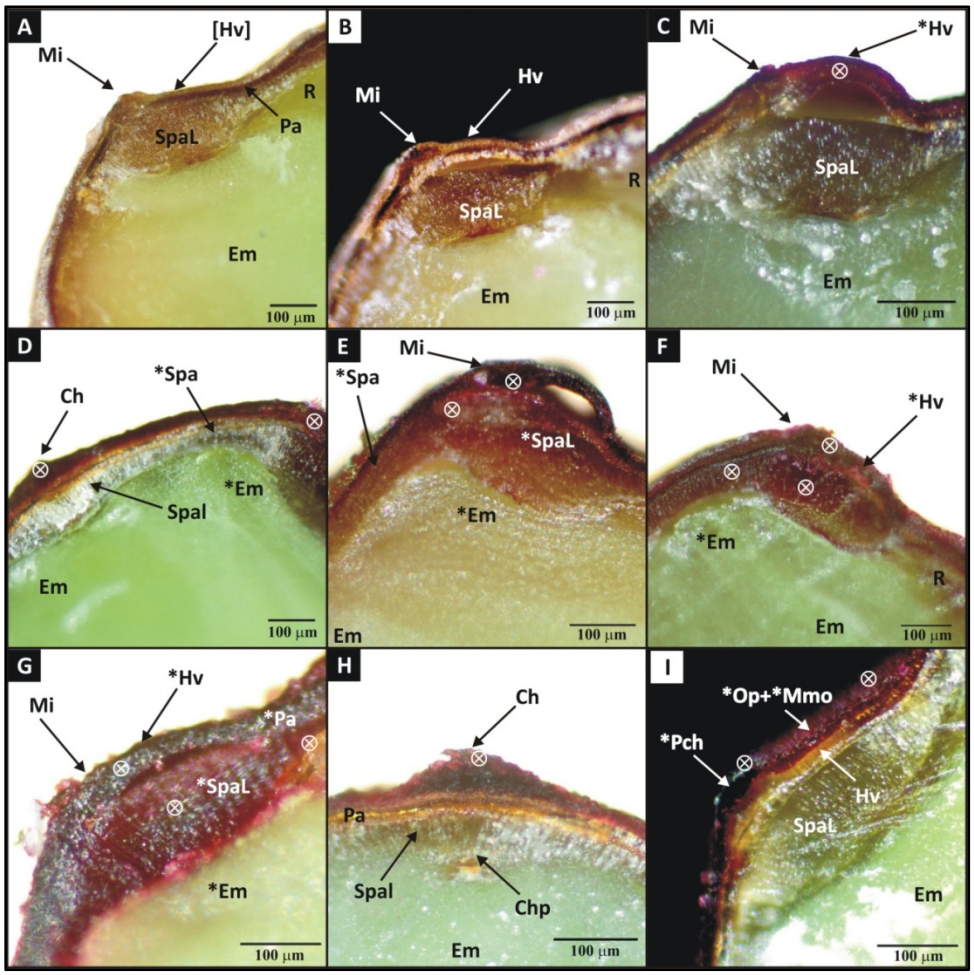



Figure 2.3. Longitudinal sections through micropylar and chalazal areas of seeds of *G. carolinianum* allowed to imbibe acid fuchsin for different periods of time: (A) micropylar region of an impermeable seed before imbibition; (B) micropylar region of a permeable seed before imbibition; (C), (E), (F) and (G) micropylar region of permeable seeds with opened water-gap after 5 min, 2 h, 3.5 h and 4 h, respectively; (D) both chalazal and micropylar regions of a permeable seed after 1.5 h; (H) chalazal region of a permeable seed after 1.5 h; (I) micropylar region of an impermeable seed after 4 h. Abbreviations: Ch, chalaza; Chp, chalazal plug; Em, embryo; Hv, hinged valve (opened); [Hv], hinged valve (closed); Mi, micropyle; Mmo, multi-layered middle parenchyma cells; Op, outermost polygonal parenchyma cell layer; Pa, palisade cells; Pch, parenchyma cells of micropyle; R, radicle; Spa, subpalisade cells; SpaL, elongated subpalisade cells of micropylar region; Spal, elongated subpalisade cells of chalazal region. A cross in a circle indicates presence of acid fuchsin dye in different cell layers; an asterisk indicates regions that have imbibed.

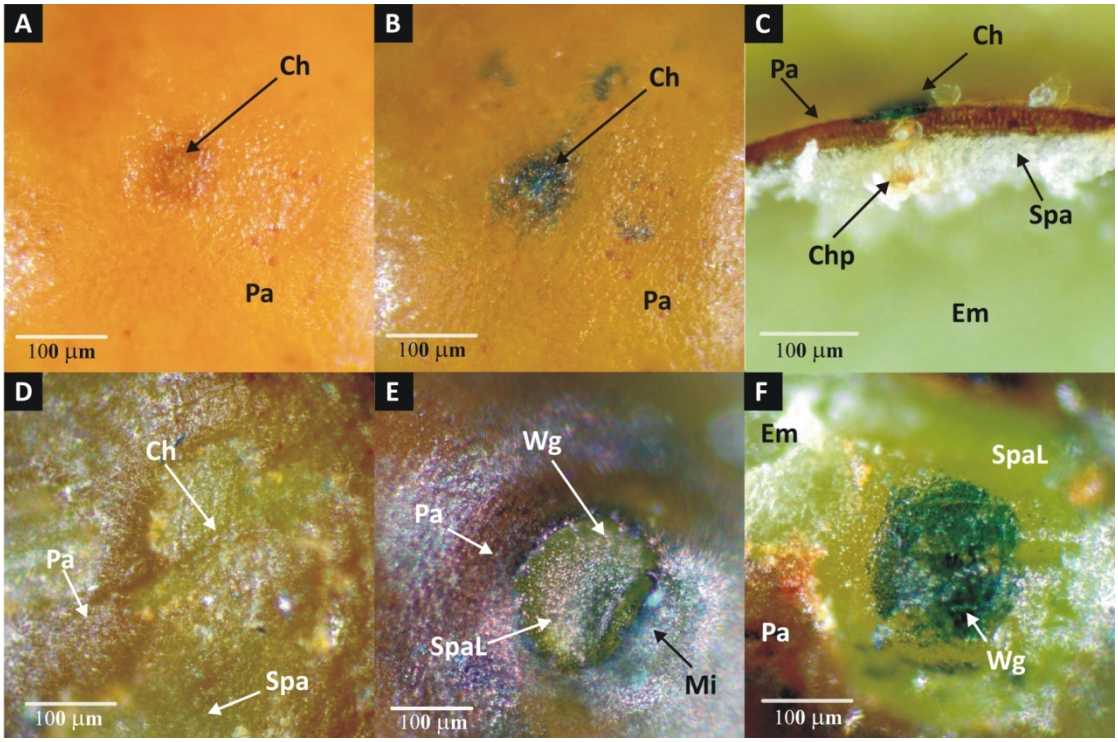



Figure 2.4. Light micrographs of micropylar and chalazal regions of seeds of *G. carolinianum* allowed to imbibe methylene blue for different periods of time: (A) and (B) surface view of chalazal end of a seed before and 1.5 h after imbibition; (C) longitudinal section through chalazal region 1.5 h after imbibition; (D) chalazal region of a seed 1.5 h after imbibition (palisade layer removed to expose subpalisade layer); (E) micropylar region of a seed after 10 min imbibing the dye (hinged valve removed); (F) micropylar region of a seed after 30 min (palisade layer removed to expose elongated subpalisade layer). Abbreviations: Ch, chalaza; Chp, chalazal plug; Em, embryo; Mi, micropyle; Pa, palisade cells; Spa, subpalisade cells; SpaL, elongated subpalisade cells of micropylar region; Wg, water-gap.

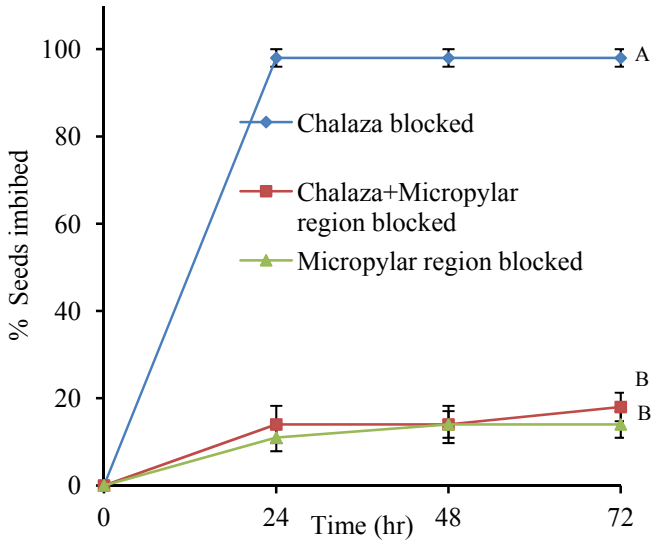



Figure 2.5. Percentage of *G. carolinianum* seeds which imbibed (mean ± s.e.) during 72 h of incubation at 20/10 °C with the chalaza blocked, the chalaza + micropylar region blocked or the micropylar region blocked. Different letters indicate significant differences between treatments ($P < 0.05$).

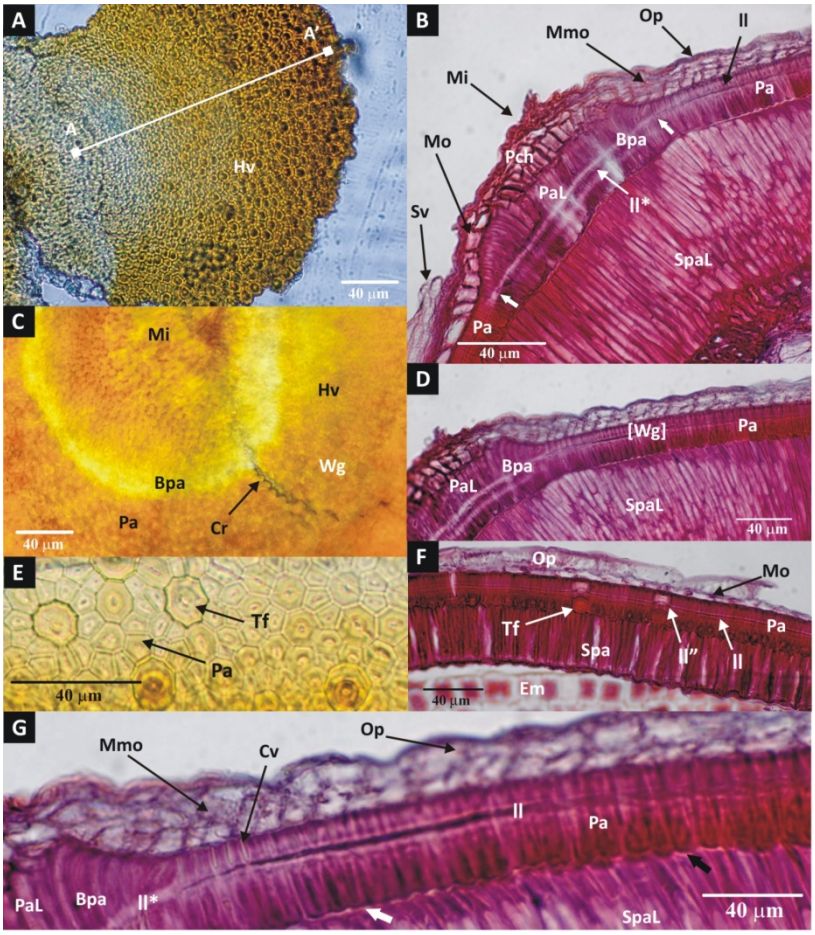



Figure 2.6. Light micrographs of *G. carolinianum* seeds: (A) top view of water-gap (the line A–A' indicates the increasing size of the palisade cells); (B) longitudinal section through micropylar region of a dormant seed (the two short thick white arrows demarcate the widened light line in the micropylar region); (C) periclinal section through the water-gap with the hinged valve; (D) longitudinal section of water-gap; (E) periclinal section of the palisade layer away from the water-gap; (F) longitudinal section of the seed coat away from the water-gap; (G) close-up of a longitudinal section of a water-gap (the short thick white or black arrow indicates subpalisade cells with smooth or rough outer periclinal walls, respectively. Abbreviations: Bpa, bent palisade cells; Cr, crack demarcates the margin of the water-gap; Cv, cell lumen; Em, embryo; Hv, hinged valve; ll, light line; ll*, widened light line in the micropylar region; ll'', raised light line in the tanniferous cells; Mi, micropyle; Mmo, multi-layered middle parenchyma cells; Mo, single layer of middle parenchyma cells; Op, outermost polygonal parenchyma cell layer; Pa, palisade cells; PaL, elongated palisade cells of micropylar region; Spa, subpalisade cells; SpaL, elongated subpalisade cells of micropylar region; Sv, seed-coat vascular tissue; Tf, tanniferous cells in palisade layer; Wg, water-gap open; [Wg], water-gap closed.

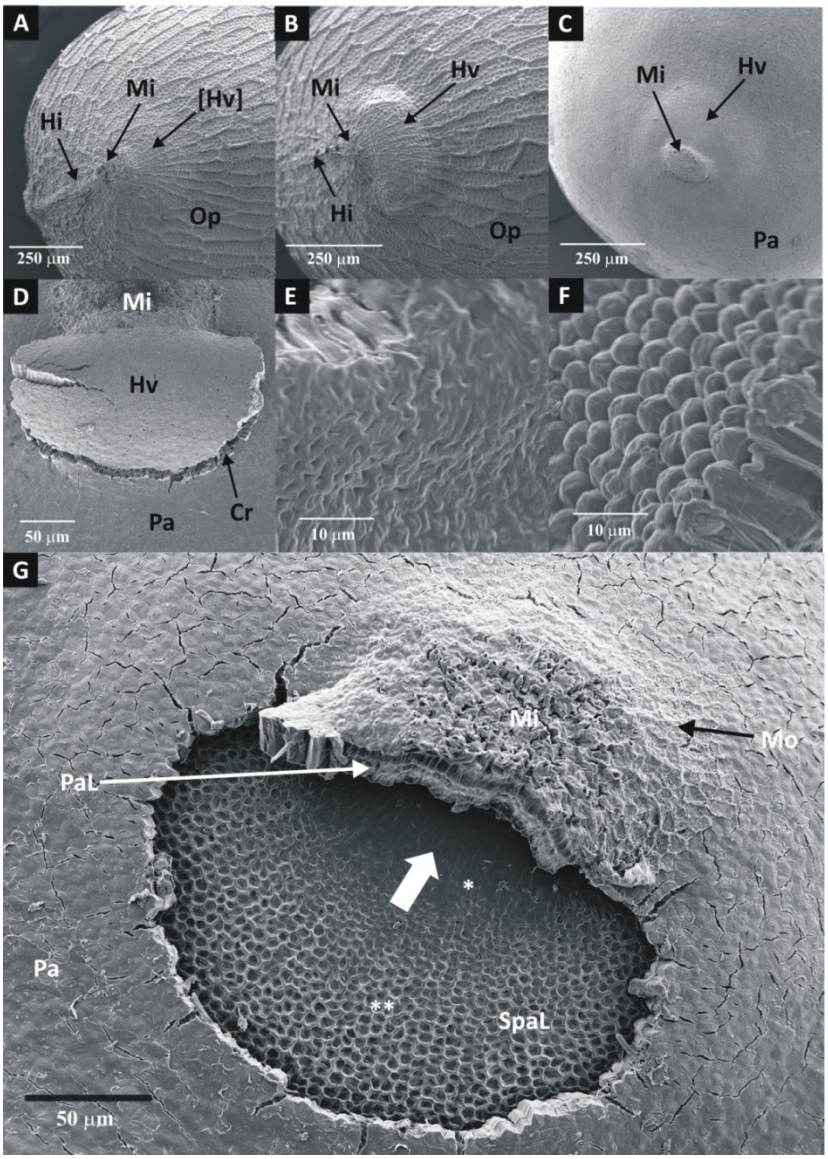
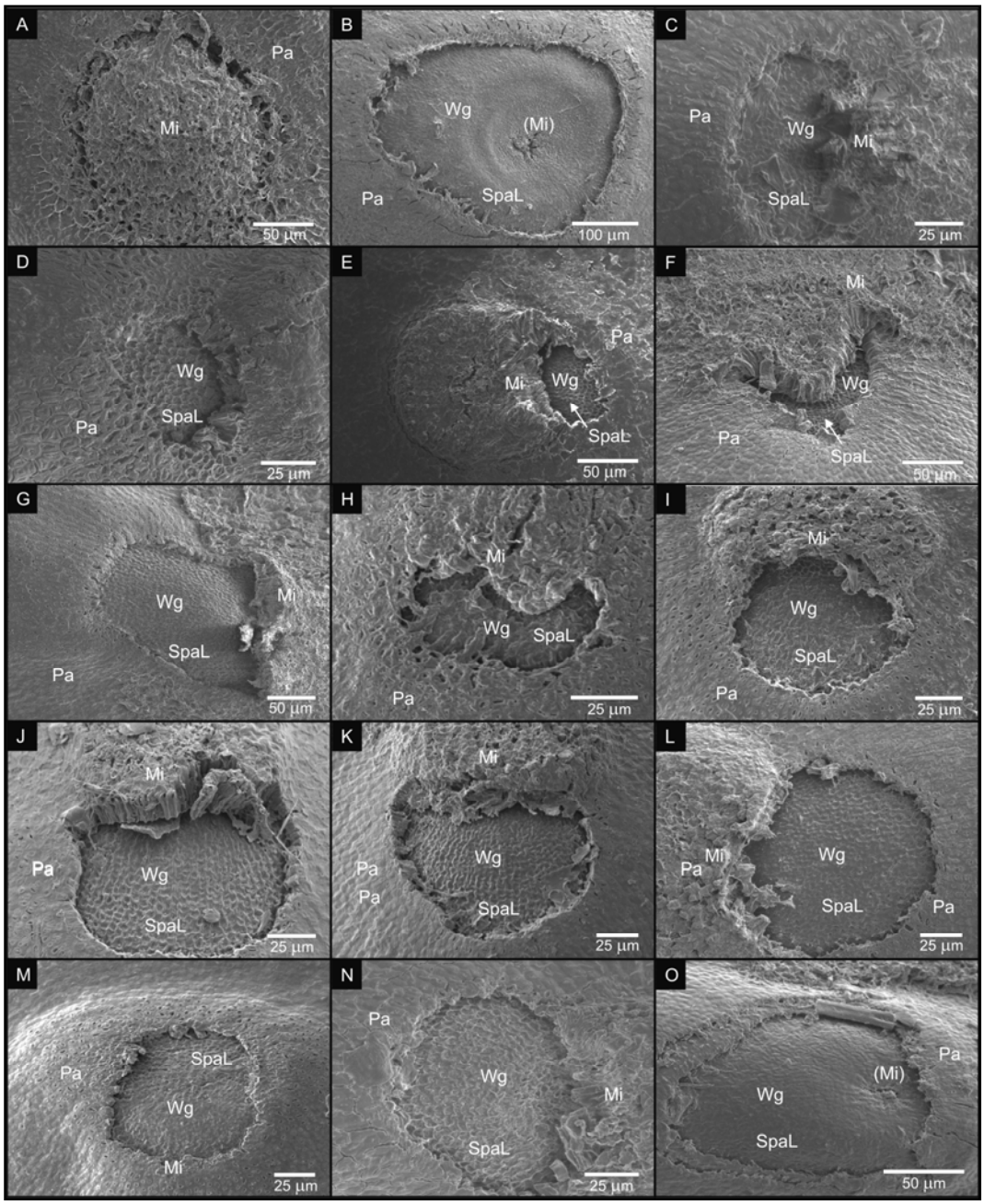



Figure 2.7. Scanning electron micrographs of *Geranium carolinianum* seeds: (A) micropylar area of a dormant seed; (B) micropylar area of a non-dormant (heat-treated) seed immersed in water for 10 min; (C) micropylar area of a non-dormant seed without outer permeable cell layers; (D) raised hinged valve of a non-dormant seed without outer permeable cell layers (soaked in water for 10 min); (E) relatively smooth inner periclinal cell walls of water-gap palisade cells near the micropyle; (F) convex-shaped inner periclinal cell walls of water-gap palisade cells near radicle end; (G) water-gap opening of a non-dormant seed without outer permeable cell layers and hinged valve dislodged (soaked in water for 20 min). Abbreviations: Cr, crack demarcates the margin of the water-gap; Hi, hilum; Hv, hinged valve (opened); [Hv], hinged valve (closed); Mi, micropyle; Mo, single layer of middle parenchyma cells; Op, outermost polygonal parenchyma cell layer; Pa, palisade cells; PaL, elongated palisade cells of the micropyle; SpaL, elongated subpalisade cells; *, subpalisade cells with smooth outer periclinal cell wall; **, subpalisade cells with concave outer periclinal cell wall. The large white short arrow in (G) indicates the region of initial water uptake.


A
Pa
Mi
50 µm
B
Wg
(Mi)
Pa
SpaL
100 µm
C
Pa
Wg
Mi
SpaL
25 µm
D
Wg
Pa
SpaL
25 µm
E
Pa
Mi
Wg
SpaL
50 µm
F
Mi
Wg
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SpaL
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G
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Figure 2.8 (continued)

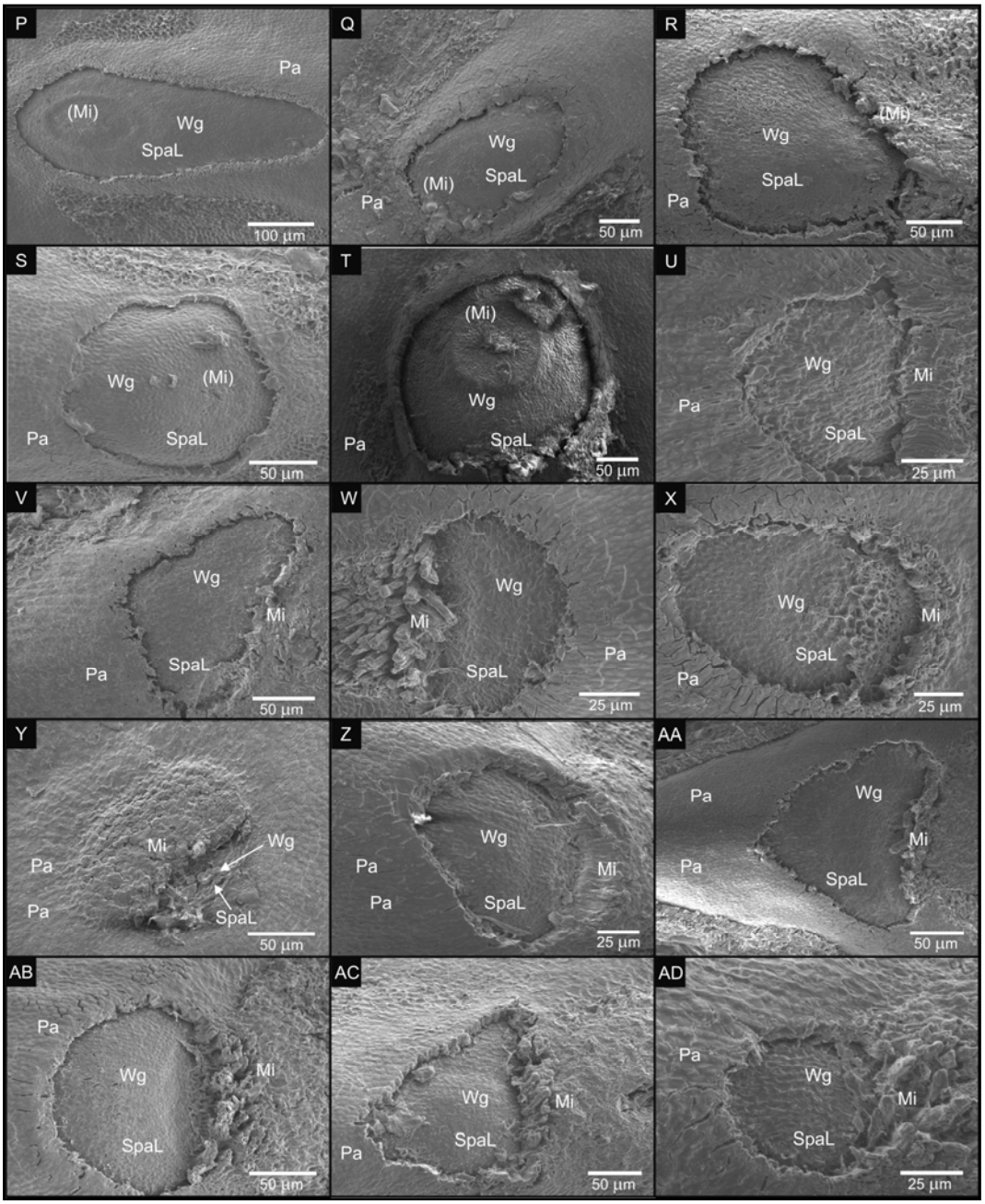

Figure 2.8. Scanning electron micrographs of water-gaps of Geraniaceae species: (A) *H. Pimpinellifolia*; (B) *C. macrophylla*; (C) *G. grandistipulatum* ; (D) *G. sessiliflorum*; (E) *G. Disectum*; (F) *G. bohemicum*; (G) *G. tuberosum*; (H) *G. polyanthes*; (I) *G. ocellatum*; (J) *G. molle*; (K) *G. pusillum*; (L) *G. pyrenaicum*; (M) *G. lucidum*; (N) *G. madrense*; (O) *E. taxanum*; (P) *E. ciconium*; (Q) *E. cicutarium*; (R) *E. manescavii*; (S) *E. moschatum*; (T) *M. angustifolia*; (U) *P. alternans*; (V) *P. crithmifolium*; (W) *P. nanum*; (X) *P. vitifolium*; (Y) *P. australe*; (Z) *P. dolomiticum*; (AA) *P. mirrhifolium*; (AB) *P. alchemilloides*; (AC) *P. quinquelobatum*; (AD) *P. tongaense*. Mi, Micropyle; (Mi) location of the micropyle before it is dislodged due to imbibition. Abbreviations: Pa, palisade cells; SpaL, elongated subpalisade cells of the micropylar region; Wg, water-gap.

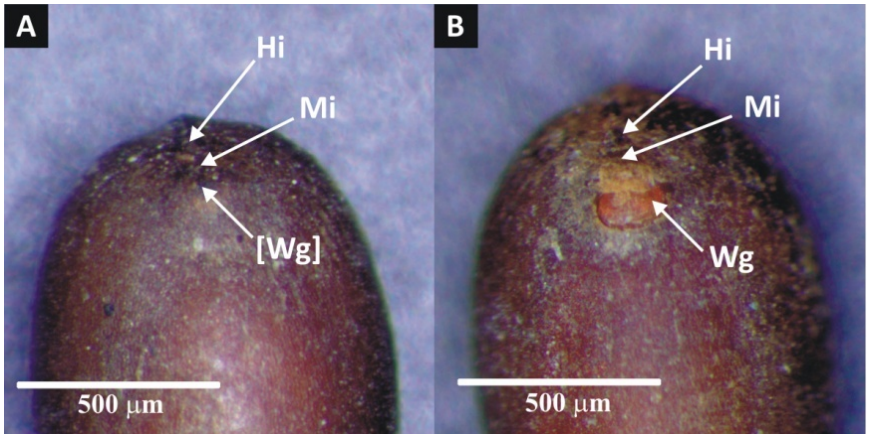


Figure 2.9. Micropylar region of *G. carolinianum* seeds exhumed after 4 months of burial in soil at 2 cm depth: (A) impermeable seed; (B) permeable seed. Abbreviations: Hi, hilum; Mi, micropyle; Wg, water-gap open; [Wg], water-gap closed.

# CHAPTER 3

## Acquisition of physical dormancy and ontogeny of the micropyle-water gap complex in developing seeds of *Geranium carolinianum* L. (Geraniaceae)

## INTRODUCTION

Seeds or fruits with physical dormancy (PY) acquire water impermeability during the final stages of maturation drying (Baskin and Baskin, 1998; Li *et al*., 1999*a*; Jayasuriya *et al*., 2007*b*; Qu *et al*., 2010). One or more water-impermeable palisade cell layers in the seed- or fruit-coat along with closed micropyle, hilum and chalaza form physical barriers for water entry, thus causing PY (Egley, 1989; Baskin and Baskin, 1998; Baskin *et al*., 2000). Initial uptake of water on breaking of PY occurs at the water gap, a morpho-anatomically specialized area in the seed or fruit coat(s) of species with PY (Baskin *et al*., 2000; Jayasuriya *et al*., 2007*a*; Gama-Arachchige *et al*., 2010). Throughout the dormancy period, the water gap, which acts as an environmental signal detector, remains occluded by various specialized structures that must be disrupted or dislodged for the seeds to become permeable (Baskin *et al*., 2000).

PY is known to occur in seeds or fruits of 18 angiosperm plant families and is unknown in gymnosperms (Nandi, 1998; Horn, 2004; Baskin *et al*., 2000; Baskin 2003; Koutsovoulou *et al*., 2005; Baskin *et al*., 2006; APG III, 2009; Tsang, 2010). Acquisition of PY during seed development has been studied in several families, including Anacardiaceae (*Rhus aromatica*, *Rhus glabra*) (Li *et al*., 1999*a*), Bixaceae (*Bixa orellana*) (Yogeesha *et al*., 2005), Convolvulaceae (*Ipomoea lacunosa*)

(Jayasuriya *et al*., 2007*a*), Cucurbitaceae (*Sicyos angulatus*) (Qu *et al*., 2010), Fabaceae (*Vicia faba*, *Peltophorum pterocarpum*) (Ellis *et al*., 1987; Mai-Hong *et al*., 2003 ) and Malvaceae (*Sida spinosa*) (Egley, 1976). However, acquisition of PY in Geraniaceae has not previously been investigated.

Water gap anatomy, morphology, origin and location differ among families as well as within the same family (Baskin *et al*., 2000). In addition, the anatomy and morphology of the water-gap region differ from that of the rest of the seed/fruit coat. Twelve different water-gap types have been characterized to date in seven of the 18 angiosperm families with PY (Baskin and Baskin, 1998; Li *et al*., 1999*b*; Baskin *et al*., 2000; Baskin, 2003; Jayasuriya *et al*., 2007*a*, 2008; Hu *et al*., 2008; Turner *et al*., 2009; Gama-Arachchige *et al*., 2010). However, developmental studies have been carried out on only a few of these water-gap types: the bulge adjacent to the micropyle in Convolvulaceae (Jayasuriya *et al*., 2007*a*), the carpellary micropyle in Anacardiaceae (Li *et al*., 1999*a*), the chalaza in Malvaceae–Malveae (Winter, 1960; Egley and Paul, 1982), the lens in Fabaceae–Caesalpinioideae (Manning and Van Staden, 1987) and the raphal scar in Cannaceae (Grootjen and Bouman, 1988).

Various aspects of development in the physically dormant seeds of Geraniaceae have been described by several authors. Boesewinkel and Been (1979) documented seed development in Geraniaceae and described development of the ovule, embryo and the seed coat. Development of the light line in the Geraniaceae seed coat has been described by Meisert *et al*. (2001) using transmission electron micrographs. However, the development of the water gap in Geraniaceae (hinged valve gap) remains

uninvestigated although its anatomy and morphology have been characterized by Gama-Arachchige *et al*. (2010).

The current study focuses on *Geranium carolinianum*, an herbaceous winter annual of Geraniaceae that is native to eastern North America (Piper, 1906; Small, 1907; Aedo, 2000). The species is a widely distributed weed in North America (Spencer, 1976; Haragan, 1991) and has been reported to be a naturalized weed in China, Japan, northern Europe, South America and Taiwan (Peng, 1978; Xu and Aedo, 2008; Nishida and Yamashita, 2009). Flowers of *G. carolinianum* open early in the morning and are imperfectly proterandrous, i.e. the stigma becomes receptive a few hours prior to anthesis. By noon, the flowers are selfed unless insect-pollinated. Seeds mature within 2–3 weeks after pollination. Usually five seeds are borne in a fruit, which are ultimately released by an explosive mechanism (Robertson, 1893; Dubay and Murdy, 1983).

Freshly matured seeds of *G. carolinianum* exhibit PY and a low level of physiological dormancy (PD). Also, a small fraction of innately permeable seeds is produced. PY is caused by a layer of water-impermeable palisade cells and tightly closed micropyle and chalaza (Gama-Arachchige *et al*., 2010).

The objectives of the current study were to (1) determine the stage of acquisition of PY by the developing seeds of *G. carolinianum*; (2) identify the stages of physiological changes in developing seeds; and (3) compare the simultaneous development of the water-gap region, seed coat, chalaza and micropyle.

## MATERIALS AND METHODS

### *Growing plants*

One hundred rosettes of *Geranium carolinianum* growing on the campus of the University of Kentucky, Lexington, KY, USA, were transplanted into 15-cm-diameter plastic pots filled with garden soil in March 2010 and kept on benches inside a non-heated greenhouse. The plants were watered as necessary.

### *Pollination*

In mid-April 2010, flowers were selfed by touching their stigmata with anthers with the aid of a needle around 1100 h. Subsequently, each pollinated flower was tagged to identify the date of pollination. To prevent seed dispersal after 18 d after pollination (DAP), rostra of fruits were tied with a piece of thread.

### *Ovule and seed collection*

Flower buds at different stages of development were collected. After pollination, fruits were collected daily, from 0 to 21 DAP. The collected material was placed in ZipLoc bags and taken to the laboratory immediately. Another set of 50 fruits from each DAP was collected, stored in cloth bags and dried for 2 weeks inside a non-heated greenhouse.

### *Measurement of seeds*

Thirty ovules/seeds (hereafter seeds) were separated from collected fruits of each DAP. The length and width of each seed were measured using a dissecting microscope with a calibrated micrometer eyepiece. From each DAP, five replicates of

five seeds each were weighed (fresh mass). Subsequently, the weighed seeds were oven dried at 105 °C to achieve a constant mass (dry mass). Weights were recorded to the nearest 0.0001 g. Seed moisture content was calculated (fresh mass basis).

*Morphological changes in seeds during development*

Using a dissecting microscope (Zeiss STEMI SVII) equipped with an Olympus DP25 digital camera, micrographs of developing seeds (0–20 DAP) were taken to compare morphological changes that occur during seed development.

*Imbibition and germination of developing seeds*

Experiments were conducted to determine percentage imbibition, germinability and acquisition of PY in developing seeds of *G. carolinianum*. Two hundred seeds each from 0 to 21 DAP were selected. Embryos were isolated from 100 seeds of each sample under a dissecting microscope using forceps and a razor blade. The other 100 seeds were left intact. Five replicates of 20 isolated embryos and of intact seeds were incubated on moist sand at 20/10 °C (12 h/12 h) under a 14 h/10 h daily light/dark period. Photon irradiance during the light phase was approx. 40 μmol $m^{-2}$ $s^{-1}$, 400–700 nm and the light source was cool white fluorescent tubes.

Seeds were considered germinated when the radicle emerged at least 2 mm in intact seeds or when it grew at least 2 mm beyond the tip of cotyledons in isolated embryos. The number of imbibed intact seeds and germinated seeds (both embryos and intact) was counted after 4 weeks.

*Imbibition of mature seeds with different moisture contents*

To determine the seed moisture content at which impermeability is acquired, 50 seeds at 18 DAP were removed from fruits and ten each were transferred to wire mesh screens placed over water inside five humid (closed) boxes. They were placed inside an incubator at 25 °C for 3 h to maintain 99 % relative humidity (RH) in order for the seeds to reach the maximum possible seed moisture content (to compensate for desiccation). The seeds were then placed in open Petri dishes and weighed (initial mass) using an electric balance. They were allowed to dry on the same balance under laboratory conditions (approx. 23 °C and 50–60 % RH) until the mass reached the value corresponding to the desired moisture content, as calculated from the following formula (Baalbaki *et al*., 2009):

$$W_F = [(100 - MC_i) - (100 - MC_a)] \times W_I$$

where $W_F$ is the final mass of seeds per replicate, $W_I$ is the initial mass of seeds per replicate, $MC_i$ is the initial seed moisture content and $MC_a$ is the adjusted seed moisture content. The initial moisture content ($MC_i$) of seeds at the same developmental stage (18 DAP) was calculated after allowing the seeds to reach the maximum possible seed moisture content. This procedure was followed to achieve 40, 35, 30, 25, 20, 15, 14, 13, 12, 11, 10, 9 and 8 % seed moisture content.

Five replicates of ten seeds each from the seeds with the above adjusted moisture content values were placed inside Petri dishes lined with moist filter papers and sealed with Parafilm. The number of imbibed seeds in each sample was counted daily for 4 weeks.

*Imbibition and germination of slow-dried developing seeds*

To determine the developmental stage at which seeds achieve impermeability if they are subjected to drying, 200 seeds were selected from those dried inside the non-heated greenhouse (0–21 DAP), and 100 of them were scarified mechanically with a razor blade at places on the seed coat away from the micropylar and chalazal ends without damaging the embryo. The other 100 seeds were left intact. All seeds were germinated as described above. The number of imbibed and germinated seeds were counted after 4 weeks.

*Anatomical changes in seeds during development*

To study anatomical changes in the ovules and seeds during development, ten randomly selected flower buds at different stages of development and fruits 0–20 DAP were kept fixed in formaldehyde – acetic acid until used. They were then dehydrated in a series of tertiary-butanol and embedded in paraffin wax. Subsequently, 12-µm longitudinal sections of the buds and fruits were cut using a microtome (LEICA RM 2135). Sections were stained with 1 % safranin and 2 % fast green solutions. The micropylar and chalazal regions and the seed coat away from micropyle and chalaza were observed and photomicrographed using an Olympus BX40 light microscope and an Olympus DP25 digital camera. Drawings of different types of mature palisade cells and subpalisade cells in the seed coat were made using the photomicrographs. To examine the anatomy of unstained seeds, longitudinal sections through the chalaza and the micropyle of mature seeds were taken using a VIBRATOME 1500 sectioning system and photomicrographed using the same microscope and camera.

*Statistical analysis*

Percentage germination and imbibition data were normalized by arcsine-transformation prior to analysis. Normalized germination and imbibition data were analysed by one-way ANOVA, and Duncan's multiple range test was used to determine significant differences between each treatment ($P < 0.05$). All analyses were carried out using SAS ver. 9.2 software. Non-transformed data were used for graphical representations.

## RESULTS

*Measurement of seeds*

Immediately after pollination (0 DAP), seed width was slightly greater than seed length (Fig. 3.1A). After 2 DAP, this was reversed, making seed length greater than seed width. Thereafter, both seed length and width showed a similar pattern of increase. Both parameters increased up to 7 DAP and then remained more or less the same until 16 DAP. Subsequently, following a slight increase, the length and width declined until 19 DAP but did not change thereafter.

Seeds 0–3 DAP could not be weighed due to their extremely low mass. From 4 DAP onwards, seed moisture content declined throughout the period of development (Fig. 3.1B). The decline was gradual from 4 DAP (93.1 ± 0.4 %) to 18 DAP (50.4 ± 1.0 %) and rapid from 18 DAP to 19 DAP (12.4 ± 1.3 %). Thereafter, the moisture content decreased gradually and reached 11.1 ± 0.9 % by 21 DAP. Average dry mass

increased until 14 DAP, when the seed reached physiological maturity and it remained the same afterwards.

*Morphological changes in seeds during development*

Developing seeds undergo a series of morphological changes from the ovule stage to the fully matured stage. Changes are apparent with respect to seed shape, colour and coat transparency.

During the early stages of development, seeds of *G. carolinianum* exhibited dramatic changes in shape. At 1 DAP, seeds were trigonal but became wedge-shaped by 3 DAP (Fig. 3.2A, B). At 4 DAP, seeds were bullet-shaped (Fig. 2C). They changed into an oblong shape due to increasing width at 6 DAP (Fig. 3.2D) and remained so until 18 DAP. By 20 DAP, when the seeds were ready for dispersal, they were ovoid (Fig. 3.2J).

At 0 DAP, ovules assumed a dull greyish green colour. They gradually turned into a brighter green around 12 DAP (Fig. 3.2E) which persisted until 14 DAP (Fig. 3.2F). By this stage, the micropylar area had become clearly visible as a small white patch close to the radicle end. An orange–brown tinge appeared at the radicle end by 15 DAP (Fig. 3.2G) which gradually spread throughout the seed and turned the whole seed coat orangey brown by 18 DAP (Fig. 3.2H, I). When ready for dispersal at 20 DAP, seeds assumed a dark mauve colour (Fig. 3.2J).

In developing and mature seeds, the seed coat remained translucent. After 15 DAP (Fig. 3.2G), with deposition of tannin in cells of the palisade layer, opaque spots appeared throughout the seed coat.

*Imbibition and germination of developing seeds*

From 0 to 18 DAP all the seeds imbibed but by 19 DAP, only 37 ± 16 % did so (Fig. 1C). By 20 DAP, all the seeds were impermeable. A low percentage (6 ± 1 %) of developing seeds gained germinability on 9 DAP (Fig. 3.1D). Thereafter, germinability gradually increased to its maximum on 16 DAP (97 ± 2 %). However, the seedlings produced from 9 to 12 DAP were abnormally small. After 18 DAP there was a rapid decrease in percentage germination, and by 20 DAP none germinated. This rapid decrease in percentage germination coincided with the decrease in imbibition 19 DAP (Fig. 3.1C).

Similar to intact seeds, isolated embryos started to germinate 9 DAP, yet at a higher percentage (51 ± 12 %; Fig. 3.1E) than intact seeds (6 ± 1%; Fig. 1D). From 13 DAP, germination of isolated embryos (43 ± 9 %) increased gradually to the maximum (100 ± 0 %) by 16 DAP, and remained the same until 21 DAP. As seen in fresh intact seeds, seedlings produced from 9 to 12 DAP were abnormally small.

*Imbibition of mature seeds with different moisture contents*

All the seeds having moisture contents from 40 to 13 % imbibed (100 %; Fig. 3.3A). However, the percentage of imbibed seeds declined to 94 ± 6 % when the moisture content was 12 % and on further drying, all the seeds became impermeable at a moisture content of 11 % or less. At a moisture content of 13 %, all the seeds imbibed

within 24 h (Fig. 3.3B), but those dried to 12 % moisture content required 11 d for 94 ± 3 % of them to imbibe. On further drying to 11 % moisture content, none of the seeds imbibed.

*Imbibition and germination of slow-dried developing seeds*

Seeds up to 8 DAP could not be used for experiments as their seed coats cracked on drying. Dried developing seeds showed a rapid reduction in imbibition from 9 DAP (100 %) to 13 DAP (0 %; Fig. 3.4A). Thereafter, all the seeds remained completely impermeable. Accordingly, the germination of dried developing seeds remained low, from only 25 ± 6 % on 9 DAP to 0 % by 13 DAP, indicating complete seed impermeability (Fig. 3.4B).

All the dried and scarified developing seeds were permeable throughout the experiment (Fig. 3.4C). The germination of dried and scarified developing seeds at 9 DAP was low, but markedly increased by 12 DAP (Fig. 3.4D). From 15 DAP onwards, germination was 100 %.

*Anatomical changes in seeds during development*

Development of integuments in ovules.

Formation of an outer integument (o.i.) and an inner integument (i.i.), each with two cell layers originating from the dermatogen, can be identified in developing ovules of unopened flower buds (Fig. 3.5A). However, a cluster of cells forms in the tip of the o.i. Fully developed ovules become six-seriate by periclinal cell division of the inner cell layer of each integument (Fig. 3.5B). At this stage, cells of the outermost layer of

the o.i. and of the middle and innermost layers of the i.i. can be distinguished from the other cell layers, owing to their larger cell size.

Seed coat development.

From 1 DAP to 4 DAP, cells of the integuments continue to enlarge without noticeable differentiation (figures not shown). By 6 DAP, a significant degree of cell differentiation has taken place (Fig. 3.5C). Cells of the outer parenchymal layer of the o.i. become enlarged and in certain places in the seed coat, the middle parenchymal layer of the o.i. becomes multi-layered. By this stage, cells of the innermost layer of the o.i. and outermost layer of the i.i. are longitudinally elongated, forming the palisade layer and the subpalisade layer. Cells of the former are smaller than those of the latter and bear conspicuous nuclei closer to the lower periclinal wall (Fig. 3.5C). These palisade cells enlarge and achieve full size by 8 DAP (Fig. 3.5D). The light line is feebly visible at a level approximately one-third below the outer periclinal wall of the cells of the palisade layer. Also, fine short crystals appear in the palisade cells. During this stage, the inner parenchymal cells of the i.i. start to contract due to the compression exerted by the developing embryo (Fig. 3.5D).

By 13 DAP, the outermost and the middle parenchyma of the o.i. have started to collapse and the light line in the palisade layer has become more conspicuous (Fig. 3.5E). Cytoplasm of palisade cells appears to be confined to the region near the inner periclinal wall of cells. Deposition of tannin in certain enlarged cells of the palisade layer becomes visible at this stage. In these tanniferous cells, the light line appears to be curved slightly outwards. Cell walls of the subpalisade layer have thickened.

The seed coat is fully developed by 18 DAP. In the palisade layer, cell walls are thickened (Fig. 3.5F). Lumens containing crystals have developed in the region close to the inner periclinal wall of palisade cells (Fig. 3.6A). In tanniferous cells, tannin deposition has become extensive (Fig. 3.6A). Middle and inner parenchyma of the i.i. have become compressed. By 20 DAP, a golden yellow colour can be observed in the palisade layer in unstained specimens, while all the other layers are colourless (Fig. 3.7A–D).

Development of the micropyle and water gap.

Development of the water gap takes place in a region of the integuments opposite to the hilum and adjacent to the micropyle. In developing ovules, due to anticlinal cell division, integuments gradually surround the nucellus, forming the micropyle (Fig. 3.8A–C). Soon after pollination, cells of the micropylar region start to differentiate. At 01 DAP, cells of the inner layer of the o.i. (pro-palisades) and outer layer of the i.i. (pro-subpalisades) start to elongate radially (Figs 3.9A and 3.10A). At 3 DAP, pro-subpalisades start to divide periclinally (Figs 3.9B and 3.10B), resulting in multiple layers by 4 DAP (Figs 3.9C and 3.10C). Due to elongation, pro-palisades and pro-subpalisades have acquired the typical palisade cell shape. However, directly interior to the micropylar opening, some of the subpalisade cells do not elongate but divide periclinally to form a stack of much shorter subpalisade cells. Other subpalisade cells are much longer than the palisade cells. At this stage (Fig. 3.9C), the palisade cells close to the micropyle divide periclinally, adding 1–2 layers of parenchyma cells towards the middle layer of the o.i. Also the middle parenchyma layer immediately above the water-gap palisades has become multilayered. Cells of the outermost layer of the o.i. are smaller near the micropyle than they are in the rest of the seed coat

(Figs 3.9C and 3.10C). By 6 DAP, the upper part of the elongated palisade cells of the micropyle bends sideways (Figs 3.9D and 3.10D).

At 8 DAP, walls of palisade and subpalisade cells have thickened, and the cytoplasm in those cells appears to be confined to the centre of the cells (Fig. 3.10E). Cell width is less in water-gap palisades adjacent to the bent palisades and increases towards the radicle end of the seed coat. Similarly, cell width of subpalisades gradually increases outwards from the micropyle. At this stage, the light line appears faintly as a discontinuous line in the palisades of the water-gap region. Close to the micropyle, the middle layer of the i.i. is compressed (Figs 3.9E and 3.10E).

At 14 DAP, the light line appears as a continuous and conspicuous line, and at the stretched palisades of the micropyle, it appears as two broad lines (Figs 3.9F and 3.10F). Consequently, cytoplasm in these cells exists in three zones. Most of the cytoplasm is seen in the zone above the light lines, and smaller amounts of cytoplasm can be seen in zones between and below the lines. However, in the water gap, the light line appears as a single line in the palisades, with cytoplasm restricted to the portion below the light line. Lumens are visible in the upper portion of the palisades. By this stage, the inner periclinal walls of water-gap palisades are flat near the micropyle and become convex towards the radicle end (Wpa-I and II; Fig. 3.6A). Accordingly, the outer periclinal walls of water-gap subpalisades are flat and concave (SpaL-I and II; Fig. 3.6B). In the subpalisade layer, lumens have appeared at the expense of the cytoplasm (Figs 3.9F and 3.10F).

By 18 DAP, the light line is more resolved (Figs 3.9G and 3.10G). Lumens in palisades are prominent, and deposits of these cells appear to be pushed towards the inner periclinal wall. In the micropyle, the parenchymal cells that originated from the palisade cells assume a rectangular shape in longitudinal section (Fig. 3.10G). The two light lines in the stretched palisades in the micropyle appear to be broader by this stage. Stacked shorter subpalisade cells directly below the micropylar opening are clearly visible by this stage (Spas; Fig. 3.6B). The middle layers of the i.i. appear to be compressed further (Figs 3.9G and 3.10G).

When the seeds are ready for dispersal 20 DAP, the outer and middle parenchyma layers of the o.i. are noticeably compressed (Figs 3.9H and 3.10H). In stretched palisades of the micropyle, the golden yellow colour is paler than in other regions (Fig. 3.7A, B). The palisade layer resembles an atoll by this stage, due to the differential cell length of the middle (PaL-I and II; Fig. 3.6A) and bent palisades (Bpa; Fig. 3.6A) of the micropyle. By this stage, sparse development of chlorophyll is evident in several cells of the middle layer of the i.i., which align with the micropylar opening. Such chlorophyll development is not seen anywhere else in the seed coat (Fig. 3.7A).

Throughout the development and even after dispersal, the exostome remains open at the outer parenchymal layer of the o.i. (Fig. 3.10C). It becomes closed at the middle parenchymal and palisade layers of the o.i. in later stages of development, while the endostome is closed at all the layers of the i.i. after pollination. Due to maturation drying, which commences around 19–20 DAP, palisade cells shrink, sealing the micropylar opening and the water gap (Figs 3.9H and 3.10H).

Development of the chalaza.

At initial stages of development of the integuments, a cell mass that is not differentiated into integument tissues can be identified between the bases of the two integument primordia (Fig. 3.8A, B). This undifferentiated cell mass (hypotase) forms the chalazal opening in developing and mature seeds. In developing ovules, the chalaza and micropyle are located at opposite ends of the same axis. After pollination, their relative locations shift and orient their axes 90° to each other.

In mature ovules, the vascular tissue can be identified developing along the middle parenchyma of the o.i. This middle parenchymal layer consists of multiple cell layers in the region between the hilum and chalaza. This condition is prominent near the chalazal end. In ovules at 0 DAP, cells of the chalazal opening do not show any noticeable differentiation (Fig. 3.11A). Radial elongation of pro-palisades and pro-subpalisades is apparent by 2 DAP (Fig. 3.11B). However, these cell layers are not continuous at the chalazal opening. Nevertheless, the undifferentiated cells in the opening divide increasing the cell number, and they are arranged compactly. These chalazal parenchyma cells filling the discontinuation of the palisade layers are radially stretched (Fig. 3.11B).

By 3 DAP, pro-palisades, pro-subpalisades and chalazal parenchyma are stretched further (Fig. 3.11C). Accumulation (presumably) of suberin, which persists hereafter in the middle parenchymal layers of the o.i. and nucellar tissue adjacent to the chalazal opening, is visible. These deposits are contained in enlarged parenchyma cells. Due to cell elongation, pro-palisades and subpalisades appear as typical

palisades by 6 DAP (Fig. 3.11D). As in the micropyle, chalazal palisades and subpalisades are also stretched more than those in the rest of the seed coat but to a lesser extent than those in the micropyle. Moreover, these palisades are oblique near the chalazal opening.

By 10 DAP, thickening of the radial walls of palisade cells causes the cytoplasm to appear in a bowling pin shape (Fig. 3.11E). The light line is discernible as a discontinuous line in the palisade layer, but is absent in palisade cells adjacent to the chalazal opening. The chalazal opening has become narrow 17 DAP. The light line appears to be continuous in the palisade layer and is absent in the chalazal opening and in palisades immediately adjacent to the opening (Pal, Fig. 3.6A; Fig. 3.11F). Cell wall thickening in the palisades has become extensive, restricting the deposits to the cell base. Cytoplasm of subpalisades appears to be depleted, and large lumens are visible in cells (Fig. 3.11F).

In mature seeds, the uniseriate outer parenchymal layer and the multilayered middle parenchymal layer of the o.i. are compressed and crushed. This causes suberin deposits in the middle parenchymal cells near the chalazal opening to compress towards palisades, forming a plug from the outside (Fig. 3.11F). Similarly, the chalazal opening is clogged from the inside of the subpalisades with suberized cells of the hypotase. Chalazal parenchyma cells are crushed due to contraction of palisades and subpalisades. Consequently, the chalazal opening is sealed tightly (Fig. 3.8C, D).

## DISCUSSION

The time span of development of seeds of *G. carolinianum* from pollination to dispersal, according to the current study, was 20 d. This result is in agreement with the study of Dubay and Murdy (1983) on the same species. During the period between 2 and 7 DAP, active cell division and cell expansion were indicated by a rapid increase in seed length and width. Seeds attained physiological maturity at 14 DAP, when the highest dry matter content was recorded. The steep decline of moisture content from 18 to 19 DAP signifies the loss of vascular supply to the seed and the commencement of maturation drying (Fig. 3.12). Consequently, seed size declined rapidly from 17 to 19 DAP due to the shrinkage of tissues.

Extensive cell division in the region opposite to the chalaza of ovules and young seeds results in a dramatic change in seed shape. Until 15 DAP, the seed coat remains translucent, revealing the colour of the embryo sac or embryo. The greyish green colour of the seed at 0 DAP turns into a brighter green with the growth and chlorophyll development of the embryo. By 15 DAP, an orangey brown colour develops in the seed coat, probably due to oxidation of polyphenolic compounds in the palisade layer (Marbach and Mayer, 1975; Werker, 1997). This masks the green colour of the embryo. However, the seed coat remains translucent even after this stage, yet to a lesser extent. Compaction and compression of the palisade layer after maturation drying and probably the chemical changes taking place in the seed coat (Werker, 1997) cause its colour to turn mauve. Tannin deposits in tanniferous cells of palisade layer appear as brown opaque spots scattered throughout the seed coat (Boesewinkel and Been, 1979).

Colour change of the seed coat during development is reported in other PY species (Werker, 1997). Nevertheless in PY species, seeds with their mature colour developed can still be permeable even though the seed coat is already impermeable, due to incompletely sealed openings. The micropyle, hilum and chalaza have been reported to be the openings through which water loss takes place during the final stages of maturation drying, and these very sites have been reported to allow water entry as well, i.e. into seeds with its mature colour developed (Egley *et al*., 1983; Jayasuriya *et al*., 2007*b*). In *Ipomoea lacunosa*, approx. 45 % of seeds with their mature colour developed remained permeable due to incomplete sealing of hilar fissure (Jayasuriya *et al*., 2007*b*). In *Sida spinosa*, permeability of seeds even after the development of mature colour is caused by an incompletely sealed chalaza (Egley *et al*., 1983). At the end of maturation drying, these permeable seeds become impermeable with the completion of sealing of the openings. In *G. carolinianum*, the micropyle and/or chalaza might be the sites of final water loss. However, it was not determined in the current study due to high rate of drying and small size of seeds, which make it impossible to block the micropyle and chalaza individually.

The simultaneous and significant reduction of imbibition and germination percentages indicates that seeds of *G. carolinianum* start to acquire PY on 19 DAP, and by 20 DAP all the seeds are impermeable. Maturation drying, which occurs at the same stage, plays a role in causing impermeability of the seed coat. When the seed moisture content was 11.4 % at 19 DAP, 37 % of the seeds imbibed. According to seed moisture experiments, none of the seeds imbibed at a seed moisture content of 11 %. Therefore, the critical seed moisture content at the acquisition of PY is 11 %. This critical moisture content varies among species with PY. However, most of the

determined critical moisture content values lie within the range 3–15%. For example, this value is 3 % in *Lupinus arboreus* (Hyde, 1954) and *Abelmoschus esculentus* 'Lousiana Green Velvet' (Standifer *et al*., 1989), 4.7 % in *Ormosia arborea* (Brancalion *et al*., 2010), approx. 7 % in *Peltophorum pterocarpum* (Mai-Hong *et al*., 2003), 8.9 % in *Lupinus varius* (Quinlivan, 1970) and in the endocarp of *Rhus glabra* (Li *et al*., 1999*a*), 11 % in *Crotolaria spectabilis* (Egley, 1979), 12 % in *Gossypium hirsutum* (Patil and Andrews, 1985) and *Bixa orellana* (Yogeesha *et al*., 2005), 13 % in *Ipomoea lacunosa* (Jayasuriya *et al*., 2007*b*), 13.6 % in the endocarp of *Rhus aromatica* (Li *et al*., 1999*a*), approx. 14% in *Trifolium pratense* and *Trifolium repens* (Hyde, 1954), 14.3 % in *Sida spinosa* (Egley *et al*., 1983) and 14.6 % in *Sicyos angulatus* (Qu *et al*., 2010).

Until the critical moisture level is reached, imbibition is not completely hindered. At 12 % moisture content, i.e. when the moisture content is slightly above this critical value, the number of imbibed seeds increases slowly, showing a considerable resistance to water uptake. This may be an indication of incomplete sealing of openings and/or partial impermeability of the seed coat. A similar relationship between seed moisture content and impermeability was reported by Quinlivan (1970) in seeds of three species of *Lupinus*. In *Lupinus varius*, at 10.5 % moisture content, the percentage of imbibed seeds increased slowly for 12 weeks to reach 95 %. At 8.9 % moisture content, the percentage of imbibed seeds was 10 % during the same period. The results were similar for the two other species of *Lupinus* in that study.

Germination of fresh, intact seeds of *G. carolinianum* does not occur until 9 DAP, presumably due to incomplete development of the embryo. Although seeds are

germinable from 9 DAP until PY is achieved, seedlings produced from seeds harvested 9 DAP to the stage of physiological maturity (14 DAP) are abnormally small. This indicates that developing embryos cannot produce normal seedlings until they reach physiological maturity at 14 DAP. When the seed coat is removed, a higher germination percentage of the isolated embryos than of intact seeds was observed from 9 to 12 DAP, as a consequence of elimination of the obstruction for the protrusion of the radicle imposed by the seed coat. A similar increase in germination percentage after scarification of developing seeds was observed in *Abutilon theophrasti* (Winter, 1960) and *Sida spinosa* (Egley, 1976). In *G. carolinianum*, maximum germination (100 %) of both intact seeds and isolated embryos was attained 16 DAP, 2 d after physiological maturity. However, these embryos or intact seeds at permeable stages did not begin germination within the first 2 weeks of incubation. This is evidence for the small amount of PD in developing seeds after physiological maturity as reported in mature fresh seeds of *G. carolinianum* by Baskin and Baskin (1974) and Gama Arachchige *et al*. (2010).

Slow drying can cause the time of acquisition of PY to be shifted to an earlier stage of development. Slow-dried intact seeds acquired impermeability at 13 DAP, i.e. 1 d prior to physiological maturity. Comparably, in *Trifolium ambiguum*, acquisition of PY in slow-dried seeds occurred at a developmental stage approx. 20 d earlier than in untreated seeds (Hay *et al*., 2010). Slow-dried scarified seeds of *G. carolinianum* germinated to 33 % on 9 DAP. Thus, some seeds of *G. carolinianum* are desiccation-tolerant from at least 9 DAP, and all the seeds were desiccation-tolerant from 12 DAP onwards. Therefore, as expected, seeds become desiccation-tolerant before they achieve impermeability. Germination from 9 to 11 DAP is improved by slow drying

when compared with fresh intact seeds. A similar result was observed with slow-dried seeds of *Abelmoschus esculentus* (Damir, 1997) at early stages of development and in *Vicia sativa* (Samarah *et al*., 2004) during middle and later stages of development. After 12 DAP, none of the dried intact seeds of *G. carolinianum* germinated as they were already impermeable. However, dried scarified seeds gave almost 100 % germination 12 DAP onwards, a significant increase in germination when compared with that of fresh isolated embryos. Thus, the improved germination after slow-drying is masked by the early occurrence of PY.

Ontogeny of integuments and the seed coat of *G. carolinianum* is comparable with that of *G. pratense* (Boesewinkel and Been, 1979). Similar to *G. pratense*, integuments of the ovule of *G. carolinianum* originate from the dermatogen. The origin and development of each cell layer in integuments is also similar in these two species. As yet, the development of the water gap has not been investigated in any species of Geraniaceae.

Palisade cells form the main barrier for water uptake in the seed coat in Geraniaceae (Schulz *et al*., 1991; Meisert *et al*., 1999). The innermost cell layer of the o.i. of the ovule develops into palisade cells in the seed coat in *G. carolinianum*. This endotestal origin of the seed coat palisades of Geraniaceae is an exception as palisade cells of all the other seeds of families with PY are either exotestal or exotegmic in origin. In Cannaceae, Convolvulaceae, Cucurbitaceae, Fabaceae and Sapindaceae, palisade cells are exotestal in origin whereas in Bixaceae, Cistaceae, Cochlospermaceae, Dipterocarpaceae, Malvaceae, Rhamnaceae and Sarcolaenaceae they are exotegmic in origin (Baskin *et al*., 2000). In Geraniaceae, the subpalisade layer is exotegmic in

origin and it provides mechanical support to the seed coat (Boesewinkel, 1997; Meisert, 2002). The current study revealed that cells in the palisade and subpalisade layers in mature seeds are of various forms in terms of their overall shape, size and deposits, depending on their location in the seed coat (Fig. 3.6A, B).

In the micropyle and chalaza of *G. carolinianum*, cell division and differentiation take place to a greater extent than in the rest of the seed coat. Consequently, these sites possess more cell layers and particular anatomical features. In the i.i. of the seed coat at the micropyle, the middle layer and the outer layer (subpalisade layer) become multilayered, and palisade cells are radially stretched. Further, the current study reveals that the formation of multiple layers of rectangular-shaped parenchyma cells (originating from palisades) contribute to the formation of a dome-shaped structure at the micropyle of *G. carolinianum*. This type of specialization is necessary to strengthen and seal this opening to maintain water impermeability (Werker, 1997). In *G. carolinianum*, chalazal palisades and subpalisades stretch, possibly providing the mechanical strength required to prevent the seed coat from collapsing during desiccation. The gap between palisade cells of the chalaza is filled with a chalazal plug (= suberized stopper, *sensu* Boesewinkel and Been, 1979) originating from the hypotase. In Malvanae, the chalaza is a highly anatomically specialized structure that is blocked by lignified cells at maturity and subsequently, at the breaking of dormancy, this same site acts as the water gap (Simpson, 1940; Nandi, 1998). Conversely, although the chalaza is an anatomically specialized location in the seed coat of *G. carolinianum*, it does not act as the water gap (Gama-Arachchige *et al*., 2010).

In *G. carolinianum*, the water gap develops near the micropyle (Gama-Arachchige *et al*., 2010). As these two structures are located very close to each other, a clear demarcation of the two regions is difficult. Therefore, the ontogeny of both regions was studied collectively. The palisade layer which forms the 'hinged valve' of the water-gap opening is endotestal in origin, similar to the seed coat. In the course of development of the micropylar area, palisade cells take five different morphological forms (Fig. 3.6A). Some of the palisades of the water gap are longer than those in the rest of the seed coat. Similarly, longer palisades are encountered in the lens (the water gap) in *Indigofera parviflora* and *Trifolium repens* (Manning and Van Staden, 1987; Martens *et al*., 1995). However, the water-gap palisades of *G. carolinianum* are unique due to the presence of elongated bent palisades. As in the bulge of *Ipomoea lacunosa* (Convolvulaceae) (Jayasuriya, 2007a) and the raphal scar of *Canna tuerckheimii* (Cannaceae) (Grootjen and Bouman, 1988), palisades similar in length to those at the rest of the seed coat are present in the water gap of *G. carolinianum*. However, cell width of these palisades is less than that of normal palisades (Fig. 3.6A). Moreover, the absence of tanniferous cells in the palisade layer as opposed to the rest of the seed coat adds to the uniqueness of the water gap.

Meisert *et al*. (2001) studied the development of the light line of Geraniaceae and documented that it was generated by later deposition of electron-dense substances in the already existing secondary cell wall. Also, they observed the development of a multiple light line pattern within idioblast (tanniferous) cells. In the current study, development of two broad light lines was observed in the stretched palisades of the micropyle. Around 14 DAP, the cytoplasm of stretched palisades appears to exist in three zones. This could be due to the deposition of secondary cell-wall material as two

bands. Subsequent deposition of electron-dense material on these thickenings as documented by Meisert *et al*. (2001) may be the cause for these double light lines.

At the water gap, development of palisade and subpalisade cells takes place in such a way that a perfect fit at the walls of contact of these two cell layers is ensured by the curvature of the walls. Near the micropyle end of the water gap, this contact is weaker as the contacting walls are flat (Fig. 3.6A, B). This is probably to aid in opening the water gap on dormancy-break as palisades and subpalisades of the water gap start to separate at the micropyle end, indicating sensitivity of the site to dormancy-breaking treatments (Chapter 5). Analogously, in *Indigofera parviflora*, the lens is a specialized area of intrinsic sensitivity to PY-breaking treatments in the seed coat (Manning and Van Staden, 1987).

From the results of the current study, the major events of development in seeds of *G. carolinianum* can be summarized (Fig. 3.12). After reaching physiological maturity at 14 DAP, seeds remain permeable for approx. 06 d until they achieve PY. The effect of drying on PY is highlighted as artificial slow-drying shifts the stage of acquisition of PY to earlier stages of development. The effects of other environmental conditions on PY during seed development are yet to be studied. A marked level of cell differentiation that makes these sites different in anatomy from the rest of the seed coat is evident at the micropyle and chalaza. The micropyle of *G. carolinianum* is an anatomically complex structure, and the water gap is an extension of the micropyle. Therefore, it is highly possible that the micropyle is also involved in breaking of PY. Thus, the term 'micropyle–water-gap complex' is more appropriate than simply 'water gap'.

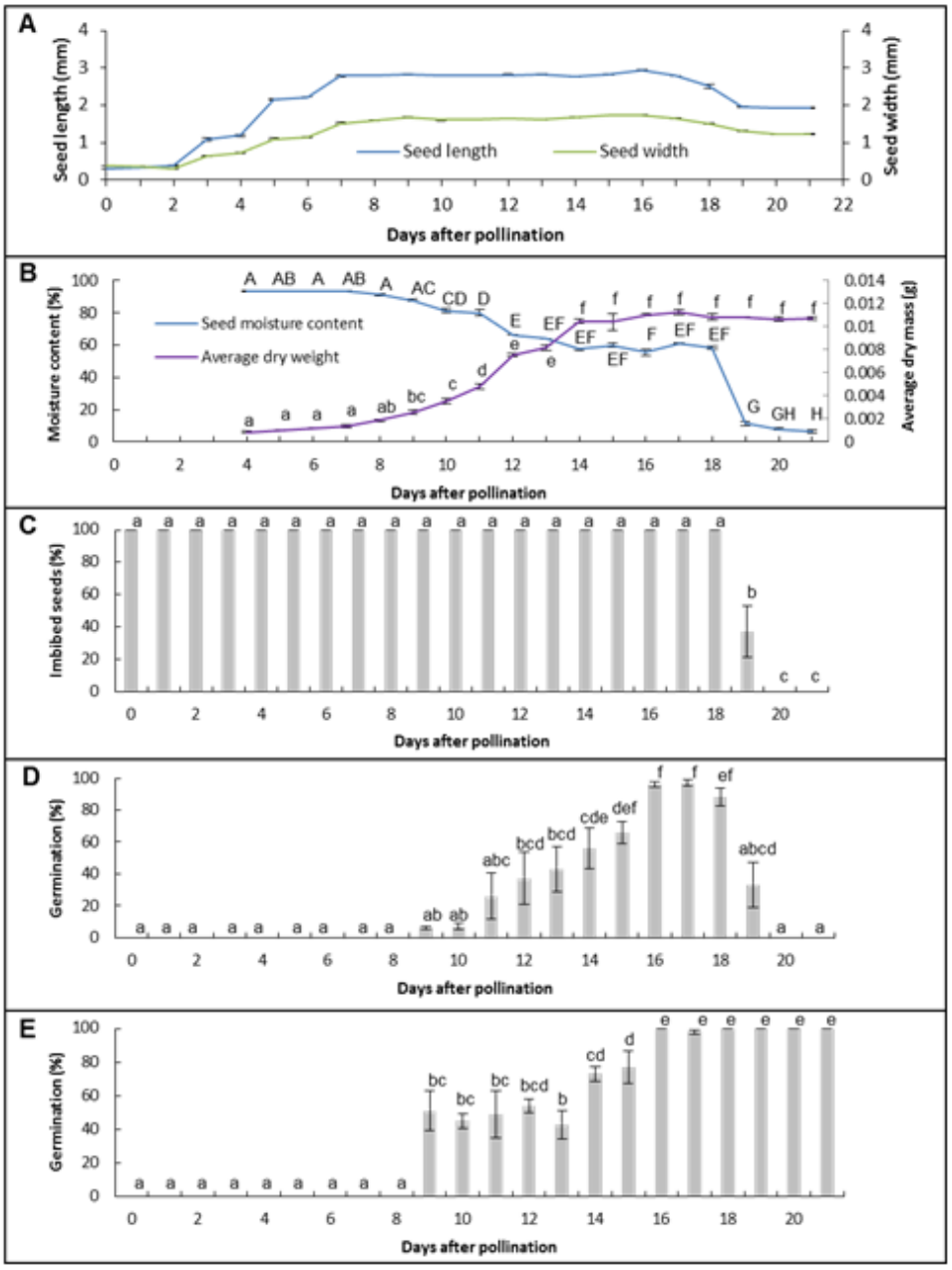


Figure 3.1 Length, width, moisture content, accumulation of dry matter, imbibition and germination of intact seeds and isolated embryos during seed development in *G. carolinianum*: (A) seed length and width (mean ± s.e.); (B) moisture content and dry matter accumulation (mean ± s.e.); (C) percentage of fresh intact seeds that imbibed (mean ± s.e.); (D) germination of fresh intact seeds (mean ± s.e.); (E) germination of isolated embryos (mean ± s.e.). Different letters indicate significant differences between values ($P < 0.05$).

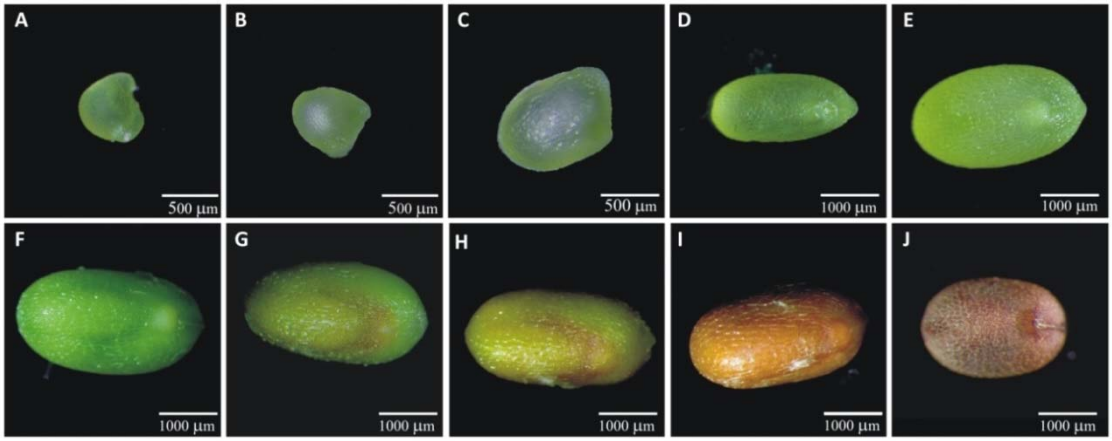


Figure 3.2. Morphological and colour changes in developing seeds of *G. carolinianum*: (A) 1 DAP, (B) 3 DAP, (C) 4 DAP, (D) 6 DAP, (E) 12 DAP, (F) 14 DAP, (G) 15 DAP, (H) 16 DAP, (I) 18 DAP, (J) 20 DAP.

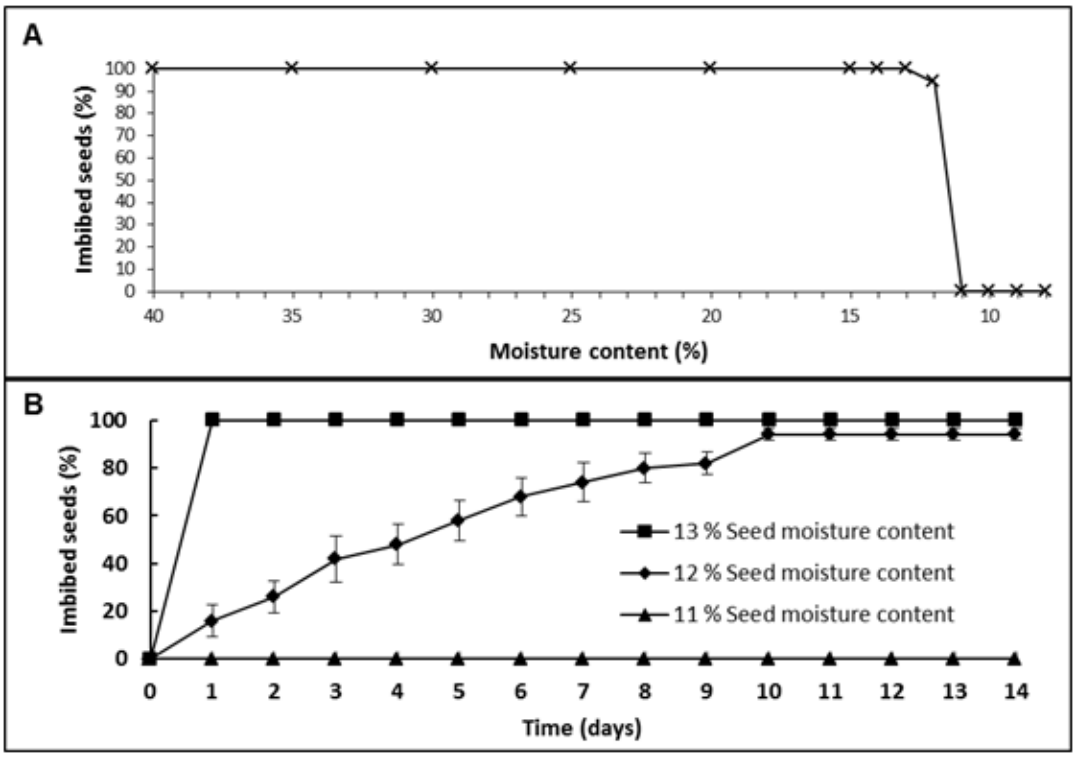


Figure 3.3. Effect of seed moisture content on imbibition of developing seeds of *G. carolinianum* harvested at 18 DAP: (A) relationship between seed moisture content and imbibition (mean ± s.e.); (B) imbibition of seeds with different moisture contents during 14 d (mean ± s.e.) under ambient temperature.

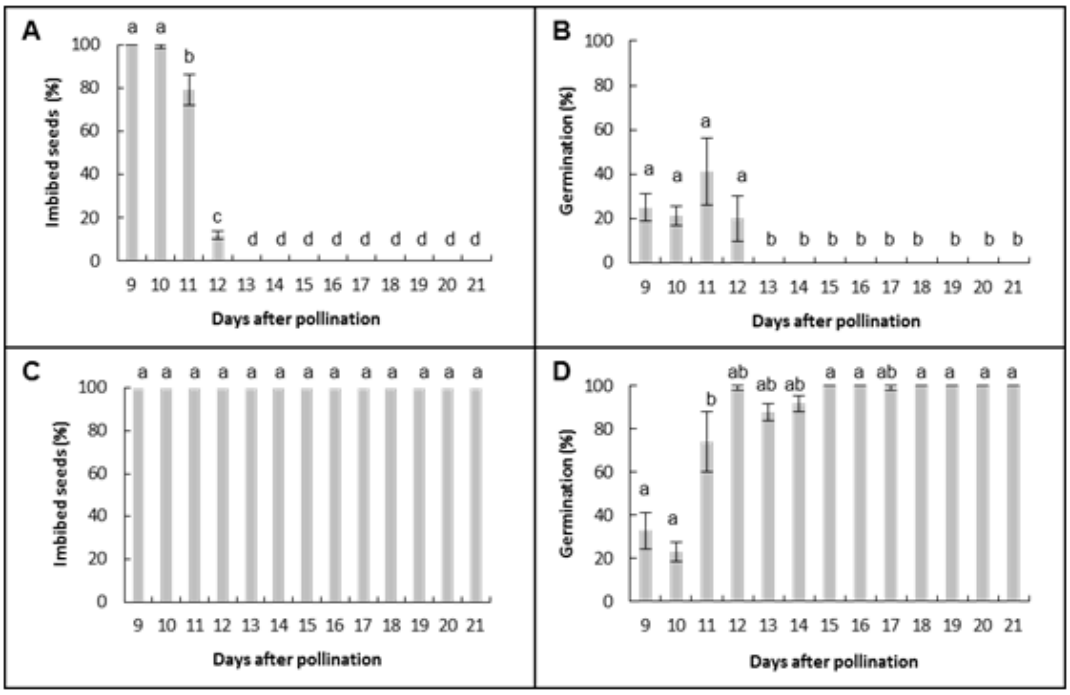


Figure 3.4. Effect of slow-drying on imbibition and germination of developing seeds of *G. carolinianum*: percentage of slow-dried intact seeds that (A) imbibed and (B) germinated (mean ± s.e.); percentage of slow-dried scarified seeds that (C) imbibed and (D) germinated (mean ± s.e.). Different letters indicate significant differences between values ($P < 0.05$).

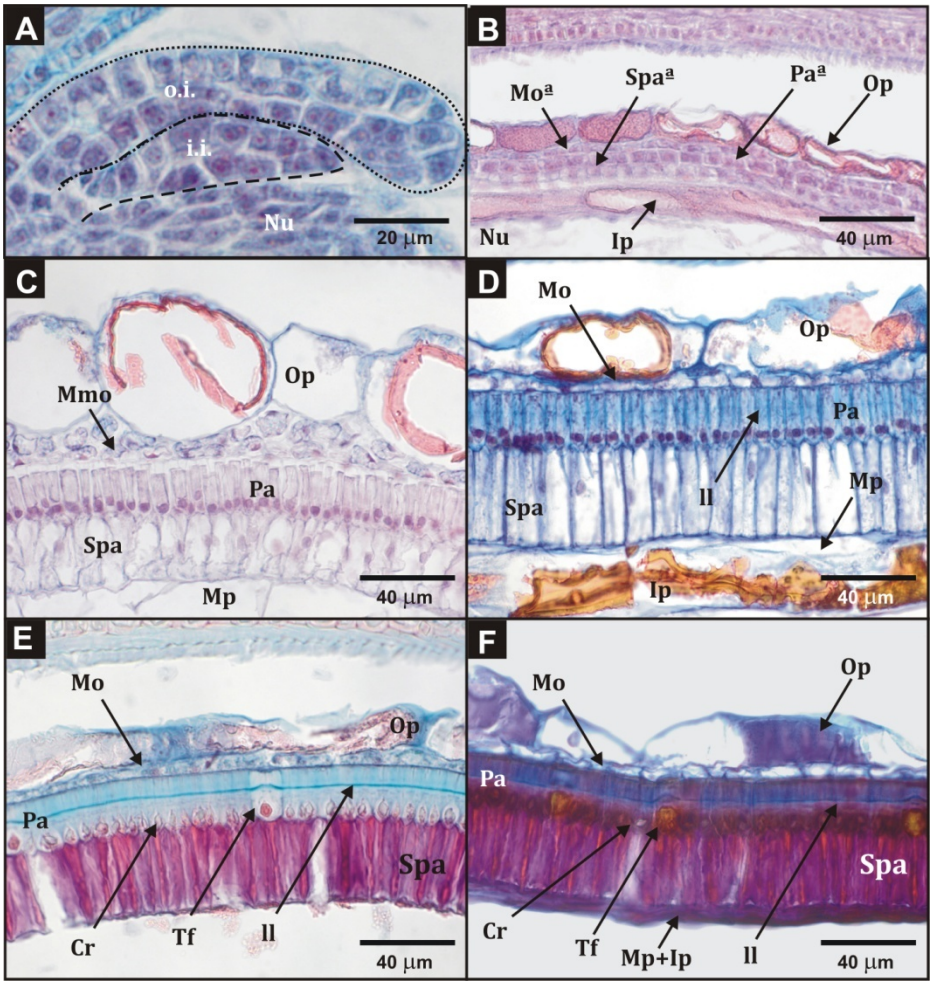


Figure 3.5. Longitudinal sections of the seed coat in developing seeds of *G. carolinianum*: (A) ovule stage-I, (B) 1 DAP, (C) 6 DAP, (D) 8 DAP, (E) 13 DAP, (F) 18 DAP. Abbreviations: Cr, crystals; i.i., inner integument; Ip, parenchyma cells of the inner layer of the i.i.; ll, light line; Mmo, parenchyma cells of the middle multi-layers of the o.i.; Mo, parenchyma cells of the middle layer of the o.i.; Mo$^{a}$, pro-parenchyma cells of the middle layer of the o.i.; Mp, parenchyma cells of the middle layer of the i.i.; Nu, nucellus; o.i., outer integument; Op, parenchyma cells of the outer layer of the o.i.; Pa, palisade cells; Pa$^{a}$, pro-palisade cells; Spa, subpalisade cells; Spa$^{a}$, pro-subpalisade cells; Tf, tanniferous palisade cells.

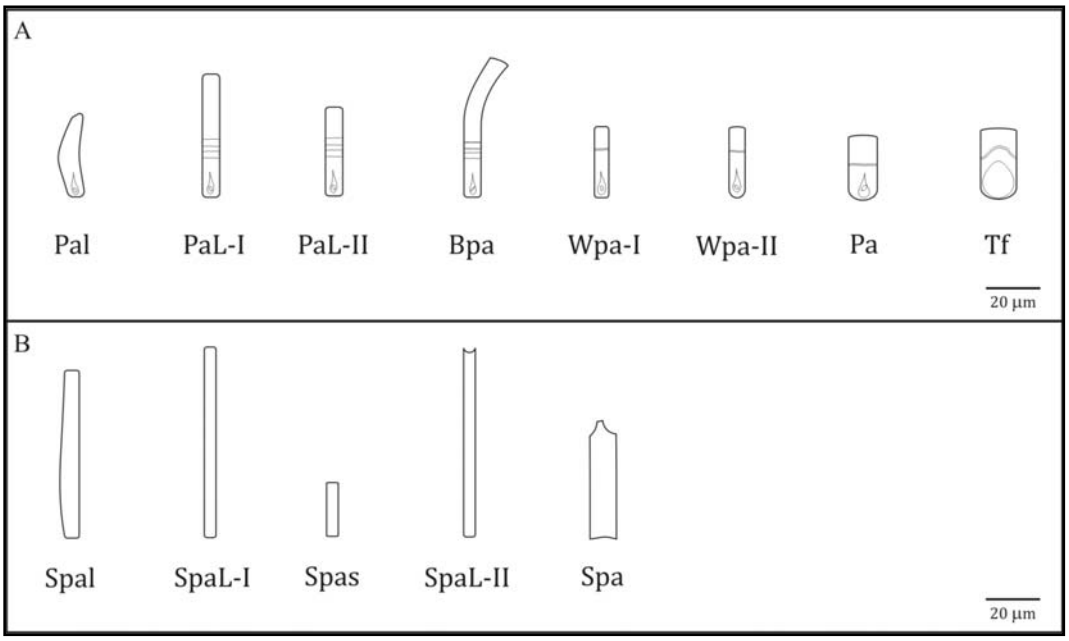


Figure 3.6. Types of palisade and subpalisade cells in the mature seed coat of *G. carolinianum*: (A) palisades; (B) subpalisades. Abbreviations: Bpa, bent palisade cells of the micropyle; Pa, palisade cells; PaL-I, PaL-II, elongated palisade cells of the micropyle; Pal, elongated palisade cells of the chalaza; Spa, subpalisade cells; Spal, elongated subpalisade cells of the chalaza; SpaL-I, SpaL-II, elongated subpalisade cells of the micropyle; Spas, short subpalisade cells of the micropyle; Tf, tanniferous palisade cells; Wpa-I and II, palisade cells of the water gap.

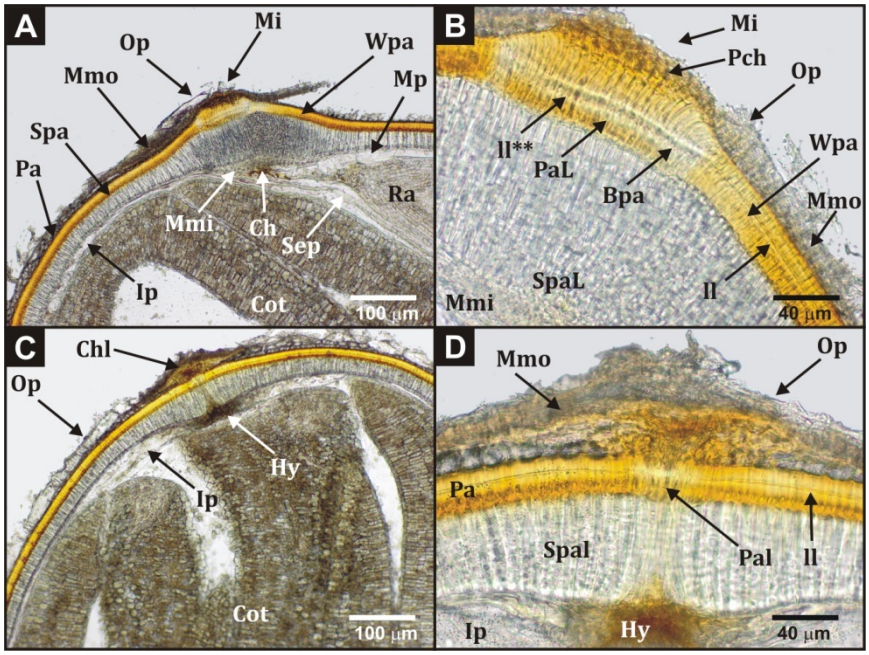


Figure 3.7. Longitudinal sections of the micropyle and chalazal region of a mature seed of *G. carolinianum*: (A) Micropylar region; (B) Close-up of the micropylar region; (C) Chalazal region; (D) Close-up of the chalazal region. Bpa, bent palisade cells of the micropyle; Ch, chlorophyll; Chl, chalaza; Cot, cotyledon; Hy, hypostase; Ip, parenchyma cells of the inner layer of the i.i.; ll, light line; ll**, broad double light line; Mi, micropyle; Mmi, parenchyma cells of the middle multi-layers of the i.i.; Mmo, parenchyma cells of the middle multi-layers of the o.i.; Mp, parenchyma cells of the middle layer of the i.i.; Op, parenchyma cells of the outer layer of the o.i.; Pa, palisade cells; PaL, elongated palisade cells of the micropyle; Pal, elongated palisade cells of the chalaza; Pch, Parenchyma cells of the micropyle; Ra, radicle; Sep, septum; Spa, subpalisade cells; SpaL, elongated subpalisade cells of the micropyle; Spal, elongated subpalisade cells of the chalaza; Wpa, palisade cells of the water gap.

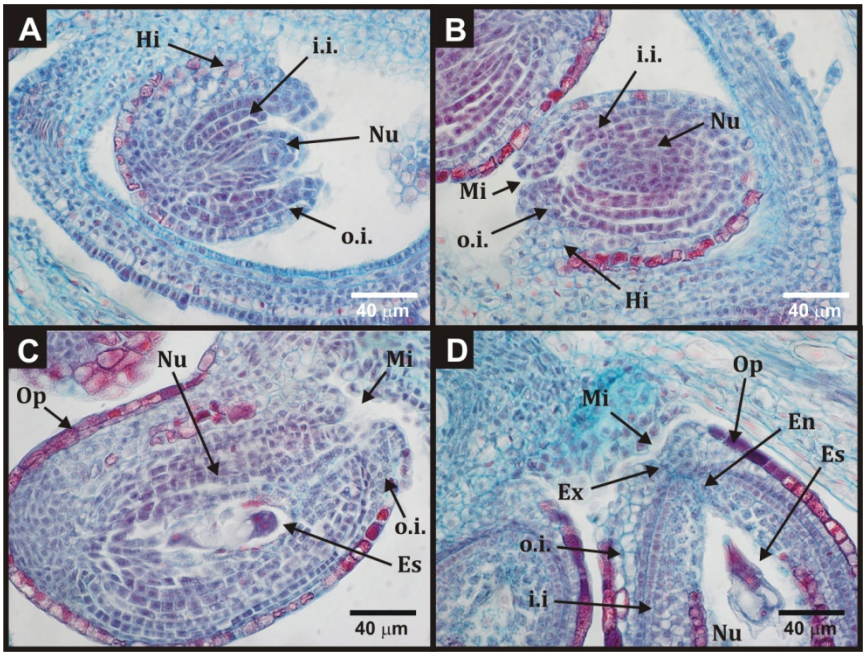


Figure 3.8. Longitudinal sections of the micropylar region in developing ovules of *G. carolinianum*: (A) Ovule stage-I; (B) Ovule stage-II; (C) Ovule stage-III; (D) 00 DAP; En, endostome; Es, embryo sac; Ex, exostome; Hi, hilum; i.i., inner integument; Mi, micropyle; o.i., outer integument; Op, parenchyma cells of the outer layer of the o.i.

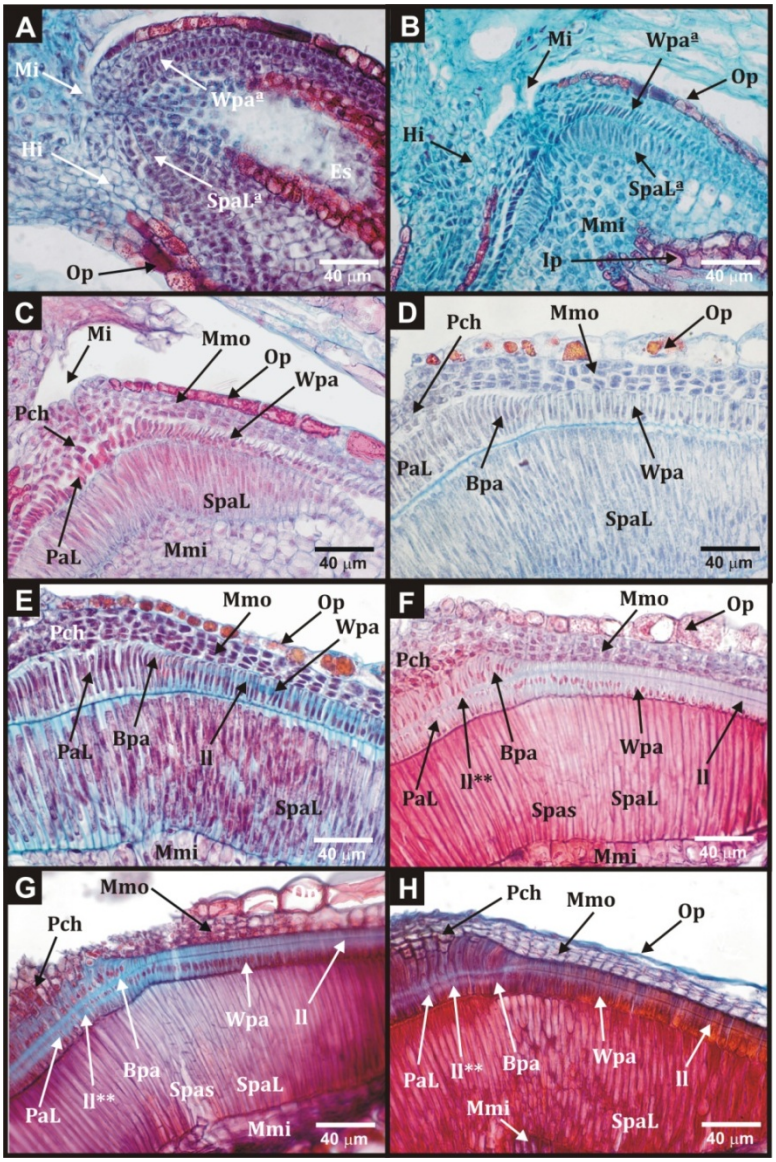


Figure 3.9. Longitudinal sections of the micropylar and water-gap regions in developing seeds of *G. carolinianum* from 1 to 20 DAP: (A) 1 DAP, (B) 3 DAP, (C) 4 DAP, (D) 6 DAP, (E) 8 DAP, (F) 14 DAP, (G) 18 DAP, (H) 20 DAP. Abbreviations: Bpa, bent palisade cells of the micropyle; Es, embryo sac; Hi, hilum; Ip, parenchyma cells of the inner layer of the i.i.; ll, light line; ll**, broad double light line; Mi, micropyle; Mmi, parenchyma cells of the middle multi-layers of the i.i.; Mmo, parenchyma cells of the middle multi-layers of the o.i.; Mmo[a], pro-parenchyma cells of the middle multi-layers of the o.i.; Nu, nucellus; Op, parenchyma cells of the outer layer of the o.i.; PaL, elongated palisade cells of the micropyle; Pch, parenchyma cells of the micropyle; SpaL, elongated subpalisade cells of the micropyle; SpaL[a], pro-subpalisade cells of the micropyle; Spas, short subpalisade cells of the micropyle; Wpa, palisade cells of the water gap; Wpa[a], pro-palisade cells of the water gap.

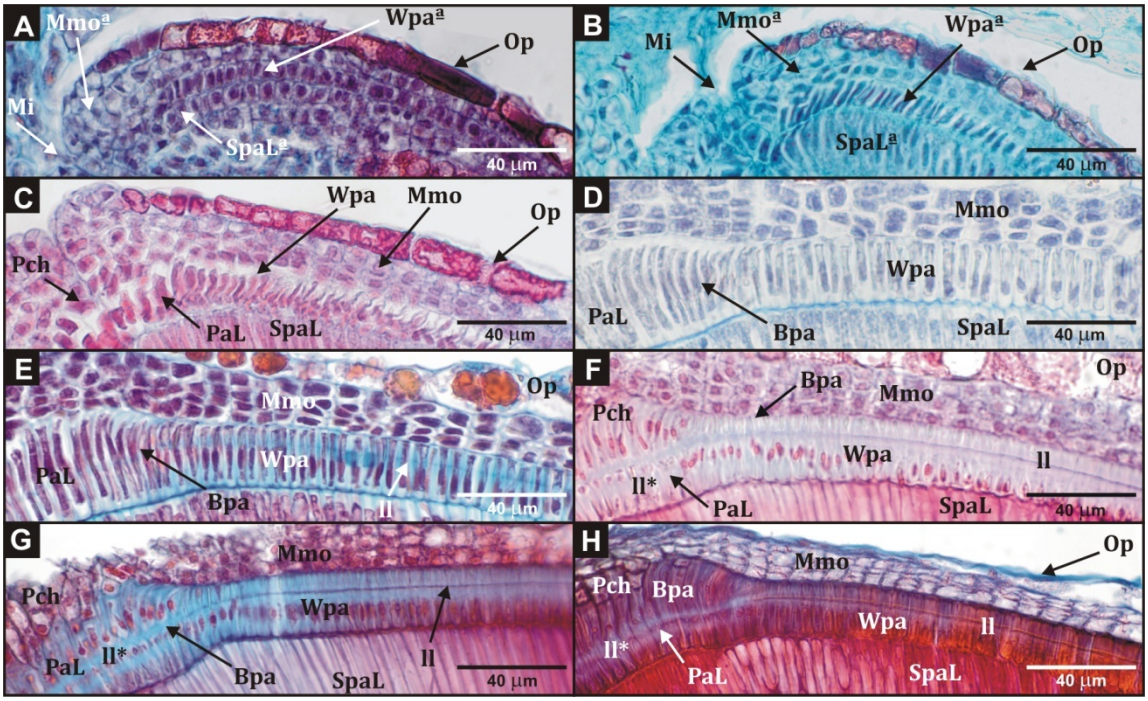


Figure 3.10. Longitudinal sections of the water gap in developing seeds of *G. carolinianum*: (A) 1 DAP, B) 3 DAP, (C) 4 DAP, (D) 6 DAP, (E) 8 DAP, (F) 14 DAP, (G) 18 DAP, (H) 20 DAP. Abbreviations: Bpa, bent palisade cells of the micropyle; ll, light line; ll**, broad double light line; Mi, micropyle; Mmo, parenchyma cells of the middle multi-layers of the o.i.; Mmo[a], pro-parenchyma cells of the middle multi-layers of the o.i.; Op, parenchyma cells of the outer layer of the o.i.; PaL, elongated palisade cells of the micropyle; Pch, parenchyma cells of the micropyle; SpaL, elongated subpalisade cells of the micropyle; SpaL[a], pro-subpalisade cells of the micropyle; Wpa, palisade cells of the water gap; Wpa[a], pro-palisade cells of the water gap.

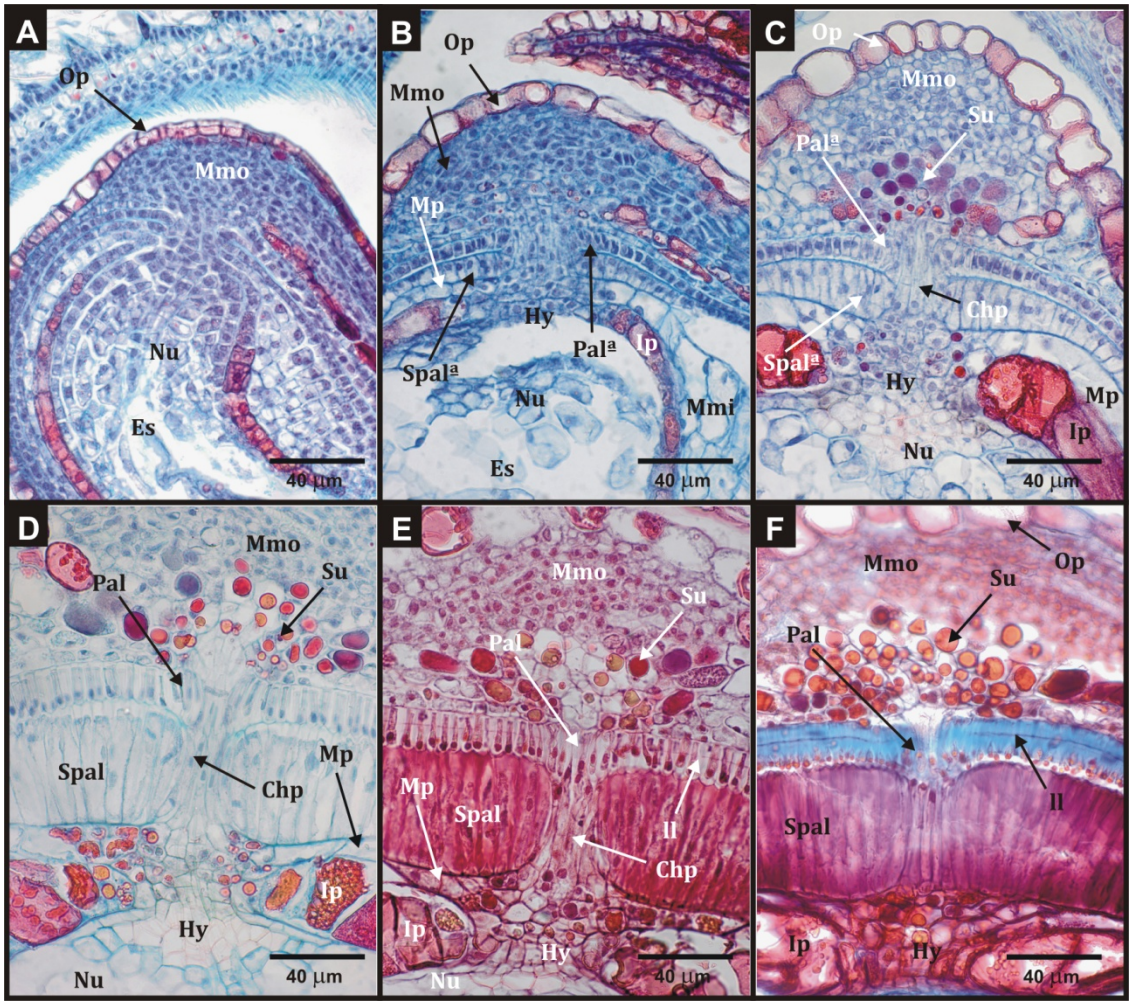


Figure 3.11. Longitudinal sections of the chalazal region in developing seeds of *G. carolinianum*: (A) 0 DAP, (B) 2 DAP, (C) 3 DAP, (D) 6 DAP, (E) 10 DAP, (F) 17 DAP. Abbreviations: Chp, parenchyma cells of the chalaza; Es, embryo sac; Hy, hypostase; Ip, parenchyma cells of the inner layer of the i.i.; ll, light line; Mmi, parenchyma cells of the middle multi-layers of the i.i.; Mmo, parenchyma cells of the middle multi-layers of the o.i.; Mp, parenchyma cells of the middle layer of the i.i.; Nu, nucellus; Op, parenchyma cells of the outer layer of the o.i.; Pal, elongated palisade cells of the chalaza; Pal[a], pro- palisade cells of the chalaza; Spal, elongated subpalisade cells of the chalaza; Spal[a], Pro-subpalisade cells of the chalaza; Su, suberin deposits.

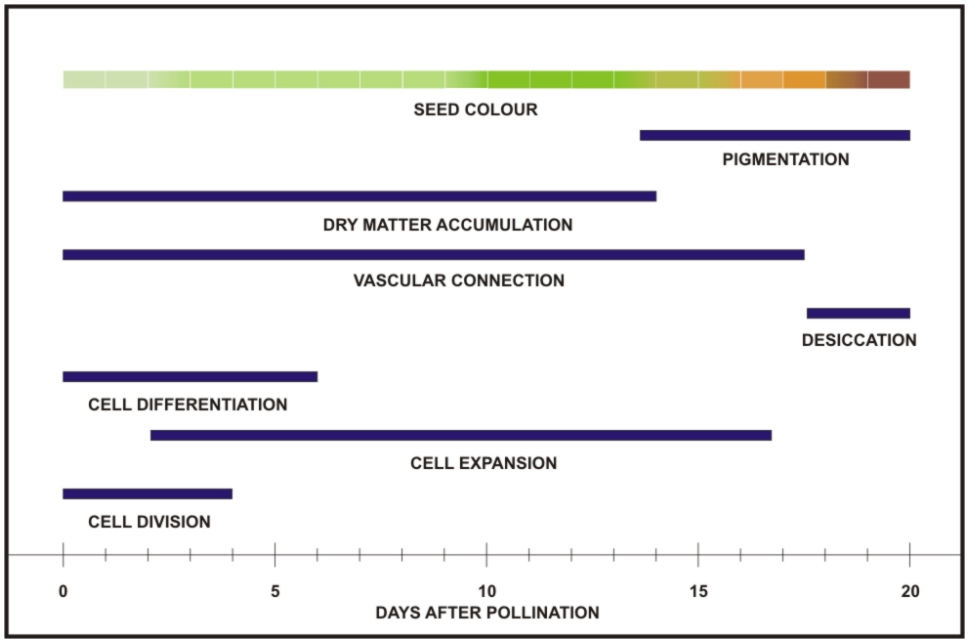


Figure 3.12. Timeline summary of the events of seed development in *G. carolinianum*.

# CHAPTER 4

## Timing of physical dormancy-break in two winter annual species of Geraniaceae by a stepwise process

## INTRODUCTION

Breaking of physical dormancy (PY) in seeds at the onset of autumn is of survival advantage for winter annuals in that it provides them with favourable conditions for germination and establishment of seedlings (Taylor, 1996*a*, *b*). In the absence of physiological dormancy (PD), breaking of PY may lead to immediate germination of seeds upon imbibition (Baskin and Baskin, 1998). Germination of winter annual species in summer may result in loss of seedlings due to prevailing drought conditions (Baskin and Baskin, 1971). Therefore, in winter annuals with PY, timing of PY break must be set to synchronize with the onset of autumn.

Taylor (1981) presented a temperature-dependent two-stage conceptual model for breaking of PY. In the first or pre-conditioning stage, the seeds are made sensitive to the second or the PY-breaking stage (Taylor, 2005). This two-stage model is known to occur in seeds of several annual species of Fabaceae [*Medicago polymorpha* (Taylor, 1996*a*, *b*), *Ornithopus compressus* (Taylor and Revell, 1999), *Trifolium subterraneum* (Taylor, 1981), *Melilotus albus*, *Medicago lupulina*, *Lotus corniculatus* and *Trifolium repens* (Van Assche *et al*., 2003)] and Convolvulaceae [*Ipomoea lacunosa* (Jayasuriya *et al*., 2008*a*), *Ipomoea hederacea* (Jayasuriya *et al*., 2009*c*) and *Cuscuta australis* (Jayasuriya *et al*., 2008*b*)]. The two-stage model consists of two distinct temperature- and/or moisture-dependent processes (Taylor, 2005; Van Assche

and Vandelook, 2006; Jayasuriya *et al*., 2009*a*). Involvement of two stages in breaking of PY can be used to explain the PY-breaking behaviour and timing of germination under natural conditions (Taylor, 2005).

*Geranium carolinianum* and *Geranium dissectum* are herbaceous winter annual species of Geraniaceae. *Geranium carolinianum* is native to eastern North America (Piper, 1906; Small, 1907; Aedo, 2000), while *G. dissectum* is native to Europe (Aedo *et al*., 1998*b*; Rhoads and Block, 2007) and is an introduced species in North America (Piper and Beattie, 1915). Both species are widely distributed weeds in North America and usually grow in disturbed habitats such as roadsides, old fields, waste places, gardens, and fallow and cultivated fields (McCready and Cooperrider, 1984; Abbas *et al*., 1995; Wilson and Clark, 2001). Moreover, both species are reported to be naturalized weeds in many parts of the world including Australia, China, Great Britain, Japan, Italy and South America (Mueller, 1885; Dunn, 1905; Macbride, 1949; Peng, 1978; Aedo *et al*., 1998*b*, 2005; Benvenuti *et al*., 2001; Xu and Aedo, 2008; Nishida and Yamashita, 2009).

As in most species of Geraniaceae, PY is known to occur in seeds of *G. carolinianum* and *G. dissectum* (Meisert, 2002; Van Assche and Vandelook, 2006; Gama-Arachchige *et al*., 2010). Freshly matured seeds of both species also exhibit shallow non-deep PD, thus the seeds have combinational dormancy (PY + PD). However, the shallow PD is lost during a short after-ripening period (Baskin and Baskin, 1974; Gama-Arachchige *et al*., 2010). The water-gap (small opening) formed in the water-impermeable seed or fruit coat during breaking of PY allows the seed to take up water. Opening of the water-gap involves dislodgment or disruption of water-gap

structures that act as environmental 'signal detectors' for germination (Baskin *et al*., 2000). The hinged-valve gap (water-gap) of the seeds of Geraniaceae is located near the micropyle. On breaking of PY, the changes in the palisade layer of the water-gap region are externally visible as a colour change from dark brown to brownish orange (Gama-Arachchige *et al*., 2010). Thus, seeds with a colour change in the water-gap region are permeable (Gama-Arachchige *et al*., 2010).

In a study of the ecological factors involved in breaking of PY in *G. carolinianum*, Baskin and Baskin (1974) concluded that PY breaking takes place under dry or alternate wet–dry conditions at high summer temperatures. They concluded that the water-impermeable seed coat, conditional dormancy of the freshly matured embryo and the inability of seeds to germinate at high summer temperatures delay germination of seeds until autumn. However, examination of a sample of seeds from their study revealed that the seeds they used were *G. dissectum*, not *G. carolinianum* (N.S. Gama-Arachchige *et al*., unpubl. res.). Furthermore, a new preliminary study showed that unlike the seeds used by Baskin and Baskin (1974), PY of *G. carolinianum* can be broken under wet conditions, further supporting the fact that the seeds they used were *G. dissectum* (N.S. Gama-Arachchige *et al*., unpubl. res.). In a study of germination ecology of several species of Geraniaceae, including *G. dissectum*, Van Assche and Vandelook (2006) showed that subsequent drying of exhumed impermeable seeds in a desiccator for 1 week at approx. 20 °C markedly stimulated germination at 23 °C [see table 7 in Van Assche and Vandelook (2006)]. Meisert (2002) observed that seeds of certain species of Geraniaceae, including *G. dissectum*, became permeable under dry storage for 5 years at room temperature. Seeds of *G. bicknellii*, *G. bohemicum* and *G. lanuginosum* germinated (>90 %) after

exposure to wet heat at 55–95 °C (Granstrom and Schimmel, 1993), suggesting that those three species do not require drying for the breaking of PY.

Occurrence of a temperature-dependent process with two steps in the breaking of PY is unknown in Geraniaceae. Furthermore, none of the previous studies has clearly explained the environmental factors involved in the timing of PY break and germination of *G. carolinianum* under field conditions.

Therefore, the objectives of the current study on G. *carolinianum* and *G. dissectum* were to (1) determine the number of steps involved in the PY-breaking processes; (2) identify the temperature and moisture regimes that activate the dormancy-breaking process at each stage; and (3) develop a conceptual model for dormancy break and germination phenology under field conditions.

## MATERIALS AND METHODS

### *Seed collection*

Stems of *Geranium carolinianum* bearing mature fruits were collected at Spindletop Farm, Lexington, KY, USA, on 9 June 2010 (GC 2010) and 1 June 2011 (GC 2011). Similarly, stems of *G. dissectum* were collected from the same location on 30 May 2010 (GD 2010) and 20 May 2011 (GD 2011). They were covered with a mesh cloth and allowed to dry for 3 d inside a non-heated greenhouse. Seeds released naturally were collected and stored at room temperature (approx. 23 °C and 50–60 % relative humidity, dry storage) until used. According to imbibition tests, >98 % of fully matured seeds of both species were impermeable (data not shown). Therefore, fully

matured seeds were used for all the experiments, which were started within 2 weeks of seed collection.

In the case of alternating incubation temperatures, high and low temperatures in the incubators were supplied on a 12 h/12 h daily basis under light/dark conditions (14 h/10 h; approx. 40 μmol $m^{-2}$ $s^{-1}$, 400–700 nm, cool white fluorescent light). The same photon irradiance and 24 h continuous light were used for constant temperatures.

*Breaking of PY under dry storage*

Experiments were carried out to investigate the effects of dry storage under constant and alternating temperatures and subsequent exposure to autumn temperatures (20/10 °C) on breaking of PY.

Seeds collected in 2011 were stored dry at constant temperatures of 5, 10, 15, 20, 25, 30, 35 and 40 °C and at alternating temperatures of 15/6, 20/10, 25/15, 30/15, 30/20 and 40/25 °C in Petri dishes. From seeds stored under each temperature, a sample of 200 was retrieved every month. One hundred seeds from each sample (five replicates of 20 seeds) were incubated at the same storage temperature on sand moistened by adding distilled water heated or cooled to the respective storage temperature. The remaining 100 seeds (five replicates of 20 seeds) were incubated at the average autumn temperature (20/10 °C) on sand moistened by adding distilled water at 20 °C. The number of imbibed seeds was counted after 14 d. The same procedure was repeated for five consecutive months.

*Sensitivity of seeds of G. carolinianum to changes in temperature*

To determine the increase of sensitivity in seeds of *G. carolinianum* to the change in temperature, GC 2011 were stored dry in Petri dishes under alternating temperature of 40/25 °C (to simulate summer soil temperatures) and under constant 30 °C (approximately the average of 40/25 °C). Seven hundred seeds each were retrieved from both storage temperatures at 0 (fresh), 2 and 4 months and were incubated on moist sand at 10, 15, 20, 25, 30, 35 and 40 °C (five replicates with 20 seeds in each). The number of imbibed seeds was counted after 14 d.

*Breaking of PY by simulated natural temperatures*

To determine the effect of temperatures that the seeds of *G. carolinianum* and *G. dissectum* would experience in nature during the PY-breaking period, seeds (GC 2011 and GD 2011) were subjected to a sequence of temperature conditions simulating the average daily maximum and minimum temperatures in Lexington in June (30/15 °C), July (30/20 °C), August (30/20 °C), September (25/15 °C) and October (20/10 °C) under constant wet and dry conditions.

Wet storage.

Seeds were placed in Petri dishes filled with sand wetted with distilled water and their lids were sealed with Parafilm®. Then five replicates (with 20 seeds each) were subjected to the three temperature schemes shown in Table 4.1. Seeds were kept under each temperature for 1 month and then transferred to the next temperature. At the end of each month, the number of imbibed seeds was recorded. Distilled water was added as required to maintain the wet condition. The procedure was continued for five consecutive months.

Dry storage.

Five samples each containing five replicates of 20 seeds each from both species were placed on dry sand in Petri dishes. They were subjected to the three temperature schemes in Table 4.1. At the end of each month, one sample was watered and left under the same temperature, and all the other samples were moved to the next temperature in the sequence. This procedure was followed for five consecutive months. Each watered sample was observed for imbibition after 14 d.

*Effect of moisture regime on breaking of PY in* G. dissectum

To determine the effect of different moisture regimes on breaking of PY in *G. dissectum*, fresh mature seeds (GD 2011) were subjected to four moisture regimes in an incubator at 40 °C. Twenty samples each containing five replicates of 20 seeds were placed on sand in Petri dishes. Five samples were subjected to each of the four moisture regimes. (1) Constant wet; seeds were kept under constant wet conditions by adding distilled water weekly. (2) Constant dry; seeds were kept dry. (3) Alternate wet–dry; seeds were alternated between wet and dry (2 weeks under each condition) for 10 weeks starting with the wet condition. (4) Alternate dry–wet; seeds were alternated between dry and wet (2 weeks under each condition) for 10 weeks starting with the dry condition. In the case of alternating moisture regimes, distilled water was added once a week during wet periods and no water was given during dry periods. For all moisture regimes, at the end of each 2 week interval one sample was tested for imbibition at 40 °C, and in the case of alternate moisture regimes all the other samples were moved to the next moisture condition. Imbibition in each sample was recorded after 14 d.

*Breaking of PY under semi-natural conditions*

To determine the effect of temperature on timing of PY break under semi-natural conditions, 100 seeds (GC 2010, GD 2010, GC 2011 and GD 2011) were placed on dry sand in plastic Petri dishes (five replicates of 20 seeds). The Petri dishes then were placed in trays filled with potting soil inside a non-heated greenhouse [second week of June 2010/11 (GC 2010 and GC 2011), first week of June 2010 (GD 2010) and last week of May 2011 (GD 2011)]. The water-gap region of seeds was observed weekly under a dissecting microscope. Seeds with colour change from dark brown to brownish orange in the water-gap region were considered permeable (Gama-Arachchige *et al*., 2010). Air temperature inside the greenhouse was recorded in 30 min intervals using a Thermochron ibutton® (DS 1921G#F50), and daily maximum and minimum temperatures were obtained from the recordings.

*Effect of soil moisture regime on breaking of PY*

To determine the effect of the soil moisture regime on breaking of PY in both species, seeds (GC 2010, GC 2011 and GD 2011) were maintained under three soil moisture regimes (wet, wet–dry and dry). Five replicates each containing 300 seeds from each species were prepared separately for each moisture regime by sowing the seeds on plastic trays (30 × 30× 5 cm) filled with a 3 cm layer of dry potting soil and covering them with 0.5 cm of the same dry soil layer. The trays containing *G. carolinianum* and *G. dissectum* were placed inside a non-heated greenhouse in the second week of June (2010/11) and the last week of May (2011), respectively. Seeds maintained under the wet soil moisture regime were watered to field capacity and were covered with aluminium foil to minimize evaporation of water. Soil in those trays was kept at

or near field capacity until the end of the experiment by watering once a week. Seeds in the wet–dry regime were watered once a week to field capacity and allowed to dry under ambient conditions. Seeds in the dry regime were left without watering until the second week of October 2010/11, after which they were watered to field capacity once a week. Seed germination was checked at 7 d intervals until the end of the experiment. The air temperature inside the greenhouse was recorded at 30 min intervals using a Thermochron ibutton® (DS 1921G#F50), and daily maximum and minimum temperatures were obtained from the recordings.

*Breaking of PY under natural conditions*

To determine the timing of breaking of PY under natural conditions, three replicates of 100 seeds (GC 2010, GC 2011 and GD 2011) were placed in nylon mesh bags and buried at a depth of 2 cm in soil for 5 months [May to October (GD 2011) and June to November (GC 2010/11)] in an open area on the campus of the University of Kentucky in 2010 and 2011. The soil temperature at a depth of 2 cm was recorded at 30 min intervals using a Thermochron ibutton® (DS 1921G#F50) sealed in a plastic bag, and daily maximum and minimum temperatures were obtained from the recordings. Manual weeding of the buried area was done when necessary during the burial period. Three bags each were exhumed every month and the numbers of intact, dead, swollen and germinated seeds were counted. The micropylar region of intact seeds was observed under a dissecting microscope. Seeds with a colour change from dark brown to brownish orange in the water-gap region were considered permeable (Gama-Arachchige *et al*., 2010). Then, all the intact seeds without the colour change were placed on moist soil and incubated at 20 °C. The number of imbibed and

germinated seeds was counted after 14 d. The permeable seed fractions (initial and after exposure to 20 °C) were calculated as follows:

Initial permeable seed percentage = $[(C_i + D_i + G_i + S_i)/\text{total}] \times 100$

Final permeable seed percentage = $[(C_i + D_i + G_i + S_i + C_f + G_f + S_f)/\text{total}] \times 100$

where $C_i$ is the number of seeds with a colour change in the water-gap region at each sampling time; $D_i$ is the number of dead seeds at each sampling time; $G_i$ is the number of germinated seeds at each sampling time; $S_i$ is the number of swollen seeds at each sampling time; $C_f$ is the number of seeds with a colour change in the water-gap region after exposure to 20 °C; $G_f$ is the number of germinated seeds after exposure to 20 °C; and $S_f$ is the number of swollen seeds after exposure to 20 °C.

*Statistical analysis*

A completely randomized design was used in all experiments. Percentage imbibition and permeable fraction data were normalized by arcsine transformation prior to analysis. Data for percentage permeable seed fractions in burial experiments were compared using paired *t*-tests ($P = 0.05$). All other imbibition percentage and germination rate data were analysed by one-way analysis if variance (ANOVA). Duncan's mean separation procedure was used to compare treatments ($P = 0.05$). All analyses were carried out using SPSS ver. 19 software.

## RESULTS

*Breaking of PY under dry storage*

In both species, imbibition did not take place in seeds stored under dry conditions at constant temperatures below 15 °C even for 5 months (Fig. 4.1A, G; results for storage under 5 and 10 °C not shown). The minimum storage temperature at which imbibition occurred was 20 °C (Fig. 4.1B, H). With increasing storage temperature and time, the fraction of imbibed seeds increased (Fig. 4.1B–F, H–L). However, in *G. carolinianum*, incubation at 20/10 °C caused a significant increase in the fraction of imbibed seeds compared with imbibition at each storage temperature, whereas in *G. dissectum*, no such change in imbibition was observed.

In *G. carolinianum*, <2 % of seeds stored under 15/6 and 20/10 °C imbibed (Fig. 4.2A, B). Generally, the percentage imbibition increased with increasing storage time (Fig. 4.2C–F). Higher imbibition percentages were observed at 30/15, 30/20 and 40/25 °C than at lower alternating temperatures (Fig. 4.2D–F). At 40/25 and 30/20 °C storage temperatures, the percentage of seeds that imbibed during subsequent incubation under 20/10 °C was significantly higher than those at the corresponding storage temperature after storage for 1 and 2 months, respectively. However, at 25/15 and 30/15 °C, no such significance was observed between percentage imbibition values after 3 months of storage (Fig. 4.2C, D).

In contrast to *G. carolinianum*, no significant difference was observed in imbibition between seeds incubated under alternating storage temperatures and those incubated at 20/10 °C in *G. dissectum* (Fig. 4.2G–L). Also, imbibition was low (<9 %) in seeds stored at 15/6 and 20/10 °C (Fig. 4.2G, H). At the other storage temperatures,

imbibition increased rapidly with storage time, reaching approx. 100 % after 2–4 months of storage (Fig. 4.2I–L).

*Sensitivity of seeds of G. carolinianum to changes in temperature*

Only 0–3% of fresh seeds (0 months) imbibed at all incubation temperatures (Fig. 4.3A, D). Seeds stored at 40/25 °C for 2 months showed significant imbibition at 10 and 15 °C (>92 %; Fig. 4.3B). The highest temperature at which a significant imbibition was observed was 20 °C (16 %). At higher incubation temperatures, imbibition was very low (<4 %). After 4 months of storage, imbibition had increased at all the incubation temperatures (Fig. 4.3C). At ≤20 °C, >86 % of the seeds had imbibed, while at 25 °C, 61 % of them had done so. At incubation temperatures >25 °C, the imbibed seed fraction remained between 20 and 30 %. An identical pattern, but lower percentages, of imbibition was observed for seeds stored at 30 °C (Fig. 4.3E, F).

*Breaking of PY by simulated natural temperatures*

In *G. carolinianum*, imbibition increased drastically in seeds transferred to 25/15 °C (September), from approx. 0 to 70 % in dry-stored seeds and from approx, 0 to 30 % in wet-stored seeds (Fig. 4.4A). On transfer of seeds to 20/10 °C (October), imbibition increased to >95 % in seeds stored under both dry and wet conditions. Unlike in *G. carolinianum*, imbibition (53 %) was first observed in *G. dissectum* when dry-stored seeds were transferred to 30/20 °C (July) (Fig. 4.4D). Under the same conditions the following month (August), the fraction of imbibed seeds increased up to 100 %. In wet-stored seeds, imbibition increased only up to 16 % even after 5 months of storage under the same alternating temperature scheme.

At simulated monthly maximum temperatures under wet and dry conditions, imbibition up to 16 and 31 %, respectively, was observed in *G. carolinianum* after transfer of seeds to 25 °C (Fig. 4.4B). Subsequently, at 20 °C imbibition increased to >90 %. In the case of *G. dissectum*, all the dry-stored seeds imbibed after 2 months of storage at 30 °C, while only 28 % of the wet-stored seeds imbibed even after 5 months of storage (Fig. 4.4E). Imbibition of both species was negligible after wet and dry storage with the average monthly minimum temperature scheme (Fig. 4.4C, F).

*Effect of moisture regime on breaking of PY in* G. dissectum

After the first 2 weeks of storage under all four moisture regimes at 40 °C, a low percentage of seeds imbibed (<4 %; Fig. 4.5A–D). During the remaining 8 weeks at the constant wet regime, the percentage of imbibed seeds increased gradually to approx. 60 % whereas under the constant dry regime, it increased rapidly to 100 % (Fig. 4.5A, B). After 4 weeks of storage (following the first dry period) under the wet–dry regime, a significantly higher percentage of seeds imbibed (approx. 80 %) (Fig. 4.5C). During the following 2 weeks of wet storage, imbibition increased to 94 %. After 4 weeks of storage under the dry–wet regime (Fig. 4.5D) (i.e. after the first wet period), a significant but low percentage of seeds imbibed (7 %). A marked increase in imbibition was then observed in the following dry period (100 %).

*Breaking of PY under semi-natural conditions*

In 2010, the minimum temperature recorded inside the greenhouse during the first 12 weeks after sowing the seeds was 17.5 °C (Fig. 4.6A). During that period, no colour change was observed in the water-gap region of the seeds of *G. carolinianum* (GC

2010) (Fig. 4.6B). A colour change was first observed in 22 % of seeds in the last week of August, following a weekly minimum temperature of 19.5 °C (Fig. 4.6A, B). Thereafter, the percentage of seeds with colour change gradually increased during late summer to early autumn, coinciding with the decrease in temperature. By the first week of October (early autumn), all seeds showed a colour change.

In 2011, the minimum temperature recorded inside the greenhouse during the first 7 weeks after sowing the seeds was 17 °C (Fig. 4.6D). During that period, no colour change was observed in the water-gap region of the seeds of *G. carolinianum* (GC 2011) (Fig. 4.6E). A colour change was first observed in 5 % of seeds in the first week of August, following a weekly minimum temperature of 21 °C (Fig. 4.6D, E). Thereafter, the percentage of seeds with colour change gradually increased during late summer to early autumn, coinciding with the decrease in temperature. By the last week of September (end of summer), all the seeds showed a colour change.

In both years, the colour change in *G. dissectum* was first observed about 2 months earlier (i.e. early summer) than that in *G. carolinianum*. The percentage of seeds with colour change gradually increased during summer and reached 100 % by the end of summer (Fig. 4.6B, E).

*Effect of soil moisture regime on breaking of PY*

In both years, *G. carolinianum* kept under the constant wet and wet–dry moisture regimes began to germinate in early September (late summer) and reached maximum germination by early October (>80 %; early autumn) as the temperature began to decrease (Fig. 4.6C, F). However, germination under the wet–dry regime was lower

than that under the constant wet regime. After watering in early October, seeds kept under the dry–wet regime started to germinate within a week and reached >93 % by early November (Fig. 4.6C, F).

In *G. dissectum* (GD 2011), timing of germination under the three moisture regimes was similar to that of GC 2010 and GC 2011 (Fig. 4.6G). However, compared with *G. carolinianum*, germination of *G. dissectum* was lower under constant wet (22 %) and wet–dry (61 %) moisture regimes (Fig. 4.6G).

*Breaking of PY under natural conditions*

In *G. carolinianum* (GC 2010 and 2011), neither fresh seeds nor those exhumed after 1 month of burial (July) were permeable (Fig. 4.7B, C, J, K). When these seeds were transferred to 20 °C, only those that had been subjected to 1 month burial in 2010 became permeable (4 %). After transferring the seeds that had been buried for 2 (August) or 3 (September) months to 20 °C, a significant increase in the fraction of permeable seeds was observed (>36 and >94 %, respectively; Fig. 4.7D, E, L, M). Further, a considerable permeable fraction (>20 %) was observed in seeds exhumed after 3 months of burial (September) following the monthly minimum temperatures of 20 (2010) and 18 °C (2011) (Fig. 4.7A, E, I, M). More than 90 % of the seeds exhumed after 4 (October) or 5 (November) months of burial were permeable. However, transfer of seeds to 20 °C did not cause a significant increase in the permeable fraction ($P < 0.05$; Fig. 4.7F, G, N, O)

All the fresh seeds of *G. dissectum* (GD 2011) were impermeable and they did not become permeable even after incubation at 20 °C (Fig. 4.7P). During the 5 month

burial period, the permeable fraction of seeds increased up to 39 % (Fig. 4.7Q–U). None of the samples exhumed during the burial period showed a significant increase in permeability after incubation at 20 °C ($P < 0.05$).

## DISCUSSION

Storage of *G. carolinianum* seeds at constant temperatures ≥20 °C followed by incubation at 20/10 °C resulted in a significant increase in the permeable seed fraction compared with seeds incubated under constant temperatures ($P < 0.05$). This observation suggests the involvement of two steps in breaking of PY in *G. carolinianum* seeds. Insensitive seeds are made sensitive at temperatures ≥20 °C during step-I, followed by step-II, where sensitive seeds become permeable at lower temperatures (i.e. 20/10 °C). However, under alternating storage temperatures with the daily maximum >20 °C and minimum <20 °C (e.g. 25/15, 30/15 °C), seeds became permeable without the requirement for incubation at 20/10 °C. Also, under alternating temperatures in which the daily minimum was ≥20 °C (e.g. 30/20, 40/25 °C) a significantly lower percentage of seeds became permeable than at 20/10 °C ($P < 0.05$). Thus, it can be assumed that seeds achieve permeability when the alternating temperature itself satisfies the temperature requirements for the completion of step-I (by the daily maximum) and step-II (by the daily minimum). Therefore, the requirement for step-II is not necessarily an alternating temperature, but it has to be ≤20 °C.

Fresh insensitive seeds of *G. carolinianum* did not become permeable at any of the incubation temperatures (10–40 °C; Fig. 4.3A, D). Dry storage under 40/25 °C (mean

summer soil temperatures) or constant 30 °C for 2 months induced sensitivity. However, they became permeable only at temperatures ≤20 °C during subsequent incubation. Four months of dry storage at the same temperatures increased the sensitivity of seeds. A significantly high percentage of seeds (>61) became permeable at temperatures ≤25 °C but not at temperatures ≥30 °C (Fig. 4.3C, F). In relation to the mean temperature at step-I, sensitive seeds responded to a temperature decrease rather than an increase in step-II. Thus, sensitive seeds do not require alternating temperatures to become permeable at step-II. Furthermore, the amplitude of the decline in temperature required in step-II decreases and eventually may reach zero as seeds become more and more sensitive with time. Therefore, during the progression from the less sensitive stage to the highly sensitive stage, the temperature range at which seeds can become permeable (completion of step-II) gradually increases from low to high.

Simulation of temperatures of each month during summer and autumn provides further evidence for the occurrence of two steps in the PY-breaking process of *G. carolinianum*. During the first 3 months of storage, step-I may be completed due to high summer temperatures. However, since seeds had low sensitivity during June (30/15 °C) and were not exposed to temperatures <20 °C during July and August (30/20 °C), step-II might have been suspended during the first 3 months of summer. After exposing sensitive seeds to autumn temperatures (25/15 and 20/10 °C), seeds became permeable upon the completion of step-II. Results were similar in the simulation of constant high temperatures of each month. In this temperature simulation experiment, the storage moisture condition (wet or dry) did not affect step-I or step-II in PY breaking of *G. carolinianum*.

Based on the results of laboratory experiments, 20 °C was selected as the temperature threshold for the completion of step-II for greenhouse and burial experiments. Similar to laboratory experiments, seeds sown in the non-heated greenhouse that were exposed to hot summer temperatures showed the colour change in the water-gap (= PY break) of *G. carolinianum* when the daily minimum temperature declined to ≤20 °C in mid to late summer. In early summer, those seeds did not respond to low temperatures, possibly because they were insensitive or less sensitive to low temperatures at that time.

Increase of sensitivity to low temperature (≤20 °C) in *G. carolinianum* was also observed in seeds buried in the soil. Seeds exhumed in early summer did not respond to 20 °C, while seeds exhumed during mid to late summer responded significantly ($P < 0.05$). Even though seeds were sensitive to low temperature during mid to late summer, a majority of them did not become permeable in the soil, since the minimum soil temperature remained >20 °C. When the minimum soil temperature dropped below 20 °C in early autumn, seeds became permeable.

Seeds of *G. dissectum* become permeable under temperatures ≥20 °C (dry) without the requirement for a subsequent low temperature (20/10 °C) treatment. Similarly, the colour change in the water-gap region (= PY break) of seeds occurred much earlier in the non-heated greenhouse under dry conditions than in *G. carolinianum* in summer under high temperatures. These results suggest that in contrast to *G. carolinianum*, breaking of PY in *G. dissectum* involves only a single step under one temperature regime (≥20 °C).

According to temperature simulation and moisture regime experiments conducted in the laboratory and greenhouse, it is evident that seeds of *G. dissectum* achieve permeability regardless of the moisture conditions (dry or wet storage). However, the dry condition is much more effective than the wet condition in breaking of PY (Figs 4.4D, E and 4.5A, B). Also, the initial moisture treatment affects the PY-breaking behaviour. If the seeds are initially exposed to a wet period, subsequent drying can make a significant fraction of them permeable (Fig. 4.5C). On the other hand, exposure of seeds initially to a dry period followed by a wet period delays their becoming permeable until the next dry period (Fig. 4.5D). However, the similar fractions of permeable seeds observed under constant dry (after 4 weeks) and wet–dry (2 weeks wet followed by 2 weeks dry) storage indicate that during the wet period, seeds progress towards PY break, possibly by becoming sensitive to drying (Fig 4.5B, C).

During the 5 month period of burial in the soil at 2 cm depth, <40 % of *G. dissectum* seeds became permeable. It is possible that the moisture or the high relative humidity in soil delays permeability in >60 % of the seeds. Furthermore, Van Assche and Vandelook (2006) demonstrated that when immediately buried, fresh seeds of several species of Geraniaceae (including *G. dissectum*) remain impermeable until they are exposed to drying. This may be considered a moisture-dependent, conditional stepwise PY-breaking process (Table 4.2). However, we found that breaking of PY in *G. dissectum* can take place in a single step under any moisture regime although at a different rate. Therefore, induction of sensitivity in seeds under wet conditions may be an essential step for seeds buried in soil but not for the seeds lying on the soil surface since they are constantly exposed to dry conditions during summer. The requirement

for drying for breaking of PY may be an environmental cue that makes seeds permeable only from the upper layers of soil during summer, while seeds in the deeper moist layers maintain a soil seed bank (Van Assche and Vandelook, 2006).

Of the 18 families with PY (Baskin and Baskin, 1998; Nandi, 1998; Baskin *et al*., 2000, 2006; Baskin, 2003; Horn, 2004; Koutsovoulou *et al*., 2005), involvement of two steps in PY breaking has been demonstrated only for Fabaceae and Convolvulaceae (Table 2). Also, Van Assche and Vandelook (2006) suggested the existence of a moisture-dependent, stepwise PY-breaking process in Geraniaceae. However, our study shows that seeds of *G. dissectum* do not behave in accordance with this pattern. Therefore, we suggest it to be considered a conditional two-step PY-breaking process.

Our study is the first report of a temperature-dependent, two-step PY-breaking process in Geraniaceae (*G. carolinianum*). Neither of the steps in the PY-breaking process of *G. carolinianum* were affected by the soil moisture regime, and both of them can be completed at constant temperatures (Table 4.2). Moreover, highly sensitive seeds may not require a second temperature condition to complete step-II. Therefore, it is possible that some seeds may become permeable without the second treatment.

Winter annuals in Fabaceae that have been reported as having a two-stage PY-breaking process require alternating temperatures in both step-I and step-II (except *Trifolium subterraneum*; only in step-II) to complete the process (Table 2). However, the effect of constant low temperatures in step-II has not been tested in the studies

with Fabaceae species. Therefore, it is possible that those species may respond to the decline in temperature in early autumn below a certain threshold (as in *G. carolinianum*). PY breaking of the summer annual *Ipomoea lacunosa* requires low temperatures in the first step and high temperatures in the second step, which is the reverse of the PY-breaking requirement in the winter annual *G. carolinianum.* This pattern may ensure that the PY break takes place during spring and summer in *I. lacunosa* and during early autumn in *G. carolinianum.*

Cycling between sensitivity and insensitivity in PY breaking previously has been reported in some species of Fabaceae and Convolvulaceae (Table 4.2). Neither seeds of *G. carolinianum* nor those of *G. dissectum* demonstrated such cycling behaviour. Thus, they depend on a stepwise PY-breaking behaviour in timing their germination.

In *G. carolinianum*, breaking of PY involves two moisture-independent steps regulated by temperature (Fig. 4.8A). Low temperature is the environmental cue that triggers PY breaking in late summer and subsequent germination in early autumn. In *G. dissectum*, PY breaking takes place in early summer, either in two steps or in a single step, the number of steps being determined by the moisture condition of the environment (Fig. 4.8B). However, the inability of imbibed seeds to germinate under high temperatures delays germination until autumn (Fig. 4.9). By means of these different dormancy-breaking strategies, seedling establishment in the two species is ensured to occur under favourable environmental conditions in autumn.

Table 4.1. Temperature conditions for each month of the three storage temperature schemes

| Temperature scheme | Month | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | June (1) | | July (2) | | August (3) | | September (4) | | October (5) |
| 1. Alternating | 30/15 °C | → | 30/20 °C | → | 30/20 °C | → | 25/15 °C | → | 20/10 °C |
| 2. Constant high | 30 °C | → | 30 °C | → | 30 °C | → | 25 °C | → | 20 °C |
| 3. Constant low | 15 °C | → | 20 °C | → | 20 °C | → | 15 °C | → | 10 °C |

Table 4.2. Life form, time of germination and conditions involved in the two-step model for breaking of PY

| Family/species | Life form | Germina-tion | Breaking of PY | | Reference |
|---|---|---|---|---|---|
| | | | Step I | Step II | |
| Convolvulaceae | | | | | |
| *Cuscuta australis** | A | Summer | 15/6 °C (dry) | 35/20 °C (wet) | Jayasuriya *et al*. (2008*a*) |
| *Ipomoea hederacea** | SA | Summer | ≥25 °C | 35/20 °C (dry) | Jayasuriya *et al*. (2009*b*) |
| *Ipomoea lacunosa** | SA | Summer | ≥20 °C (wet) | 35 °C (RH >90 %) | Jayasuriya *et al*. (2008*b*) |
| Fabaceae | | | | | |
| *Medicago polymorpha* | WA | Autumn | 60/15 °C | 35/10 °C | Taylor (1996*a*, *b*) |
| *Medicago lupulina* | SA | Spring | 5 °C | 15/6 °C | Van Assche *et al*. (2003) |
| *Melilotus albus** | B | Spring | 5 °C | 15/6 °C | Van Assche *et al*. (2003) |
| *Ornithopus compressus** | WA | Autumn | 60/15 °C | 48/15 °C | Taylor and Revell (1999) |

Table 4.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| *Trifolium subterraneum* | WA | Autumn | ≥15 °C | 60/15 °C | Taylor (1981) |
| Geraniaceae | | | | | |
| *Geranium carolinianum* | WA | Autumn | ≥20 °C | ≤20 °C | This study |
| *Erodium cicutarium* | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |
| *Geranium columbinum* | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |
| *Geranium dissectum*† | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |
| *Geranium lucidum* | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |
| *Geranium molle* | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |
| *Geranium pusillum* | WA | Autumn | If seeds buried in soil (wet) | Drying | Van Assche and Vandelook (2006) |

A, Annual; B, Biennial; SA, summer annual; WA, winter annual; RH, relative humidity
* Species known to have sensitivity cycling (sensitive ↔ insensitive)
† The present study shows that seeds of *G. dissectum* can become permeable under wet conditions without drying, hence they also have a one-step PY-breaking process.

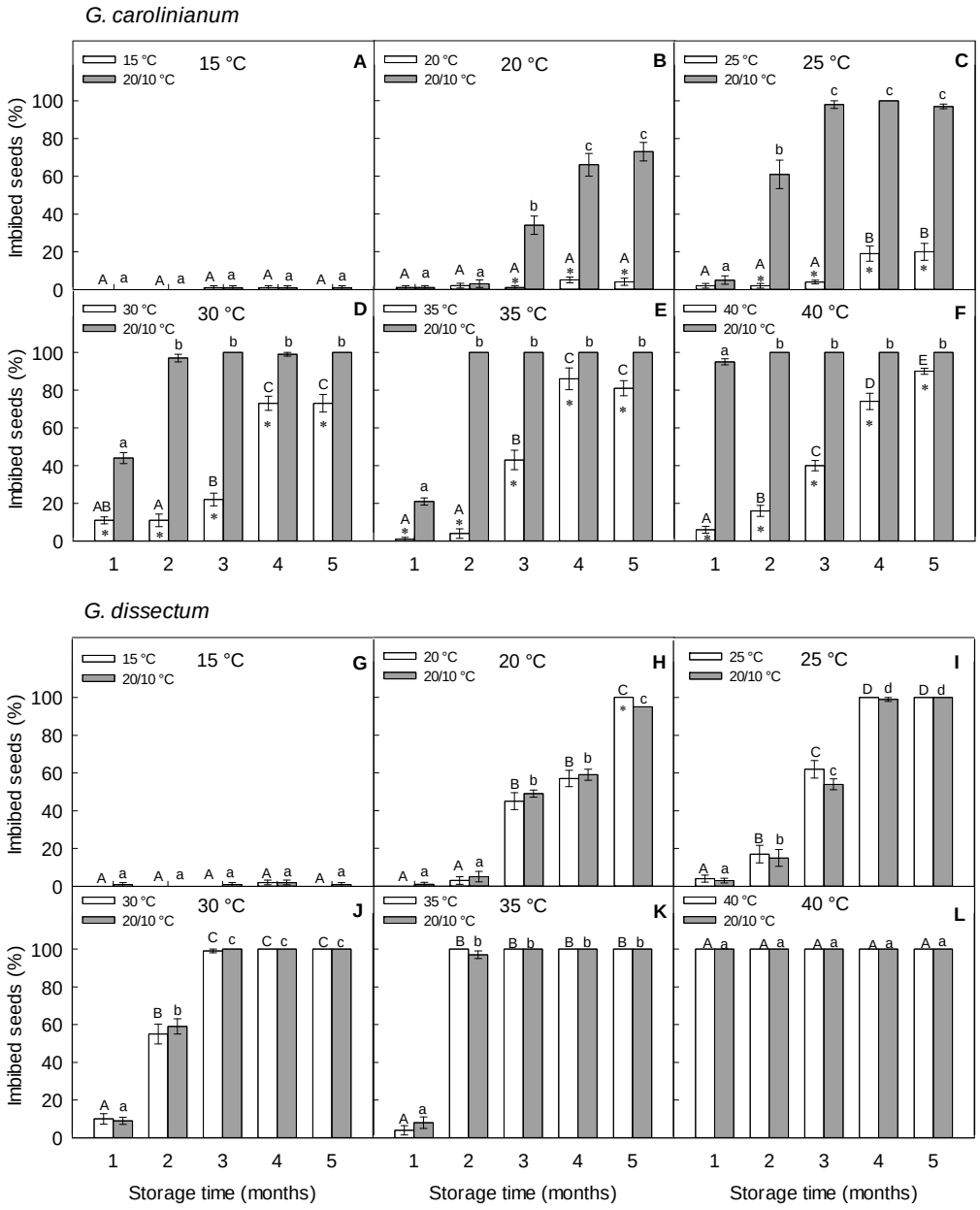


Figure 4.1. Percentage of imbibed seeds (mean ± s.e.) of (A–F) *G. carolinianum* and (G–L) *G. dissectum* at constant temperatures and at 20/10 °C after dry storage at different constant temperatures (15–40 °C) for 1 – 5 months. An asterisk indicates a significant difference in imbibition between the two incubation temperatures in each month. Different upper- and lower-case letters indicate a significant difference between different months in imbibition under a constant temperature and under 20/10 °C, respectively ($P < 0.05$).

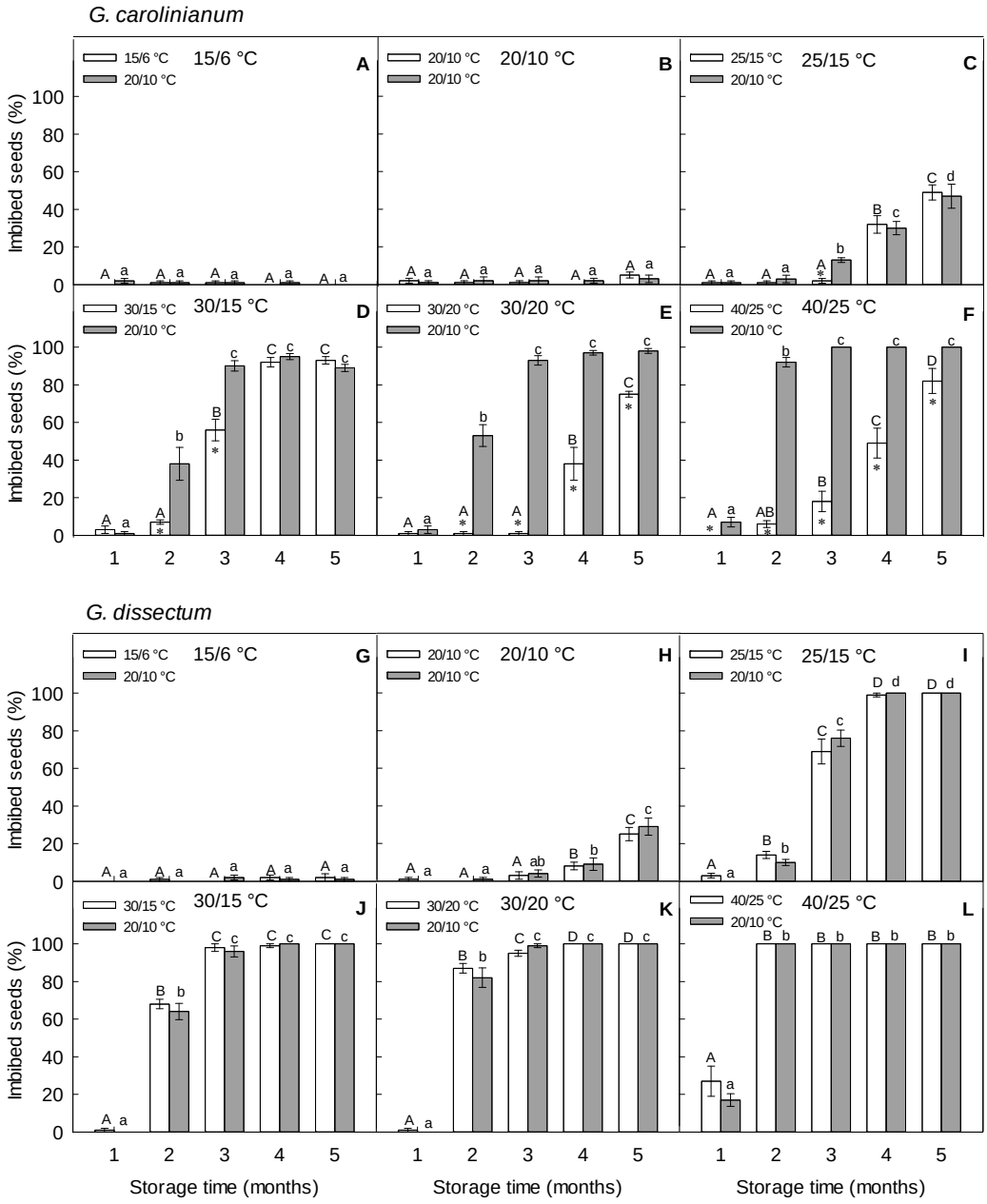


Figure 4.2. Percentage of imbibed seeds (mean ± s.e.) of (A–F) *G. carolinianum* and (G–L) *G. dissectum* at alternating temperatures and at 20/10 °C after dry storage at different alternating temperatures for 1–5 months. An asterisk indicates a significant difference in imbibition between the two incubation temperatures in each month. Different upper- and lower-case letters indicate a significant difference between different months in imbibition under a particular alternating temperature and under 20/10 °C, respectively ($P < 0.05$).

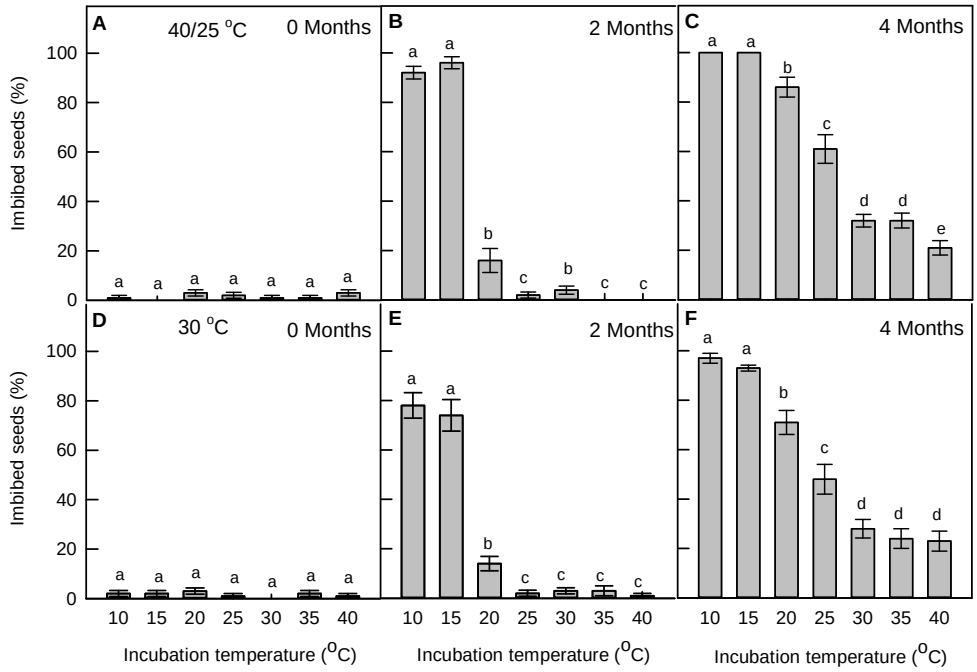


Figure 4.3. Percentage of imbibed seeds (mean ± s.e.) of *G. carolinianum* incubated at different constant temperatures after dry storage (A–C) at 40–25 °C and (D–F) at 30 °C. Different letters indicate significant differences in imbibition percentages between the incubation temperatures ($P < 0.05$).

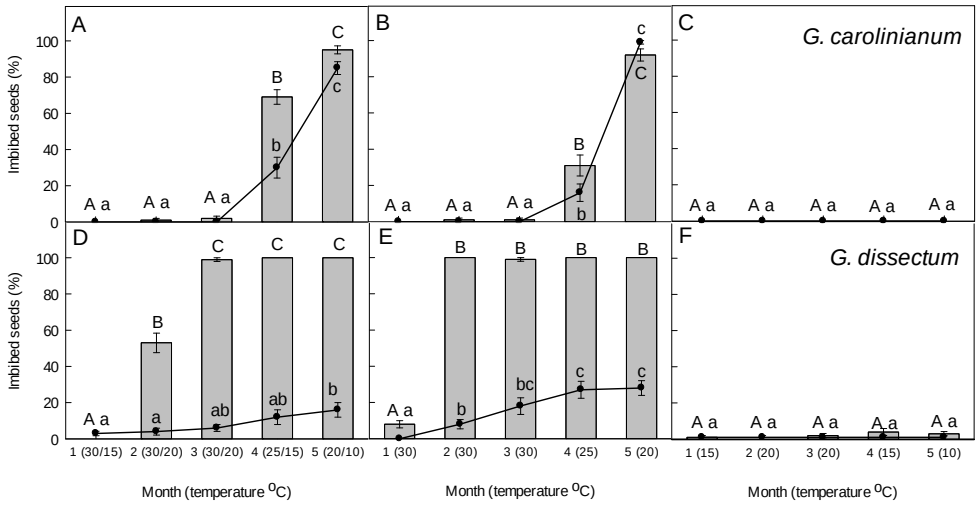


Figure 4.4. Percentage of imbibed seeds (mean ± s.e.) of (A–C) *G. carolinianum* and (D–F) *G. dissectum* stored under dry and wet conditions (as indicated) under temperature sequences: (A, D) alternating; (B, E) daily maximum; and (C, F) daily minimum temperature simulating natural temperature conditions of each month. Different upper- and lower-case letters indicate significant differences in imbibition percentages between the incubation temperatures under dry storage and wet storage, respectively ($P < 0.05$).

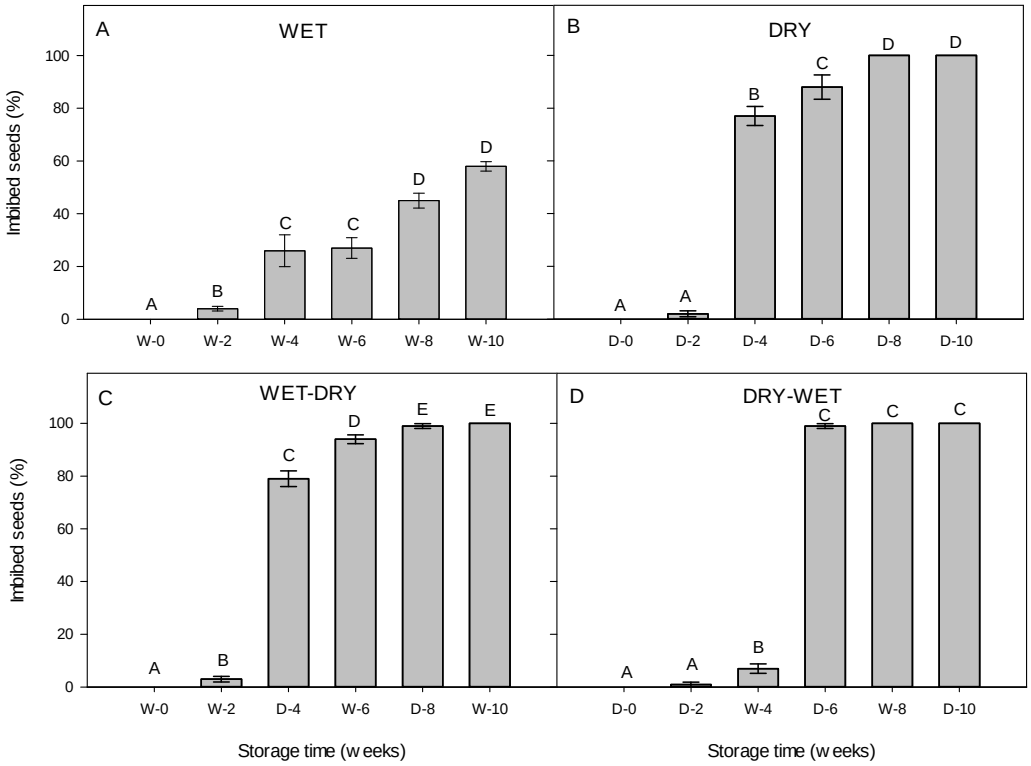


Figure 4.5. Percentage of imbibed seeds (mean ± s.e.) of *G. dissectum* at 40 °C, after storage under different moisture regimes at 40 °C. The moisture conditions of each week during the storage period are indicated on the axis: W, wet; and D, dry. Different letters indicate significant differences between imbibition percentages within each moisture regime ($P < 0.05$).

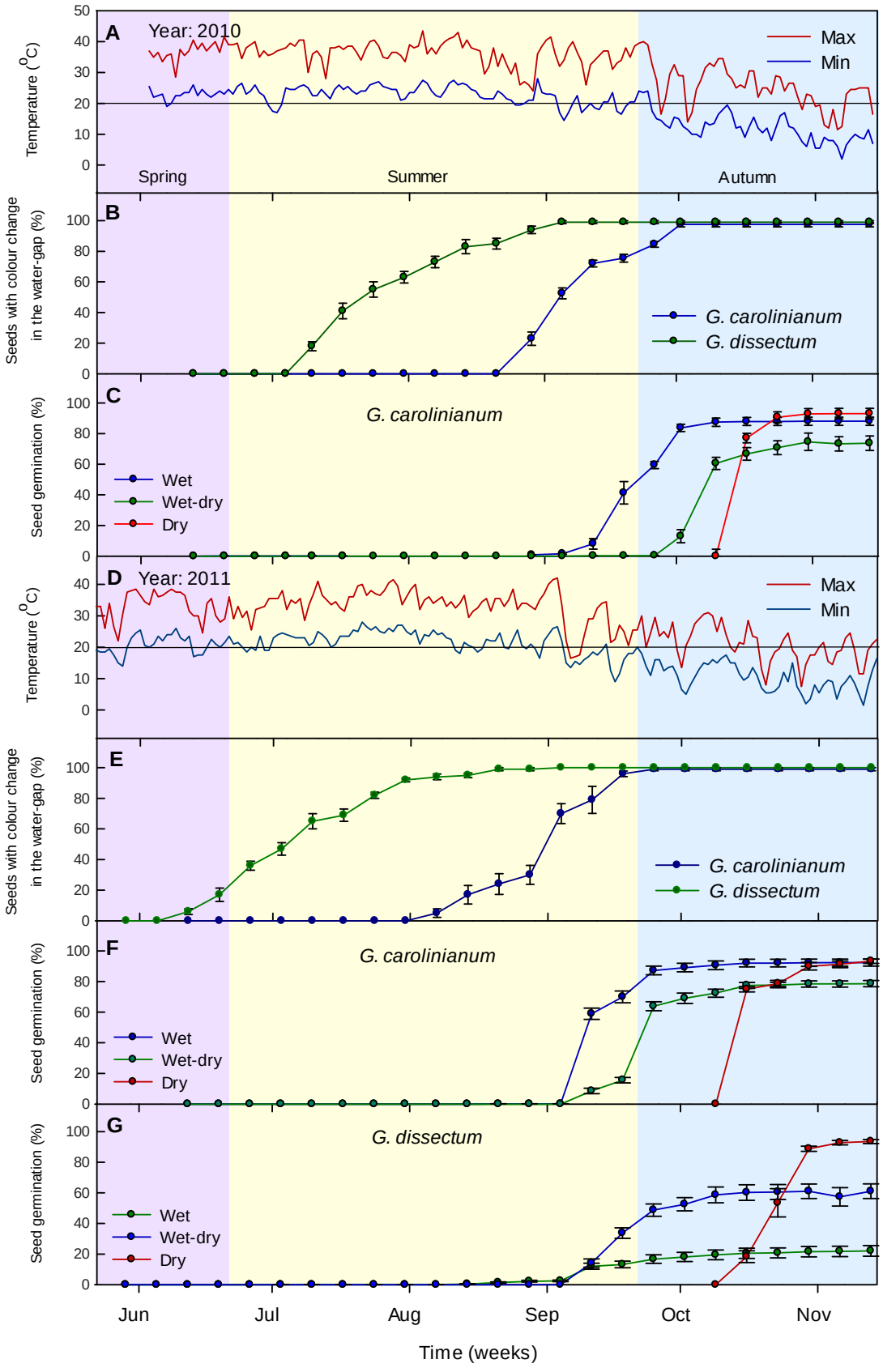
A
Year: 2010
Max
Min
Spring
Summer
Autumn
Temperature (°C)
B
Seeds with colour change in the water-gap (%)
G. carolinianum
G. dissectum
C
G. carolinianum
Wet
Wet-dry
Dry
Seed germination (%)
D
Year: 2011
E
F
G. carolinianum
G
G. dissectum
Jun
Jul
Aug
Sep
Oct
Nov
Time (weeks)

Figure 4.6. PY breaking of *G. carolinianum* and *G. dissectum* seeds under greenhouse conditions in 2010 and 2011. (A, D) Daily minimum and maximum air temperatures in the non-heated greenhouse in 2010 and 2011, respectively. Percentage (mean ± s.e.) of seeds with colour change from dark brown to brownish orange in the water-gap region of *G. carolinianum* and *G. dissectum* under dry conditions, (B) in 2010 and (E) 2011. Seed germination percentage (mean ± s.e) in *G. carolinianum* under the three moisture regimes in (C) 2010 and (F) 2011. (G) Seed germination percentage (mean ± s.e.) of *G. dissectum* in 2011 under the three moisture regimes.

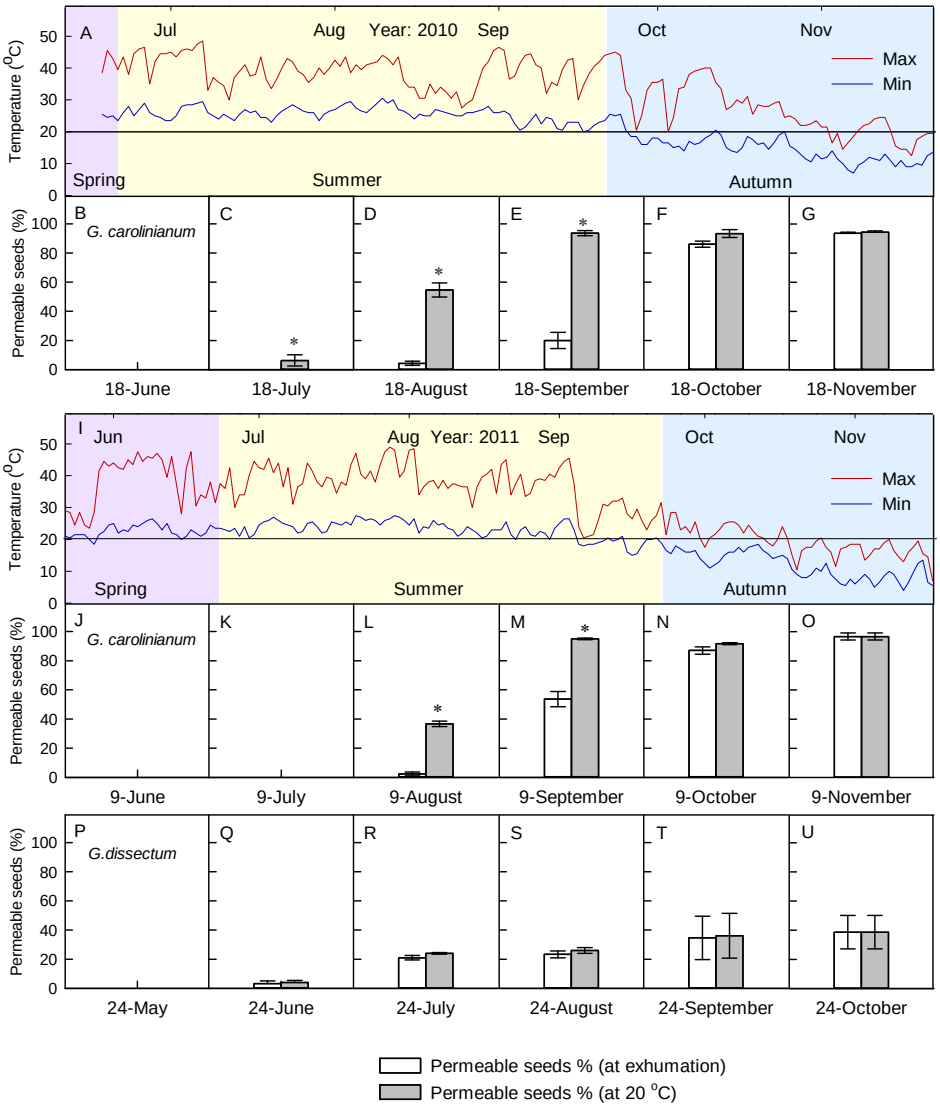


Figure 4.7. PY breaking in buried seeds of *G. carolinianum* in 2010/11 and *G. dissectum* in 2011. (A, I) Daily minimum and maximum soil temperatures at 2 cm soil depth in 2010 and 2011, respectively. Percentage (mean ± s.e.) of permeable seeds of *G. carolinianum* of (B) fresh seeds and after incubation at 20 °C; (C–G) at the time of exhuming and after incubation at 20 °C, in 2010. Percentage (mean ± s.e.) of permeable seeds of *G. carolinianum* of (J) fresh seeds and after incubation at 20 °C; (K–O) at the time of exhuming and after incubation at 20 °C, in 2011. Percentage (mean ± s.e.) of permeable seeds of *G. dissectum* of (P) fresh seeds and after incubation at 20 °C; (Q–U) at the time of exhuming and after incubation at 20 °C, in 2011 An asterisk indicates a significant difference between treatments.

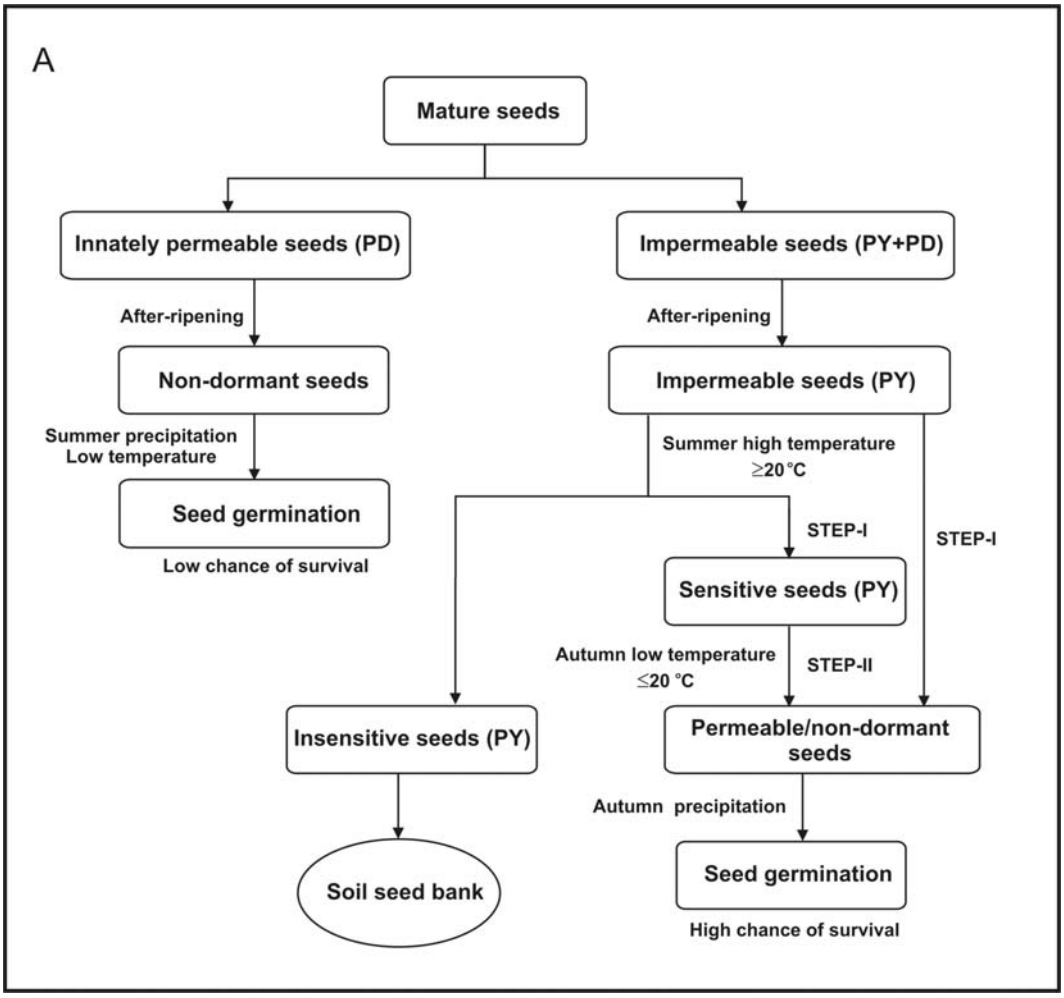
A
Mature seeds
Innately permeable seeds (PD)
Impermeable seeds (PY+PD)
After-ripening
After-ripening
Non-dormant seeds
Impermeable seeds (PY)
Summer precipitation
Low temperature
Summer high temperature
≥20 °C
Seed germination
Low chance of survival
STEP-I
STEP-I
Sensitive seeds (PY)
Autumn low temperature
≤20 °C
STEP-II
Insensitive seeds (PY)
Permeable/non-dormant seeds
Autumn precipitation
Soil seed bank
Seed germination
High chance of survival

Figure 4.8 (continued)

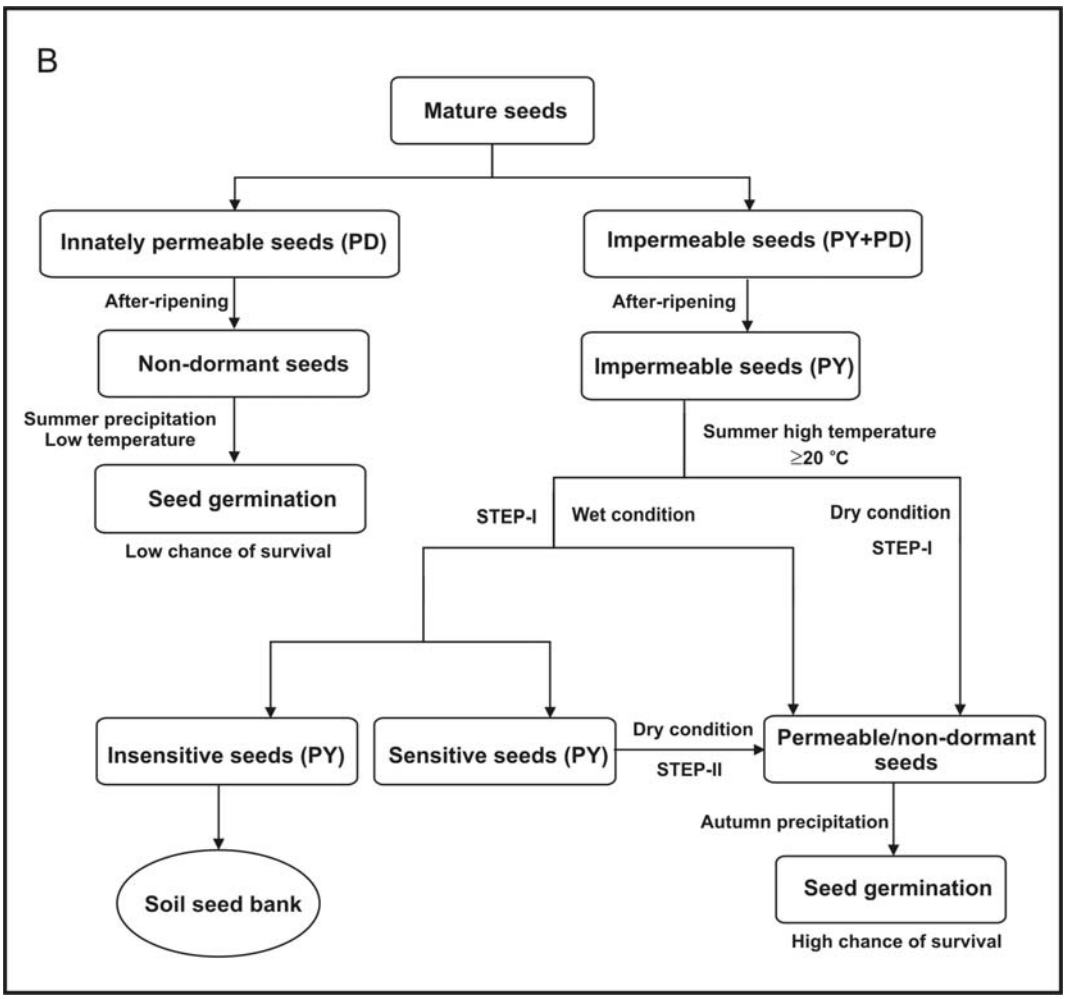


Figure 4.8. Conceptual models for breaking seed dormancy in (A) *G. carolinianum* and (B) *G. dissectum*.

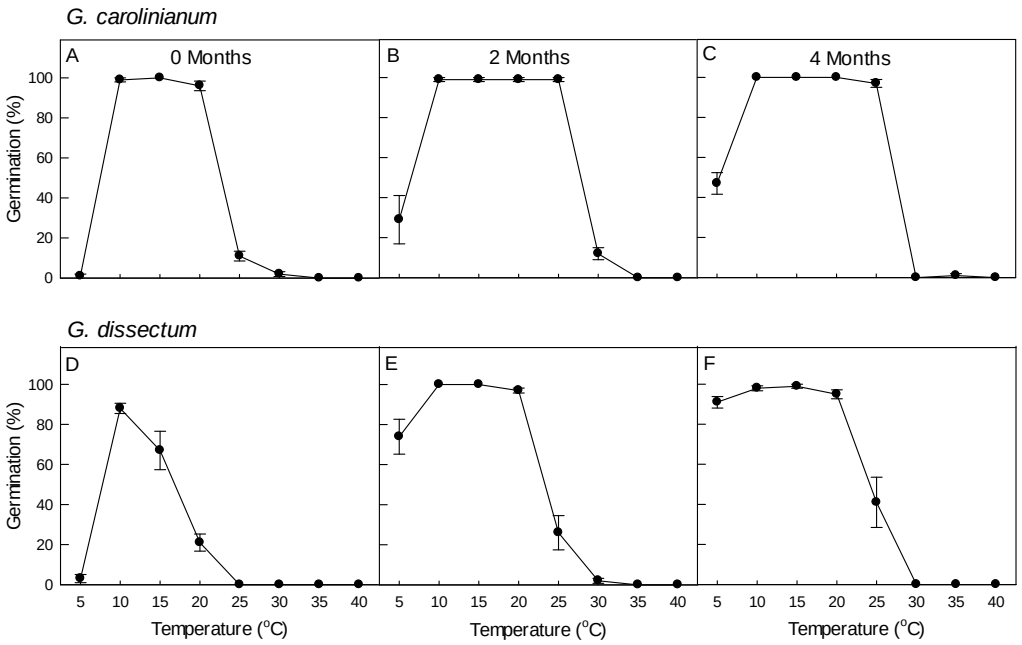


Figure 4.9. Percentage germination (mean ± s.e.) of manually scarified seeds of (A-C) *G. carolinianum* and (D-F) *G. dissectum* at different constant temperatures after dry-storage at ambient room conditions for 0, 2 and 4 months.

# CHAPTER 5

## Quantitative analysis of the thermal requirements for stepwise physical dormancy-break in seeds of the winter annual *Geranium carolinianum* L. (Geraniaceae)

## INTRODUCTION

Temperature is the primary factor involved in breaking of physical dormancy (PY) (Taylor, 2005). Depending on the species, PY-break in seeds can take place either in one step or two steps (Gama-Arachchige *et al*., 2012). The process of PY-breaking in seeds of certain annual species takes place in two steps controlled by two different temperatures (Taylor, 1981, 2005) and/or moisture regimes (Gama-Arachchige *et al*., 2012). During the first step, PY-seeds become sensitized to dormancy breaking treatment(s), yet they remain impermeable. During the second step, seeds become permeable upon exposure to the appropriate environmental conditions (Jayasuriya *et al*., 2008*a*, 2009*c*, Gama-Arachchige *et al*., 2012).

The concept of thermal time, i.e. the exposure to a temperature above a threshold level for a particular time period has been successfully applied in determining and comparing the rates of various physiological events in plants and poikilothermic invertebrates (Trudgill *et al*., 2005). This concept has been employed in describing and quantifying physiological dormancy (PD)-break by after-ripening (Bradford, 2002; Batlla *et al*., 2009) and single step PY-break (McDonald, 2000). However, the concept of thermal time has not been used for the explanation of stepwise PY-breaking processes.

*G. carolinianum* is a winter annual weed, native to eastern North America and is reported to be a naturalised weed in many parts of the world including Australia, China, Great Britain, Japan, Italy and South America Mature seeds of *G. carolinianum* exhibit PY and shallow PD (Aedo *et al*., 1998*b*; Aedo, 2000; Gama-Arachchige *et al*., 2012).

The water-gap region is a morpho-anatomically specialized area in the seed or fruit coat in species with PY that opens during PY-breaking and allows the entry of water into the seed during imbibition. In Geraniaceae, a small opening near the micropyle (hinged-valve gap) acts as the water-gap (Gama-Arachchige *et al*., 2010). PY-break in *G. carolinianum*, a temperature and time dependant process, occurs in two temperature-dependant steps. During the first step, seeds become sensitive when stored at temperatures ≥20 $^{o}$C. In the second step, sensitive seeds are made permeable when exposed to temperatures ≤20 $^{o}$C (Gama-Arachchige *et al*., 2012).

On breaking of PY, the water-gap region in seeds of *G. carolinianum* becomes visible in brownish orange colour. Application of pressure causes a similar colour change in the palisade cells of the water-gap region while making the seeds permeable (Gama-Arachchige *et al*., 2010). It has been shown that the pressure that builds up upon heating under the palisade layers of the lens in seeds of *Acacia kempeana* (Hanna, 1984) and under the bulges in seeds of *Ipomoea lacunosa* (Jayasuriya *et al*., 2008*a*) causes the water-gap palisades to pop off, forming the water-gap opening(s). However, the colour change in the water-gap region of *G. carolinianum* takes place when sensitive seeds are placed at a lower temperature than the sensitivity-inducing

temperature. Therefore, a pressure buildup under the water-gap palisades in *G. carolinianum* is unlikely.

The objectives of the current study on seeds of *G. carolinianum* were to (1) investigate the role of temperature in driving the two steps of PY-breaking, (2) establish a thermal time (degree-weeks) model to explain sensitivity induction quantitatively and (3) propose a mechanism to explain PY-breaking, focusing on the water-gap region.

## MATERIALS AND METHODS

*Seed collection and preparation*

Stems of *Geranium carolinianum* bearing mature fruits were collected from plants growing on Spindletop Farm, Lexington, KY, USA, on 1 June 2011. They were covered with a mesh-cloth and allowed to dry for 3 days inside a non-heated greenhouse. Seeds released naturally were collected and stored in a refrigerator (approx. 5 $^{o}$C, dry storage) until used. Experiments were started within two weeks of seed collection.

Step-I: Induction of sensitivity

*Sensitivity induction test*

To calculate the thermal time required for sensitivity induction, seeds were stored dry at constant temperatures of 5, 10, 15, 20, 25, 30, 35 and 40 $^{o}$C in Petri dishes for 20

weeks. Cool white fluorescent light at 400-700 nm was supplied continuously, at approx. 40 μmol $m^{-2}$ $s^{-1}$.

Due to the lack of visible changes, insensitive seeds cannot be distinguished from sensitive seeds (Gama-Arachchige *et al*., 2010). Therefore, the ability to imbibe water after exposure to low temperatures (≤20C) was selected as an indication of sensitivity (Gama-Arachchige *et al*., 2012). A sample of 100 seeds was retrieved from each storage temperature every week and incubated at 10 $^{o}$C (under same the light conditions) on moist sand in five replicates of 20 seeds each. The number of imbibed seeds was counted after 2 weeks.

To study the relationship between storage temperature and sensitivity induction, the Arrhenius plot was constructed using sensitivity induction rates 1/T50 (where, T50 = storage time taken for 50% of the seeds to become sensitive) plotted against reciprocal of storage temperature (1/T).

*Development of the model*

The model was developed based on the assumptions that seed sensitivity induction is irreversible and the base temperature to induce sensitivity is constant for all the sub-populations (Gama-Arachchige *et al*., 2012).

The induction of sensitivity (step-I) was assessed in relation to the accumulation of thermal time. The thermal time units required for induction of sensitivity were calculated using the following function (1),

$$\theta_{PY} = (T_s - T_b).t_{PY} \quad (1)$$

where $\theta_{PY}$ is the thermal time ($^{o}$Cweeks) to induce sensitivity, $T_s$ the temperature at which seeds were stored ($^{o}$C), $T_b$ the base temperature to induce sensitivity ($^{o}$C) and $t_{PY}$ the storage time (weeks).

The base temperature was estimated using the reciprocal of the time required for sensitivity induction (rate of sensitivity induction). The PROBIT procedure in SAS ver. 9.2 was applied to estimate the time required for the induction of sensitivity in sub-populations of 25%, 50% and 75% of seeds. The rates of sensitivity induction were plotted against storage temperature and a linear regression model was fitted to estimate the x-intercept for each percentile. The average value of x-intercepts was considered as base temperature (Steinmaus *et al*., 2000; Bazin *et al*., 2011).

To determine the best model that describes the distribution of $\theta_{PY}$ within a population, Gompertz, Hill, Logistic, Sigmoid and Weibull functions were applied using global curve fitting option in Sigmaplot ver. 12.0. The best fit was first examined by superimposing the curve on the data points (Motulsky and Ranasnas, 1987). Then, to select the best model the candidate models were compared with corrected Akaike Information Criterion (AICc) (function 3) that considers model complexity and modelling accuracy (Burnham *et al*., 2011; Symonds and Moussalli, 2011; Eizenberg *et al*., 2012),

$$\mathrm{AICc} = 2k + n\left[\ln\left(\frac{\mathrm{RSS}}{n}\right)\right] + \frac{2k(k+1)}{n-k-1} \quad (2)$$

where *k* is the number of fitted parameters in the model, *n* the number of observations in the model and RSS the residual sum of squares. A lower AICc value indicates better fit of the model to the observed data, with the best approximating model being the one with the lowest AICc value (Symonds and Moussalli, 2011).

The model was evaluated based root mean square error (RMSE) (function 3),

$$RMSE = \sqrt{\left(1/\mathrm{n}\right)\sum_{\mathrm{i}=1}^{\mathrm{n}}\left(y_{obs} - y_{pred}\right)^2} \qquad (3)$$

where $y_{obs}$ and $y_{pred}$ are the observed and predicted imbibition values, respectively, and *n* the number of observations in the model. Smaller RMSE values indicate better fit of the model to the observed data.

*Model validation*

The model validation was performed using the results from sensitivity induction by alternating storage temperatures and non-heated greenhouse experiments.

*Alternating temperatures:* Seeds were stored at alternating temperatures of 15/6, 20/10, 25/15, 30/15, 30/20 and 40/25 $^{o}$C in Petri dishes. High and low temperatures were supplied on a 12 h/ 12 h daily basis under light/dark conditions (14/10 h; under the same light conditions as described above). From each storage temperature, a sample of 100 seeds was retrieved every week and incubated at 10 $^{o}$C on moist sand in five replicates. The number of imbibed seeds was counted after 2 weeks.

*Non-heated greenhouse:* Twenty seeds each were placed on dry sand in 100 plastic Petri dishes, which were placed on trays filled with potting soil inside a non-heated greenhouse. Air temperature inside the greenhouse was recorded in 30-minute intervals using a Thermochron ibutton (DS 1921G#F50) and daily average temperatures were calculated. Each week, five Petri dishes were retrieved and the sand was moistened with distilled water. They were incubated at 10 $^{o}$C under the same light conditions. The number of imbibed seeds was counted after two weeks.

The thermal time units required for induction of sensitivity for the alternating temperatures 30/20, 35/20, 40/25 $^{o}$C and non-heated greenhouse experiments were calculated using function (4) and for alternating temperatures where low temperature period is ≤ 15 $^{o}$C using function (5),

$$\theta_{PY} = (T_{avg} - T_b).t_{PY} \quad (4)$$

$$\theta_{PY} = (T_h - T_b).t_{PY}/2 \quad (5)$$

where $\theta_{PY}$ is the thermal time ($^{o}$Cweeks) to induce sensitivity, $T_{avg}$ the average temperature at which seeds were stored/average daily temperature in the greenhouse ($^{o}$C), $T_h$ the high temperature period ($^{o}$C), $T_b$ the base temperature to induce sensitivity ($^{o}$C) and $t_{PY}$ the storage time (weeks).

Cumulative percentage of sensitive seeds from alternating temperature and greenhouse experiments were plotted against thermal time and the developed thermal time model (three-parameter Gompertz) was superimposed to compare the actual

thermal induction and predicted thermal time by the model. Goodness of fit of the developed model was estimated for each alternating storage temperature and non-heated greenhouse data according to RMSE values.

Step-II: Breaking of PY

*Effect of temperature on PY-break*

To determine the effect of temperature on PY-break in step-II, seeds were stored at 30 $^{o}$C in Petri dishes for 4 months to induce sensitivity. A ~2 mm layer of moulding clay was spread inside a Petri dish and 20 sensitive seeds without the colour change in the water-gap region were embedded so as to point the water-gap upwards. The open Petri dish was immersed in a temperature-controlled water bath and observed for colour change at the water-gap as an indication of PY-break. The number of seeds with a colour change was counted in 15 sec intervals for 1 hour. The procedure was repeated with five replicates for each temperature regime (5, 10, 15, 20, 25, 30, 35 and 40 $^{o}$C). Water bath temperature of 5, 10, 15 and 20 $^{o}$C was maintained using ice, while a hot plate was used to achieve the higher temperatures.

To study the relationship between incubation temperature and PY-break, the Arrhenius plot was constructed using PY-breaking rates 1/T50 (where, T50 = incubation time taken for 50% of the seeds to become permeable) plotted against the reciprocal of incubation temperature (1/T).

*Determination of separation force*

To determine the force required for separation of the water-gap palisade cell layer from sub-palisade layer during the PY-breaking step, 50 seeds made sensitive by

storing them at 25 $^{o}$C for 5 months and 50 insensitive seeds (untreated) were cut transversely into two halves. The halves with the micropyle were glued at the cut surface onto wooden blocks (Fig. 5.1). The separation force was measured with a Chatillon® DFM10 penetrometer. A probe with a blunt tip 0.2 mm in diameter was fixed to the penetrometer and the micropyle was touched with the tip of the probe. As the stage of the penetrometer was moved upwards, observations were made under a microscope until a colour change was seen at the micropyle-water-gap region, at which time the maximum force reading was recorded.

*The effect of external cooling*

To determine the internal temperature of seeds upon external cooling, 60 seeds made sensitive by storing them for five months at room temperature (approx. 23 $^{o}$C). Using a 0.45 mm drill bit, a hole was drilled in each seed up to the subpalisade layer of the water-gap end, starting at the widest point of the seed at the end opposite to the water-gap. The probe of a type K micro-thermocouple (0.432 mm width) was inserted into the drill-hole of a seed. The water-gap end of the seed was placed on the water surface in a temperature-controlled water bath and the internal temperature of the seed was recorded at 1 second intervals for 1 minute using LASCAR EL-USB-TC data loggers. The minimum temperature recorded during the 1 minute period was used for calculation. The procedure was repeated for 15 seeds each, for 0, 5, 10, 15 and 20 $^{o}$C. For 0 $^{o}$C, ice was used instead of water.

*Role of moisture in opening of the water-gap*

To evaluate the role of moisture level in opening of the water-gap, permeable (heat treated) seeds were incubated at different relative humidity levels. Eight-hundred

seeds were made permeable by them storing under 30 $^{o}$C for five months followed by exposure to 10 $^{o}$C for 24 hr. Five replicates (20 seeds each) were placed on a wire mesh platform suspended in accelerated aging plastic boxes filled with 100 ml of saturated salt solutions as follows, to maintain different RH levels: $H_2O$, 100 %; KCl, 83.5%; NaCl, 75%; MgCl, 32%; and LiCl, 11.5% at 30 $^{o}$C; $NaNO_2$, 65% at 25 $^{o}$C; $Mg(NO_3)_2$ 50.5% at 35 $^{o}$C; and CaCl, 40% at 5$^{o}$C (Weston *et al*., 1992; Fang and Moore, 1998; Baalbaki *et al*., 2009). After 24 hours of incubation, the number of seeds with a water-gap blister was recorded.

*Morphological changes during early imbibition*

To observe the morphological changes during early imbibition, 40 untreated seeds were soaked in water for two hours and the outer permeable layers were removed with a tooth-pick (Gama-Arachchige *et al*, 2010). They were made permeable by drying at 40 $^{o}$C for two months followed by exposure to 10 $^{o}$C for 24 hr. To observe the morphological changes during early imbibition, seeds were allowed to imbibe water under ambient conditions for 0 to 20 min. Three seeds each were removed from water at two min intervals for 20 min of imbibition and blotted dry. Three sensitive (impermeable) seeds (outer permeable layers removed) were used as a control. All the seeds were mounted on scanning electron microscopy specimen stubs using double-sided carbon tapes. Then, the samples were sputter-coated with gold-palladium (15 nm), scanned with an S-3200 Hitachi scanning electron microscope at an acceleration voltage of 5.0 kV and micrographs taken.

## RESULTS

Step-I: Induction of sensitivity

*Sensitivity induction test*

Seeds stored at temperatures ≤15 $^{o}$C did not become sensitive even after 20 weeks of storage (Fig. 5.2A; results for storage under 5 and 10 $^{o}$C not shown). The minimum storage temperature at which seeds became sensitive was 20 $^{o}$C. With increasing storage temperature and time, the fraction of sensitive seeds increased. The time required for 50% of the seeds to acquire sensitivity decreased exponentially with increasing temperature ($R^2$=0.98; Fig. 5.3A).

The Arrhenius plot for sensitivity induction (step-I) showed a negative relationship between the rate of sensitivity induction and the reciprocal of storage temperature. Temperature coefficient values ($Q_{10}$) were between 3.5 and 2.0 ($R^2$=0.97; Fig. 5.3B).

*Development of the model*

Linear extrapolation of the sensitivity induction rate data of three sub-populations resulted in an average x-intercept of 17.22 $^{o}$C (Fig. 5.4). Based on the RMSE values, all the candidate functions strongly fitted with the sensitivity induction data at constant temperatures (Table. 5.1). However, four-parameter Weibull and three-parameter Gompertz functions were the best two models based on AICc values with 265.002 and 265.388, respectively (Table 5.1). Therefore, the model with fewer parameters (Gompertz) was selected as the best model due to the ease of explanation of data, function (6),

$$S(\%) = 100 \times e^{-e^{-\left(\frac{x-x_0}{b}\right)}} \quad (6)$$

where S is the cumulative percentage of sensitive seeds, b the rate of increase, x the thermal time ($^{o}$C weeks) and $x_o$ the lag phase until the induction of sensitivity.

The values of parameters of the best fitted model are b=19.8380±1.2937 and $x_o$= 54.6846±0.9600 (*n*=65, RMSE=7.43; Fig. 5A). Therefore, the three-parameter Gompertz model for the sensitivity induction in PY-seeds of *G. carolinianum* can be expressed as function (7),

$$S(\%) = 100 \times e^{-e^{-\left(\frac{\theta_{PY}-54.6846}{19.8380}\right)}} \quad (7)$$

During the lag phase, sensitivity was not detected in seeds until a thermal time of 24.39 $^{o}$Cweeks was supplied (Fig. 5.5A). Thereafter, 25, 50 and 75 % of the seeds became sensitive at 48.02, 61.96 and 79.40 $^{o}$Cweeks, respectively.

*Model validation*

Seeds stored at 15/6 and 20/10 $^{o}$C alternating temperatures did not become sensitive even after 20 weeks of storage (Fig. 5.2B; results for storage under 15/6 and 20/10 $^{o}$C not shown). At 25/15 $^{o}$C, ~60% of seeds were sensitive by 20 weeks while, at all the other storage temperatures 100% of seeds were sensitive by 20 weeks.

The developed three-parameter Gompertz model fitted well for the observed values of the sensitivity induction at alternating temperatures with RMSE values ranging from

4.28 to 16.33 (Fig. 5.5B, Table 5.2). Moreover, the fitted model showed good agreement with the non-heated greenhouse data RMSE=11.91 and with the 40/25 $^{o}$C (average summer soil temperature; Gama-Arachchige *et al*., 2012) data, RMSE=12.40 (Fig. 5.5B; Table 5.2). However, the fitted model slightly over-estimated the sensitivity induction under non-heated greenhouse conditions and 40/25 $^{o}$C. Also the model slightly underestimated the sensitivity induction under 30/15, 30/20 and 35/20 $^{o}$C.

Step-II: Breaking of PY

*Effect of temperature on PY-break*

$T_{50}$ colour (minutes), the time taken for the colour change (= PY-break) in 50 % of the seed population, decreased exponentially from ~62 minutes at 25 $^{o}$C to ~15 seconds at 5 $^{o}$C (Fig. 5.3A). At temperatures ≥30 $^{o}$C, none of the seeds indicated a colour change even after 24 h.

The Arrhenius plot for PY-break (step-II) showed a positive relationship between the rate of PY-break and the reciprocal of storage temperature with temperature coefficient ($Q_{10}$) values between 0.02 and 0.1($R^2$=0.98; Fig. 5.3B).

*Determination of separation force*

The force required for the separation of water-gap palisade cell layer from the sub-palisade cell layer was significantly reduced from insensitive seeds (2.59±0.07 N) to sensitive seeds (1.60±0.05 N) ($P$< 0.05).

*The effect of external cooling*

The temperature difference between the water bath and seed interior decreased linearly with increasing water bath temperature (Fig. 5.6A).

*Role of moisture in opening of the water-gap*

The relationship between the storage relative humidity and the fraction of seeds with a water-gap blister followed a sigmoidal pattern (RMSE=4.32; Fig. 5.6B). The water-gap blister formed in seeds stored under a RH of >40%. With increasing RH, the fraction of seeds with a water-gap blister increased rapidly and reached 100% at 80% RH.

*Morphological changes during early imbibition*

No difference was observed in the morphology of water-gap palisade cells of sensitive (impermeable) and permeable (heat-treated) seeds prior to imbibition (Fig. 5.7A,B). After two minutes of imbibition, a slight rise began to appear in the water-gap palisade layer of permeable seeds and it had risen further by 4 minutes of imbibition (Fig 5.7C). Upon further imbibition, the water-gap palisades continued to rise until a crack was formed in the periphery of the raised area by eight minutes (Fig. 5.7D). On continued imbibition, the raised area detached at the crack while still hinged to the palisades at the micropyle (hinged-valve) after 12 minutes (Fig. 5.7E). By 20 minutes, the hinged-valve was completely dislodged, revealing the water-gap (Fig. 5.7F).

## DISCUSSION

Physical dormancy break in *G. carolinianum* is a moisture-independent, two-step process controlled by temperature (Gama-Arachchige *et al*., 2012). In the present study, a mathematical model was developed for the induction of sensitivity during step-I in PY-breaking in *G. carolinianum* seeds. The time required to attain sensitivity in seeds held under constant temperatures was described well by the developed three-parameter Gompertz model (RMSE=7.43). The developed model was robust enough to successfully predict the acquisition of sensitivity for alternating temperatures and semi-natural non-heated greenhouse conditions (RMSE=4.28-16.33). Thus, the developed model described the thermal requirements for sensitivity induction in each fraction of the seed population (Fig. 5.5A,B).

The parameter values of the model indicate that induction of sensitivity takes place at temperatures above the base temperature 17.22 $^{o}$C, and the higher the temperature above this value, the higher would be the rate of sensitivity induction. The base temperature for thermal models for dormancy break in PD seeds is assumed to be constant for all seed fractions of a population (Bradford, 2002). Similarly, in this study the value for the base temperature obtained by extrapolating the rates of sensitivity showed a constant value for all seed fractions (Fig. 5.4).

Mott *et al* (1981) and McDonald (2000) reported that the base temperature for PY break in several tropical and subtropical legume species growing in northern Australia ranged between 40-55 $^{o}$C. The high summer soil temperatures of those sites usually exceed this base temperature, hence a considerable amount of seeds become permeable during summer and germinate in the autumn (McDonald, 2000). Similarly, > 90% of the *G. carolinianum* seeds buried at a depth of 2 cm in an open area on the

campus of the University of Kentucky became sensitive during the summer of 2011 and germinated in autumn 2011 (Gama-Arachchige *et al.*, 2012). The average summer soil temperature at this location was ~28 $^{o}$C and therefore all the seeds can become sensitive within ~12 weeks during summer. Thus, the formation of a long term soil seed bank in Lexington is highly unlikely.

A negative correlation between the reciprocal of storage temperature and the rate of sensitivity induction during step-I can be observed in Arrhenius plots (Fig. 5.3B). In this study, $Q_{10}$ values for step-I ranged between 2.0 and 3.5. $Q_{10}$ values for chemical processes are generally in the range of 2-3 (Atwell *et al.*, 1999). Therefore, the involvement of a chemical process(es) during the sensitivity induction stage can be inferred in seeds of *G. carolinianum*. A similar observation ($Q_{10}$ values 3.4-5.1) has been obtained for PY-break in *Medicago arabica* (Van Assche and Vandelook, 2010).

Significant reduction in the force required to separate water-gap palisade cells from sub-palisade cells indicates weakening of the bond between these two cell layers during the sensitivity induction step. Zeng *et al* (2005) demonstrated that when seeds are exposed to field conditions, PY-break in several legume species is related to loss of lipids in the seed coat. Further, they suggested that the polymeric structure of lipids changes on exposure to high summer temperatures, due to weakening of hydrophobic bonds which increase the thermal degradation of lipids. In *G. carolinianum* seeds, a similar process can be expected to take place. During step-I, weakening of the polymeric lipids in the seed coat loosens the bonding between palisade and subpalisade layers. This phenomenon may be more prominent in the water-gap region, where the connection between the two layers is weak. The thermal

requirements to complete the weakening process (sensitivity induction) can be estimated from the model developed in this study. According to the results, weakening of the seed coat takes place at temperatures above 17 $^{o}$C and ~135 $^{o}$Cweeks are required for all the seeds to become sensitive.

Data from the non-heated greenhouse fitted well (RMSE=11.91) with the developed thermal time model. Therefore, this model is capable of predicting sensitivity induction under semi-natural conditions. However, further field experiments are required to test the application of this thermal time model to predict sensitivity induction under natural conditions.

*Step-II*

A positive correlation was observed for the reciprocal of incubation temperatures and the rate of step-II (PY-break) with $Q_{10}$ values between 0.02 - 0.1. As $Q_{10}$ values below 1.5 indicate purely physical processes (Clearwater *et al*., 2000), it can be assumed that a physical process is responsible for step-II. Based on the results from present study and Gama-Arachchige *et al* (2012), it was observed that the base temperature for PY-break in step-II varied with the storage temperature in step-I. Therefore, in the present study no thermal time models were developed for this step. However, further studies should be carried out to evaluate the possibility of developing a thermal time model for this step.

Sensitive *G. carolinianum* seeds can be made permeable when exposed to temperatures lower than the sensitivity induction temperature (Gama-Arachchige *et al.*, 2012). In the present study, the internal seed temperatures were always higher by

several degrees than the external temperature of the seed coat (= temperature of water bath). This temperature difference can create a tensile stress across the seed coat. Mott (1979) observed that seeds of several species of *Stylosanthes* became permeable only at the lens when in contact with a high temperature (140 - 150 $^{o}$C) metal plate for a short period (15 - 60 s). He also found that when seeds were made to contact the 145 $^{o}$C metal plate, the internal temperature of the seeds reached only ~100 $^{o}$C after 60 seconds. Since the seeds were agitated during the high heat treatment, only a very small portion of them were momentarily heated, while a larger portion remained cooler. This differential temperature might have imposed a considerable mechanical stress on the seed coat, causing the metastable (weak) palisade cells at the lens to fracture.

Based on the previous observations by Taylor (1996*a*, *b*) and Jayasuriya *et al* (2008*a*), the completion of the step-II in PY-break is much faster than the step-I in *Medicago polymorpha* and *Ipomoea lacunosa,* respectively. A similar pattern was observed in the PY-break of *G. carolinianum*. However, the completion of step-II in *G. carolinianum* takes place at a rate much faster than that in other species studied. At 5 $^{o}$C, only 15 seconds were required for 50 % of the seed population to complete step-II. Therefore, it can be assumed that step-I enables the seeds to maintain impermeability and thus survive in adverse conditions while progressing towards sensitivity. In sensitive seeds, step-II is triggered immediately on sensing the environmental cues that signal the commencement of favourable conditions for germination.

*Mechanism involved in the opening of the hinged-valve*

Sensitive *G. carolinianum* seeds cannot be distinguished from the insensitive seeds based on morpho-anatomical features (Gama-Arachchige *et al*., 2010). When sensitive seeds are exposed to temperatures lower than the sensitivity-induction temperature, a colour change can be seen near the micropyle (Fig. 5.8A-F; 5.9A, B). When a temperature difference builds up across the seed coat, palisade and subpalisade layers may shrink differentially (Fig. 5.9A). Consequently, the two cell layers may slip, forming a gap between them (Fig. 5.9B). Since the periclinal cell walls in contact in these two layers are smooth at the micropyle and gradually become corrugated towards the radicle (resulting in friction when slipping), the formation of the gap initiates at the micropyle and develops towards the radicle (Fig. 5.7F, 5.8A-F). This gap causes the incident light to refract, revealing a 'clam shell' shaped lighter colour area near the micropyle (water-gap) (Fig. 5.8F). The same colour change is imparted on applying a mechanical force at the water-gap region. According to the penetrometer experiment, this force is significantly lower for sensitive seeds than for insensitive seeds. This is a good indication of weakening of the connection between palisade and subpalisade layers in the water-gap region during sensitivity induction (step-I). A similar colour change in the water-gap region has previously been reported in *Sida spinosa*, upon application of pressure (Egley and Paul, 1981).

Opening of the water-gap is controlled by the availability of moisture during imbibition (Fig. 5.6B). A web of cracks (a few micrometers in depth) can be found in the upper periclinal walls of the palisade cell layer of mature seeds (insensitive, sensitive and permeable) (Fig. 5.7A,B). Water can enter through these cracks, causing the upper part of the palisades to swell. However, the lower part of the palisades cannot expand since the lower periclinal walls of the palisades are tightly bound to

sub-palisades throughout the seed coat in sensitive and insensitive seeds. This stops further imbibition. After PY is broken, the water-gap palisade cells can continue to swell since they are not connected to the water-gap subpalisade cells (Fig. 5.9C). This causes the deepening of the cracks in palisades and makes the cells permeable. Subsequently, as imbibition proceeds, the palisades of the whole water-gap region swell and bend outward forming a blister (hinged-valve) (Fig. 5.9D). Then, due to the tension, the water-gap palisades separate from subpalisades along the water-gap margin. Eventually, with further swelling the hinged-valve dislodges from the seed coat revealing the water-gap (Fig. 5.9E,F).

In conclusion, induction of sensitivity by temperature during the first step of PY-break in *G. carolinianum* can be best explained by the thermal time model using a three-parameter Gompertz model. The developed thermal time model is also able to predict sensitivity induction in *G. carolinianum* under semi-natural conditions. Differential thermal contraction of the palisade layer in the water-gap region may be the reason for the colour change and PY-break. Thus the water-gap region acts as a thermal sensor that detects the onset of autumn.

Table 5.1. Summary of the model selection statistics for models fitted to sensitivity induction at constant temperature storage.

| | Candidate models | Equation | RSS | RMSE | *k* | *n* | AICc |
|---|---|---|---|---|---|---|---|
| 1 | Gompertz, 3 parameter | $Y = a*exp(-exp(-(x-x_0)/b))$ | 3473.6894 | 7.43 | 3 | 65 | 265.002 |
| 2 | Sigmoid, 3 parameter | $Y = a/(1+exp(-(x-x_0)/b))$ | 3789.4080 | 7.76 | 3 | 65 | 270.656 |
| 3 | Logistic, 3 parameter | $Y = a/(1+abs(x/x_0)^b)$ | 3587.7317 | 7.55 | 3 | 65 | 267.101 |
| 4 | Logistic, 4 parameter | $Y = Y_0+a/(1+abs(x/x_0)^b)$ | 3567.8018 | 7.59 | 4 | 65 | 269.012 |
| 5 | Weibull, 4 parameter | $Y = a*(1-exp(-(abs(x-x_0+b*ln(2)^(1/c))/b)^c)))$ | 3374.2916 | 7.38 | 4 | 65 | 265.388 |

Table 5.2. Evaluation of the thermal time model for induction of sensitivity in seeds of *Geranium carolinianum* under alternating temperatures and non-heated greenhouse conditions.

| Temperature condition | *n* | RMSE |
|---|---|---|
| 25/15 | 21 | 4.28 |
| 30/15 | 17 | 16.33 |
| 30/20 | 17 | 12.14 |
| 35/20 | 17 | 9.28 |
| 40/25 | 12 | 12.40 |
| Non-heated greenhouse | 18 | 11.91 |

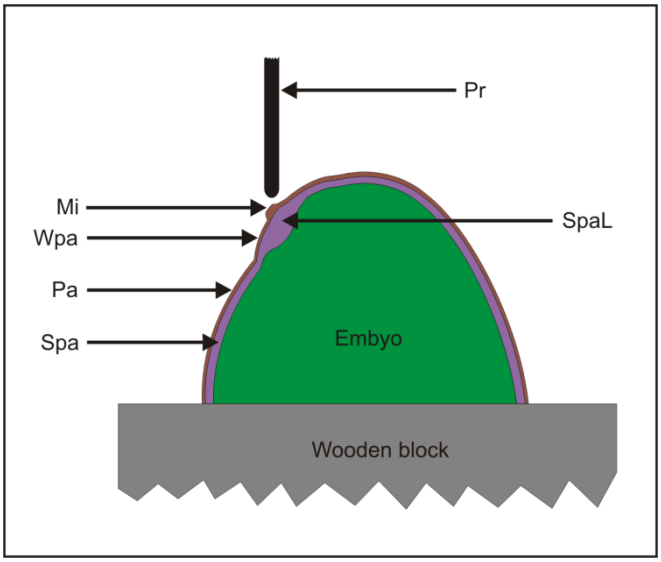


Figure 5.1. Experimental setup for the measurement of separation force of palisade cells from subpalisade cells in the water-gap of seeds of *G. carolinianum*: Mi, micropyle; Pa, palisade cells; Pr, probe of the Chatillon® DFM10 penetrometer; Spa, subpalisade cells; SpaL, elongated water-gap subpalisade cells; Wpa, water-gap palisade cells.

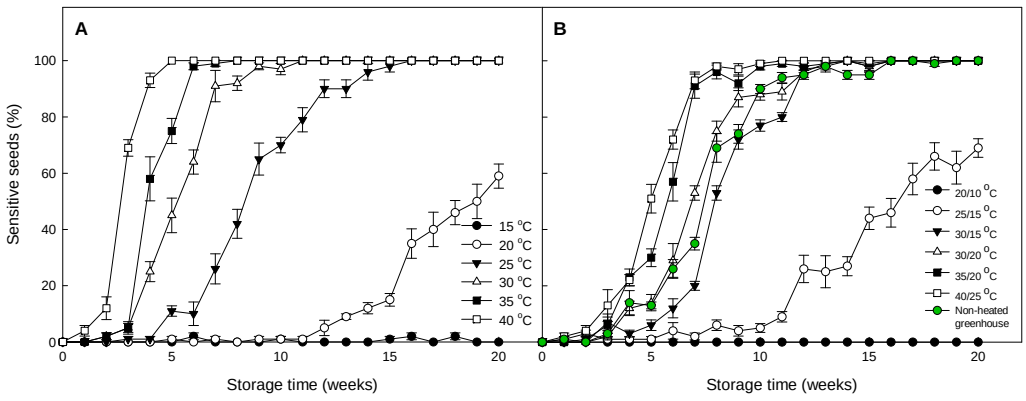


Figure 5.2. Cumulative percentage of sensitive seeds (mean ± s.e) at the end of two weeks incubation at 10 °C after dry storage at different (A) constant temperatures and (B) alternating temperatures or non-heated greenhouse conditions.

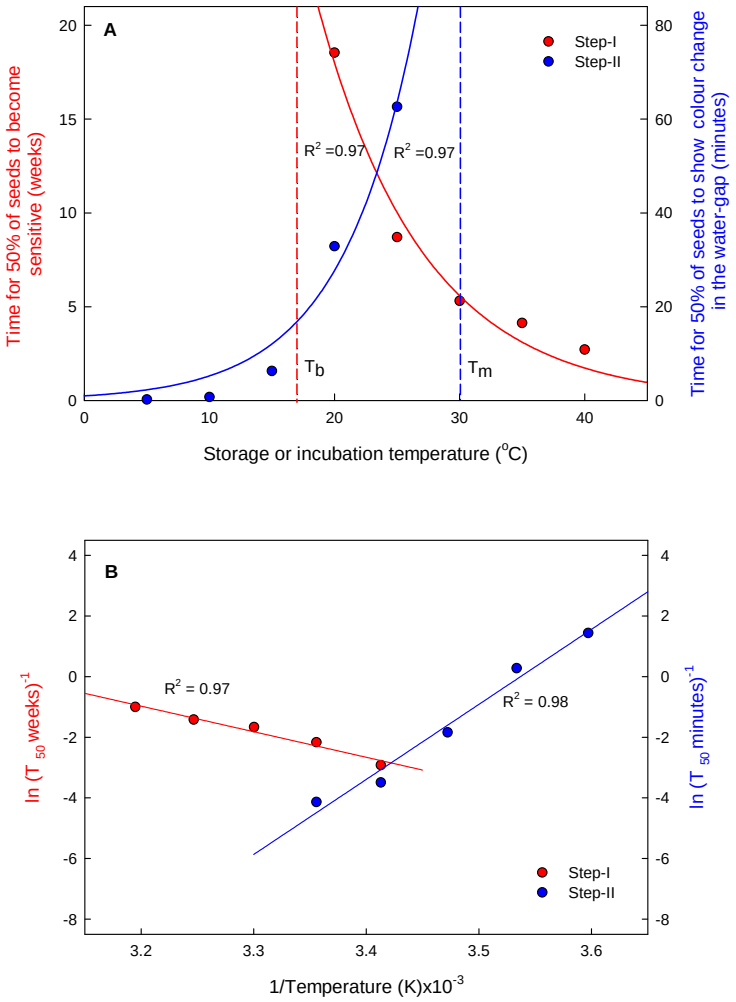


Figure 5.3. (A) Time taken for 50% of seeds to become sensitive under different storage temperatures (step-I; red line) and to show colour change in the water-gap region (step-II; blue line) under different incubation temperatures. Vertical dash lines indicate the minimum temperature limit ($T_b$) for the induction of sensitivity (step-I; red) and the maximum temperature limit ($T_m$) for the induction of colour change in the water-gap region (step-II; blue). $R^2$ values were derived from exponential regression lines. (B) Arhenius plots of sensitivity induction rate ln $(T_{50}$ weeks$)^{-1}$ (step-I; red line) and PY-break rate ln $(T_{50}$ minutes$)^{-1}$ (step-II; blue line) plotted against 1/temperature (K) x $10^{-3}$. $R^2$ values were derived from linear regression lines.

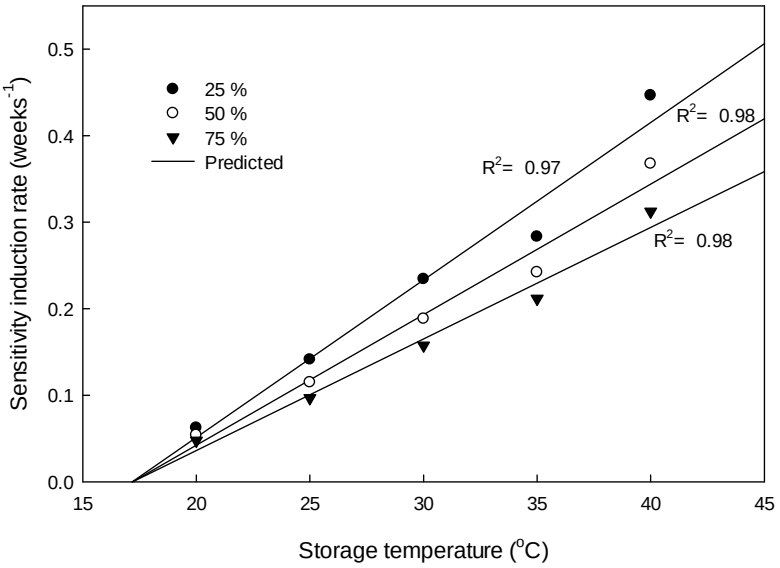


Figure 5.4. Sensitivity induction rates of seed subpopulations (25%, 50% and 75%) of *G. carolinianum* plotted against the storage temperature. Extrapolation of the linear regression to the x axis yielded the base temperature ($T_b$). $R^2$ values were derived from linear regression lines.

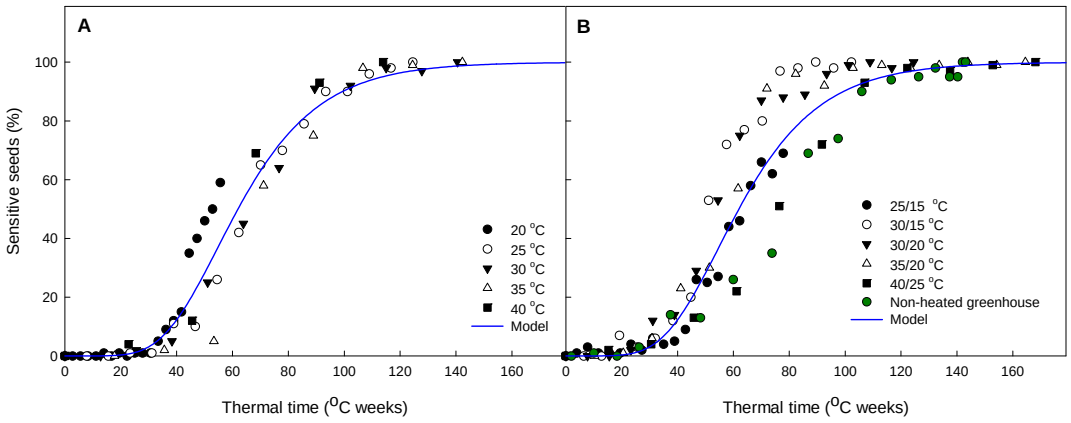


Figure 5.5. Cumulative sensitivity induction (%) of seeds of *G. carolinianum* as a function of sensitivity induction thermal time ($^{o}$Cweeks). (A) Symbols represent the observed percentage of sensitive seeds at different constant storage temperatures. The solid line corresponds to the three-parameter Gompertz model [equation (7)]. (B) Validation of the developed thermal time model for sensitivity induction (solid line) for alternating temperatures and non-heated greenhouse conditions.

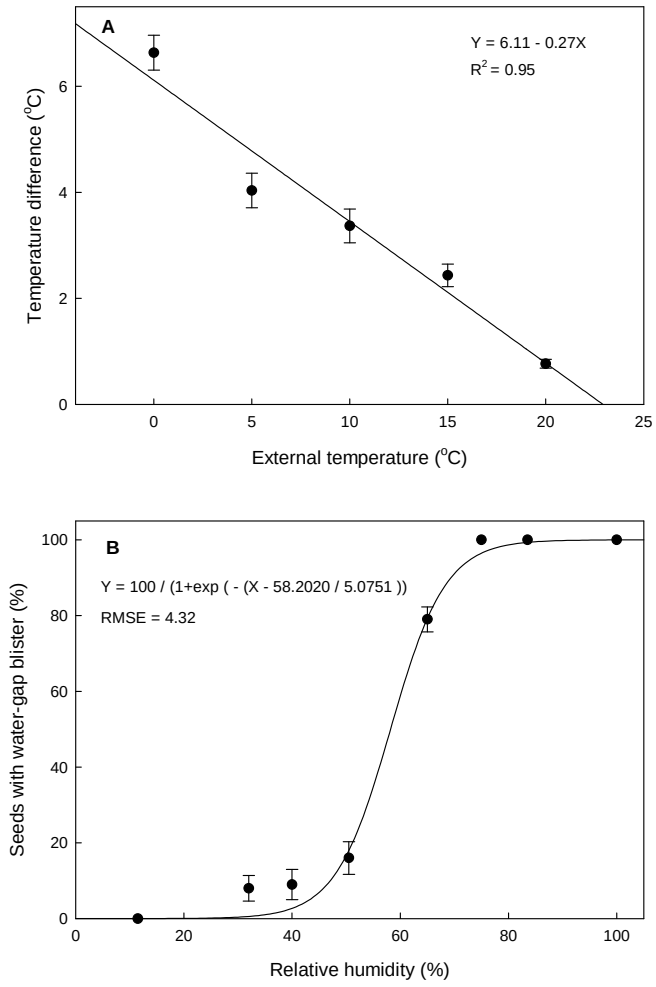


Figure 5.6. (A) Difference between internal and external temperatures of seeds of *G. carolinianum* plotted against the seed external temperature (water bath). $R^2$ value was derived from the linear regression line. (B) Percentage of seeds with water-gap blister (mean ± s.e) after incubating for 24 h under different relative humidity levels. RMSE value was derived from the sigmoidal regression line.

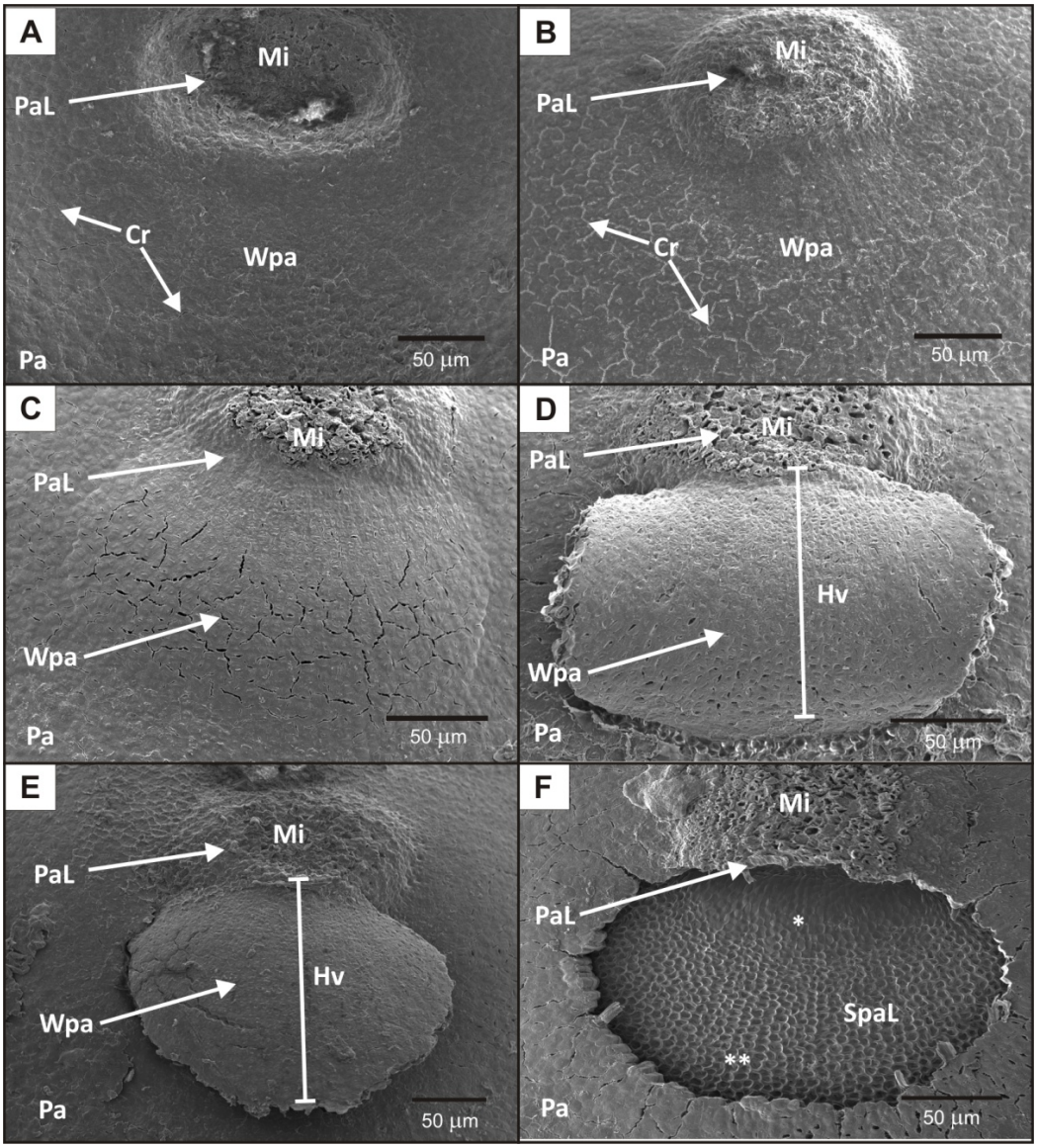


Figure 5.7. Scanning electron micrographs of the micropylar-water-gap region of *G. carolinianum* seeds without the outer permeable cell layers: (A) sensitive seed (impermeable); (B) seed with colour change in the water-gap (permeable); (C) permeable seed soaked in water for two minutes with slightly raised water-gap palisades forming a blister; (D) permeable seed soaked in water for eight minutes with raised water-gap palisades; (E) permeable seed soaked in water for twelve minutes with raised water-gap palisades (hinged-valve) still attached at the micropylar end; (F) permeable seed soaked in water for 20 minutes with revealed water-gap opening after the dislodgement of the hinged-valve. Abbreviations: Cr, cracks on the palisade layer; Mi, micropyle; Pa, palisade cells; PaL, elongated palisade cells of the water-gap; SpaL, elongated subpalisade cells of the water-gap; Wpa, water-gap palisades; *, subpalisade cells with a smooth outer periclinal cell wall; **, subpalisade cells with a corrugated outer periclinal cell wall.

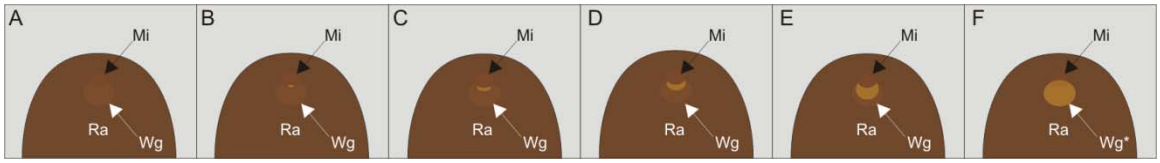


Figure 5.8. Schematic diagrams of the morphological changes in the water-gap region of sensitive seeds of *G. carolinianum* during different stages of PY-break (step-II). (A-F) The colour change starts at the micropyle and develops towards the radicle.

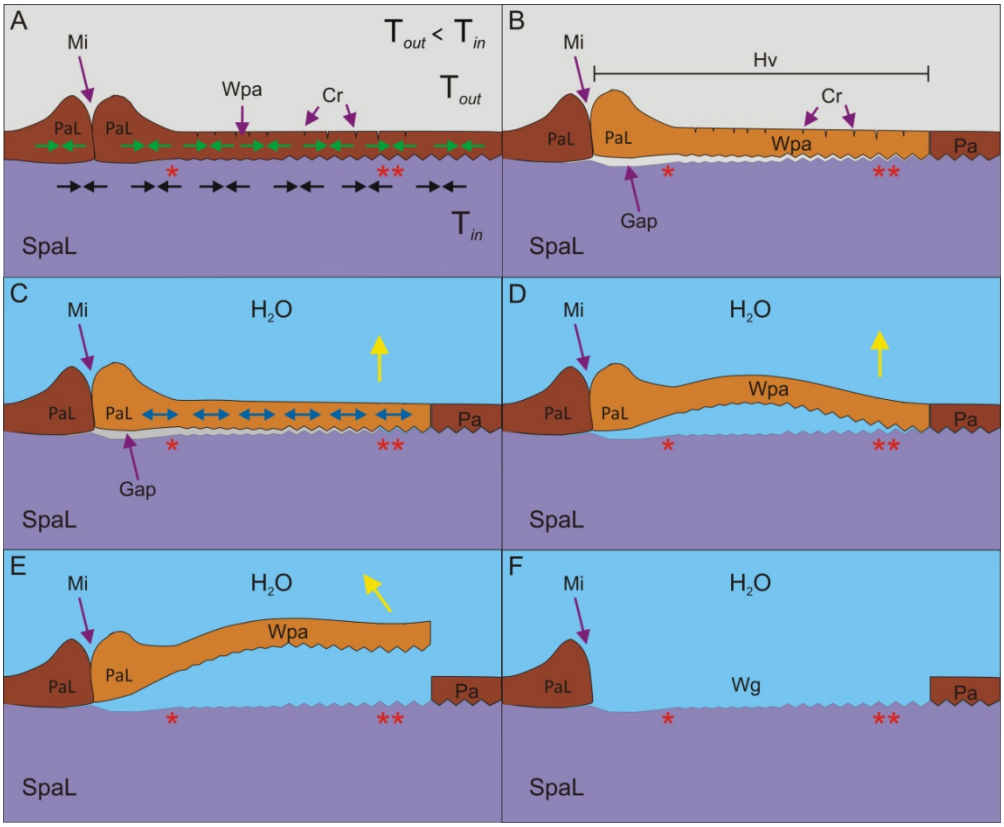


Figure 5.9. Schematic diagrams of median longitudinal sections of the water-gap region of *G. carolinianum* depicting the proposed mechanisms for PY-breaking and opening of the water-gap: (A) a sensitive seed exposed to cold temperature; arrows indicate differential shrinking of the palisade layer (green) and subpalisade layer (black); (B) a permeable seed; light-brown colour of the water-gap palisades depict the colour change visible externally after PY is broken; note the gap between the palisade and subpalisade layers. (C to F) A permeable seed during different stages of early imbibition: (C) expansion of water-gap palisades; blue arrows indicate the expansion of water-gap palisade cells due to imbibition; (D) formation of the blister; (E) formation of the hinged-valve; (F) water-gap revealed after the dislodgement of the hinged-valve. Abbreviations: Cr, cracks on the palisade layer; Hv, hinged-valve (composed of PaL and Wpa); Mi, micropyle; Pa, palisade cells; PaL, elongated palisade cells of the water-gap; SpaL, elongated subpalisade cells of the water-gap; Wpa, water-gap palisade cells; Wg, water-gap opening; *, subpalisade cells with a smooth outer periclinal cell wall; **, subpalisade cells with a corrugated outer periclinal cell wall; yellow arrows indicate the direction of dislodgment of the hinged-valve.

# CHAPTER 6

## Identification and characterization of 10 new water gaps in seeds and fruits with physical dormancy and classification of water-gap complexes.

## INTRODUCTION

Physical dormancy (PY) is caused by a water-impermeable palisade cell layer(s) in seed or fruit coats (Baskin *et al*., 2000) along with tightly sealed chalaza and micropyle openings (Gama-Arachchige *et al*., 2010). PY has been demonstrated or inferred to occur in species of 18 angiosperm plant families and it is unknown in gymnosperms (Nandi, 1998; Horn, 2004; Baskin *et al*., 2000; Baskin 2003; Koutsovoulou *et al*., 2005; Baskin *et al*., 2006; Horn, 2004; APG III, 2009; Tsang, 2010). Seeds of some species with PY also have physiological dormancy (PD); hence, they are considered to have combinational dormancy (CD). The breaking of PY involves disruption or dislodgement of 'water-gap' structures causing the seeds/fruits to become permeable. The water-gap region is a morpho-anatomically specialized area and it differs from the rest of the seed or fruit coat. Location, anatomy, morphology and origin of water-gaps can differ between and even within families (Baskin *et al*., 2000, Jayasuriya *et a*l., 2009). Twelve different water-gap regions in seven families have been characterized previously. However, the water-gaps previously have not been characterized in Biebersteiniaceae, Cucubitaceae, Fabaceae (clade Cladrastis), Lauraceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae.

Water-gap structures in seeds/fruits act as environmental signal detectors for seed germination (Baskin *et al*., 2000). The ability of water-gap structures to sense environmental conditions allows seeds with PY to become permeable just prior to the commencement of conditions favourable for germination and plant establishment (Taylor, 1996a, b; Jayasuriya *et al*., 2008a, 2009; Gama-Arachchige *et al*., 2012). The mechanisms of sensing environmental signals for PY-break by water-gap structures differ between species (Jayasuriya *et al*., 2008a, 2009; Gama-Arachchige *et al*., 2013). Since the water-gap region plays a major role in maintaining and breaking of PY and thus in plant survival and fitness via timing of seed germination, it is important to characterize the diversity of this structural complex as a basis for understanding how it functions under natural conditions. Such a study also will provide information for further investigations into how PY evolved in different plant lineages.

In the literature, the term 'water-gap' is used interchangeably to define both the opening formed during the PY-break and the whole specialized region of the seed or fruit coat (Jayasuriya *et al*., 2007; Turner *et al*., 2009; Gama-Arachchige *et al* 2010; Karaki *et al*., 2011; De Paula *et al*., 2012). Moreover, Gama-Arachchige *et al* (2011) studied the development of the water-gap region of *Geranium carolinanum* and reported that the micropylar water-gap region of *G. carolinianum* is an anatomically complex structure. They introduced the term 'water-gap complex' to define this whole water-gap region of *G. carolinianum*. However, to date no attempt has been made to define the different structures involved in early imbibition or to classify water-gap regions.

Our general objectives were to (1) identify the water-gaps in seeds/fruits of Biebersteiniaceae, Cucurbitaceae, Fabaceae (clade Cladrastis), Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Lauraceae, Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae and (2) devise a classification system for water-gap regions based on the information from previous studies and from the current study. Such a system would greatly facilitate the evaluation of evolutionary relationships between species with water impermeable seed/fruit coats with regard to the morpho-anatomy of PY and PY-break. However, fruits of Biebersteiniaceae and Lauraceae could not be obtained. Thus, water-gaps of those two families were not studied.

For some of the study species, information on PY-break and/or seed/fruit coat anatomy is available. Nandi (1998) studied the seed coat anatomy of eight species of Malvales *s.s.* (including *Bixa orellana* and *Helianthemum nummularium*) and compared the anatomical similarities of the specialized chalazal region (Bixoid chalaza). Based on the complex structure of the chalzal region, Baskin *et al* (2000) suggested that the Bixoid chalaza may act as the water gap in seeds of Bixaceae and Cistaceae. Razanameharizaka *et al*. (2006) and Turner and Dixon (2009) tested the effect of boiling water on PY breaking in the eight species of *Adansonia*. Two of the species were non-dormant and the other six species had PY. Boiling for 15 s - 5 min (depending on the species) was effective in PY-breaking. PY of seeds of *Ceanothus americanus* can be broken by exposing them to hot or boiling water (Table 1, Schramm and Johnson, 1981). Exposing seeds of *C. velutinus* to dry heat resulted in irreversible opening of the hilum (Gratkowski, 1962; Conard *et al*., 1985). Based on these observations, the hilar slit can be assumed to be the water gap in *Ceanothus* spp.

Geneve (2009) blocked the whole hilar region of seeds of *Cercis canadensis* that had been boiled for 1 min and concluded that the hilar area is the water-gap. However, the roles of pseudolens, micropyle and hilar slit in imbibition were not studied. Ohga (1926) and Shaw (1929) studied the fruit anatomy of *Nelumbo nucifera and N. lutea,* respectively, and characterized a specialised area in the fruit coat known as the protuberance. However, none of these studies focused on identification and characterization of the water-gap regions.

Thus, the specific objectives of the current study were to (1) morpho-anatomically describe the water gap for the families Fabaceae (clade: Cladrastis), Cucurbitaceae, Malvaceae (subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae), Nelumbonaceae, Rhamnaceae, Sapindaceae (subfamily Sapindoideae) and Surianaceae; (2) re-evaluate the water gap of Fabacae (section Cercideae); (3) confirm (or not) the morpho-anatomy of the water-gaps of Bixaceae and Cistaceae; and (4) devise a classification scheme for water-gap regions and their morpho-anatomical features.

## MATERIALS AND METHODS

*Seed sources*

Mature fruits and seeds for the present study were obtained from commercial and personal seed collections from Australia, China, France, Taiwan and United States of America (Table 6.1). All seeds were stored at room temperature (approx. 23 $^{o}$C and 50-60 % relative humidity, dry storage) until used. In this study only the impermeable

seeds in the seed lots were used. To select the impermeable seeds/fruits fraction, seeds/fruits of each species were allowed to imbibe in distilled water in glass beakers for 3 d at ambient room conditions and any imbibed seeds/fruits were discarded prior to experiments.

*PY- breaking*

Wet and/or dry heat treatments were used to break PY in each study species (Table 6.1). Dormancy breaking treatments were selected based on unpublished research of Gama-Arachchige *et al.*: (1) *Wet heat*, depending on availability of seeds five replicates of 1, 5 or 20 seeds were placed in a hot water bath; (2) *Wet-dry heat*, three replicates of 25 fruits of *N. nucifera* were subjected to alternating wet and dry heat by immersing them in boiling water for 5 min and then drying in an incubator at 40 $^{o}$C for 24 hr (repeated 10 times); (3) *Dry heat*, seeds of *K. paniculata* and *S. saponaria* (5 replicates of 10 seeds each) were heated in an oven at 60 $^{o}$C for 7 days and then stored in paper bags under ambient room conditions for 1 month; and (4) *Open flame*, the hilar end of the seed of *S. angulatus* was held ~5 cm over a Bunsen burner flame for 30 sec.

*Morphological changes in the seed coat after PY- breaking*

To study the ultra-morphological changes in the putative water-gap region after PY is broken, impermeable and permeable (heat treated) seeds were mounted on scanning electron microscope specimen stubs using double-sided carbon tapes. Seeds were sputter-coated with gold-palladium (15 nm), scanned with an S-3200 Hitachi scanning electron microscope at an acceleration voltage of 5.0 kV and micrographs were taken.

*Dye tracking*

Dye-tracking experiments were performed to determine the initial site of water entry into permeable seeds during imbibition. Heat-treated seeds (of all study species) were dipped in concentrated solutions of acid fuchsin or methylene blue. Three seeds each of each species were removed after 30 sec, 1 min, 5 min and 10 min intervals and their longitudinal bisections were observed for staining under a dissecting microscope (ZEISS STEMI SVII). The fruits of *N. nucifera* were examined at 1 day intervals. The pathway of the dye was observed and micrographs were taken using a digital camera (Olympus DP25).

*Blocking experiment*

To study the role of the pseudolens, the micropyle and hilar slit in the early stages of imbibition of seeds of *C. canadensis*, seeds were immersed in boiling water for 10 sec and then dried overnight at room temperature. One hundred seeds (five replicates of 20) each were blocked with Vaseline® with a sharpened toothpick at (1) hilar slit and micropyle, (2) pseudolens and micropyle and (3) pseudolens, micropyle and hilar slit. Non-blocked seeds were used as a control. Seeds were incubated in plastic Petri dishes filled with water for 7 days at 25 $^{o}$C. The number of imbibed seeds (larger in size and lighter in colour) was counted at intervals of 24 h. The final imbibition percentage data were normalized by arcsine-transformation and analyzed by one-way ANOVA using SAS ver. 9.2 software to determine significant differences between blocking treatments ($P < 0.05$).

*Light microscopy*

To compare the anatomy of the water-gap region and the general seed/fruit coat, microtome sections were used. Seeds/fruits of the study species were manually scarified and allowed to fully imbibe in water at ambient room temperature. Then, these seeds/fruits were glued to wooden blocks by applying Super-Glue® to one side of the specimen. Subsequently, depending on the species, 10-20 μm sections (longitudinal and transverse) were cut using a microtome (LEICA RM 2135). Sections were stained with 1% safranin and/or 2% fast green solutions when necessary and observed under a light microscope (Olympus BX40) equipped with a digital camera (Olympus DP25) and micrographs were taken and compared.

## RESULTS

*Morphological changes in the seed coat after PY- breaking*

Wet-heat treatment caused the chalazal cap and plug to be dislodged, hence opening the chalaza in seeds of *B. orellana* and *H. apenninum* (Fig. 6.1A,B).

The hilar slit is tightly closed in PY seeds of *S. angulatus* and after the open-flame heat treatment, it forms a concave slit-like opening (Fig. 6.1C). No other opening or morphological change was observed between impermeable and permeable seeds.

The micropyle and hilar slit are located perpendicular to each other in the hilar end of the seeds of *C. canadensis*. After 10 sec in boiling water, a blister (pseudolens) formed immediately adjacent to the micropyle (Fig. 6.1D). Moreover, the micropylar and hilar openings widened. Unlike *C. canadensis*, only the pseudolens popped open

in *B. acuminata* seeds after the PY-breaking treatment (Fig. 6.1E). In *C. kentuckea* seeds, the lens split and formed a narrow slit after the wet-heat treatment (Fig. 6.1F).

After the wet-heat treatments, a circular opening formed in the chalaza of *A. digitata* and *G. ulmifolia,* while a narrow oval-shaped opening formed in the chalaza of *B. cordifolia* (Fig. 6.1G-I).

The protuberance of impermeable *N. nucifera* was visible as a slightly raised area in the fruit wall near the persistent style (figure not shown). After the heat treatment, the outer epidermal layer and palisade layer in the protuberance became dislodged and a circular gap was formed revealing the sclerenchyma cell layer and a narrow opening to the cavity of protuberance (Fig. 6.1J).

In wet-heat treated seeds of *C. americanus,* the hilar slit ruptured and widened (Fig. 6.1K). No other visible changes on the seed coat were observed. The outermost layer of the heart-shaped aril region of *C. halicacabum* consists of spongy mesophyll cells, whereas palisade cells form the outermost layer of the other part of the seed (Fig. 6.1L). After wet-heat treatment, a slit was formed along the margin of the aril region starting at the micropyle (Fig. 6.1L). After wet-heat treatment, the hilar plug in seeds of *K. paniculata* separated from the palisade cells, thus widening the hilar rim (Fig. 6.1M). The dry-heat treatment caused the palisade cells to crack and blisters formed all over the seed (Fig. 6.1N,O). However no changes were observed on the hilar region. The hilar slit opened in seeds of *S. saponaria* after the PY-break (Fig. 6.1P). Moreover, perpendicular splits in the palisade layer formed in the hilar region. These splits extended up to the sclerenchyma layer beneath the palisade layer. After the wet-

heat treatment, a suture originated at the carpellary hilum of *S. spathulatum* and continued around the radicle side of the endocarp (Fig. 6.1Q). The suture did not form on the vascular bundle side of the endocarp.

*Dye tracking*

The time taken for entry of the dye into seeds varied among species. In *H. apenninum* and *G. ulmifolia,* dye was first observed in the chalazal opening after 30 sec (Fig. 6.2A,B; 6.3A,B), while in *B. orellana*, *B. cordifolia* and *A. digitata*, it was first observed in the chalazal opening after 1, 1 and 10 min, respectively (Fig. 6.2C-E; 6.3C-E). In *C. americanus*, *S. angulatus* and *S. saponaria*, the dye entered into seeds via the hilar slit and was observed in adjacent tissues after 30 sec (Fig. 6.2F-H; 6.3F-H). In fruits of *S. spathulatum*, the dye entered into the endocarp through the carpellary slit and carpellary-hilar suture and reached the embryo after 1 min (Fig. 6.2I; 6.3I). The dye did not enter through the vascular bundle side of the endocarp. *N. nucifera* fruits imbibed very slowly and the dye was first observed in the cavity of the protuberance after 48 hr (Fig. 6.2J;6.3J). In *C. halicacabum* seeds, the dye was first observed in the micropylar slit after 1 min (Fig. 6.2K;6.3K). Dye entered through the hilar rim in wet-heat treated seeds of *K. paniculata* and was observed in inner tissues after 3 min (Fig. 6.2L;6.3L), while in dry-heat treated seeds the dye entered through blisters (figure not shown).

During the dye tracking experiment, the pseudolens of *C. canadensis* swelled but was not dislodged (Fig. 6.2M; 6.3M). Dye was observed in the palisade cells of the pseudolens and in the upper portion of the hilar and micropylar palisade cells, but not in the pseudolens opening even after 4.5 hr (Fig. 6.2M; 6.3M). However, after 4.5 hr,

moisture was observed in the sclerenchyma cells under the pseudolens near the micropyle. Dye entered into the seeds of *B. acuminata* and *C. kentuckea* through the pseudolens gap and lens slit, respectively, and it was observed in these regions after 30 sec (Fig. 6.2N,O; 6.3N,O).

*Blocking experiment*

Blocking the pseudolens+micropyle+hilar slit of *C. canadensis* completely inhibited the water uptake during the incubation period of 120 h, while 97% of the non-blocked seeds imbibed within the first 24 h (Fig. 6.4). Imbibition in seeds with the hilar slit+micropyle or the pseudolens+micropyle blocked was slower than it was in nonblocked seeds, with the former category imbibing faster (90% by 48 h) than the latter (10% by 48 h). The rank-order of imbibition rate was non-blocked > hilar slit blocked + micropyle blocked > pseudolens + micropyle blocked > pseudolens + micropyle + hilar slit blocked.

*Light microscopy*

An epidermal layer, multiple subepidemal layers, a palisade layer and three layers of mesophyll cells comprise the seed coat of *S. angulatus* (Fig. 6.5A). The embryo is encased by the nucellar-endosperm casing. Palisade cells have narrow lumens that branch near the upper and lower periclinal walls (Fig. 6.5B). The light line runs very close to the upper periclinal wall. Near the hilar slit, palisade cells gradually become shorter (Fig. 6.5C). Furthermore, several layers of flattened subepidemal parenchyma cells are present near the hilar slit (Fig. 6.5D). Two bulges, each with two lobes, are located at the hilar end (Fig. 6.5E). These bulges are formed by loosely arranged thick-walled sclerenchyma cells (Fig. 6.5F).

The seed coat of *C. canadensis* consists of a palisade layer and several layers of sclerenchyma cells (Fig. 6.6A). Near the hilar slit, the palisade cells are slightly longer than those elsewhere in the seed coat. At the hilar slit, palisade cells are slightly shorter and curved (Fig. 6.6A,C). The light line runs through the upper one-fourth of the palisade cells and near the hilar slit and ascends to the lower one-fourth of the cell. The palisade cells of the pseudolens are similar in length to those of hilar palisades (Fig. 6.6A,B,D). A thick layer of sclerenchyma cells is present at the hilar end of the seed and vascular bundles penetrate through this layer (Fig. 6.6A). The sclerenchyma cells are tightly arranged on the pseudolens side and loosely arranged on the vascular bundle side (Fig. 6.6A,B,D).

Seed coats of both *A. digitata* and *B. cordifolia* consist of an exotegmic palisade layer that varies little throughout the seed coat (figures not shown). However this layer is discontinued at the chalazal opening. Palisade cells at the margin of the chalazal opening are slightly shorter and more curved than those in the rest of the seed coat. The chalazal plug is located below the palisade layer of the chalazal region. Tightly-packed, dark-coloured, thin-walled sclerenchyma cells of the upper portion of the chalazal plug fill the gap in the chalazal opening.

The pericarp of *N. nucifera* is composed of an epidermal layer, a subepidermal palisade layer, a wide layer of sclerenchyma and several layers of parenchyma cells (Fig. 6.7A). The protuberance organ is located in the sclerenchyma layer near the stylar end of the fruit (Fig. 6.7B). It is outlined by modified sclerenchyma cells and crystalliferous cells (Fig. 6.7B-E). The cavity of the protuberance organ is formed by

degeneration of crystalliferous cells (Fig. 6.7B,E). Its mouth is occluded by slightly shorter palisade cells and elongated sclerenchyma cells (Fig. 6.7C). Vascular bundles run through the paremchyma cell layer and one of them ends at the protuberance organ (Fig. 6.7B,F).

In *C. americanus*, a palisade layer and several layers of crushed mesophyll cells located below the palisade cells comprise the seed coat (Fig. 6.8A). Palisade cells near the hilar slit are ~1.5 times longer than those located elsewhere in the seed coat (Fig. 6.8B). In palisade cells away from hilum, the light line runs very close to the upper periclinal wall and near the hilum it gradually descends to one-third of the length of the cell.

The seed coat of *C. halicacabum* is composed of a single layer of palisade cells and multiple layers of sclerenchyma cells (Fig. 6.9A,B). The palisade cells of the aril region differ from those of the micropylar region (Fig. 6.9C,D). Palisade cells of the aril region are irregular in size, slightly convex, lack a light line, are hyaline and have small lumens containing rhomboidal oxalate crystals (Fig. 6.9C). Moreover, several layers of parenchyma cells can be seen attached to the outer periclinal walls of the palisade cells of this region. The micropylar palisade cells are columnar, uniform with large lumens containing a brownish material and possess a light line (Fig. 6.9D). The sclerenchyma layer is thinner near the micropyle due to the presence of the radicle (Fig. 6.9E). Near the micropyle, a slightly discernible marking (rupture line) separates the micropylar sclerenchyma cells from hilar sclerenchyma (Fig. 6.9F). Blisters were observed on imbibing seeds and were formed due to rupturing of palisade cells at the light line (Fig. 6.9G).

The seed coat of *K. paniculata* consists of a palisade cell layer and multiple layers of sclerenchyma cells, except at the hilar region (Fig. 6.10A-D). The light line of the palisade cells runs through the outer one-third of the cell (Fig. 6.10D). The hilar-micropylar region is closed with a hilar plug formed by sclerenchyma cells and mesophyll cells (Fig. 6.10A-C). A part of the plug protrudes outwards of the palisade layer. The plug is formed by a mass of sclerenchyma cells of which the region embedded in the seed coat is lined by an outer annulus of several layers of spongy mesophyll cells. The bottom of the plug is lined by crushed remnant cells of the endosperm (Fig. 6.10A). An opaque spongy mesophyll cell mass is located at the micropylar region, closer to the bottom of the hilar plug (Fig. 6.10B,C). A vascular bundle runs through the hilar plug and continues beneath the sclerenchyma layer, diverging from the micropylar side. Blisters on the seed coat were observed in dry-heat treated seeds (Fig. 6.10E,F). Microtome sections through these blisters showed that they are formed at the light line of the palisade layers due to the formation of cracks (Fig. 10E); these cracks continued to form ruptured areas in the palisade cells (Fig. 6.10F).

The seed coat of *S. saponaria* is composed of a palisade cell layer and multiple layers of sclerenchyma cells (Fig 6.11A). At the hilar slit, absence of these cell layers results in a dome-shaped cavity (Fig. 6.11B). The palisade cells near the hilum are almost twice the length of those away from the hilum (Fig. 6.11B,C). They gradually become shorter towards the hilar slit (Fig. 6.11B). The light line of the palisade cells runs near the center of the cells in the hilar region but closer to the upper periclinal wall

elsewhere (Fig. 6.11A, B). Blisters similar to those in seeds of *K. paniculata* were observed in seeds of *S. saponaria* after dry heat treatment (Fig. 6.11D,E).

The pericarp of *S. spathulatum* consists of two discernible areas: (1) The outer mesocarp with spongy mesophyll cells bound on the outside by a thin exocarp (not shown in figures) and on the inside by crystalliferous cells and (2) the inner endocarp with a single palisade layer that has a thick light line and a broad layer of irregularly oriented hyaline sclerenchyma cells (Fig. 6.8C). The inner endocarp epidermis is bound by radially elongated, brownish parenchyma cells (Fig. 6.8D,E). The endocarp discontinues at the carpellary-hilar opening. The sclerenchyma cells of the endocarp suture are brownish in colour. The circumlinear endocarp suture is formed by rupturing of palisade cells and by slightly yellowish coloured sclerenchyma cells of the endocarp (Fig. 6.8E).

## DISCUSSION

In the present study, 10 new water-gaps were identified and characterized morphologically and anatomically in seven families and two water-gaps in Bixaceae and Cistaceae previously hypothesized to exist by Baskin *et al* (2000) were confirmed. Information on all water-gaps known to occur in angiosperms is summarized on Table 6.2. Based on the location, anatomy and morphology, there are 24 different kinds of water-gap regions in 16 families; water-gaps in fruits of Bibersteiniaceae and Lauraceae remain to be characterized (Table 6.2). New names were assigned to certain water-gaps that had been previously reported in the literature for clarity and to avoid ambiguity. Circular water-gaps with plug-like structures

occluding the opening were given the name ‘oculus’ (eye), circular water-gaps occluded by lid-like structures formed from palisade cells ‘gap’ and narrow linear water-gaps ‘slit’. Jayasuriya *et al* (2009) characterized the water-gap regions of Convolvulaceae and called it the bulge gap adjacent to the micropyle, except for *Cuscuta*, in which the water-gap is the hilar slit. However, the two bulges are located on the opposite ends of the hilar slit. Thus they are more closely located to the hilum than to the micropyle and could possibly be an extension of the hilum itself. For these reasons, the water-gap was renamed “bulge gap adjacent to the hilum”.

The lens gap acts as the water-gap in most PY species in Fabaceae. The lens and micropyle in this family are usually located on the opposite side of the hilar slit (Lersten *et al*., 1992). However, in the case of seeds of subfamily Caesalpinioideae tribe Cercideae the lens is located next to the micropyle on the same side of the hilar slit; thus, this structure is called the ‘pseudolens’ (Lersten *et al*., 1992). Based on the results of dye tracking and blocking experiments, it was shown that pseudolens acts as the water-gap in seeds of *C. canadensis* and *B. accuminata*. However, in *C. canadensis* the micropyle and hilar slit are also responsible for initial water imbibition. Therefore, all three structures are involved in initial water uptake. This is the first report on the role of pseudolens as the water-gap, thus adding a new kind of water-gap to the family Fabaceae. Moreover, similar to other clades in Papilinoideae except Genistoids *s.l.* (where water-gap is the hilar slit), the lens slit acts as the water-gap in the clade Cladrastis.

In the present study, three new water-gaps were identified in the subfamily Sapindoideae (Sapindaceae). Three of the four kinds of water-gap regions in this

family are associated with the hilum, and only the water-gap of tribe Paullinieae is associated with the micropyle (Turner *et al*., 2009; Table 6.2). In seeds of *K. paniculata,* dry and wet heat treatments act differently on PY-break, causing the formation of two different openings in the seeds. Wet-heat dislodged the hilar plug allowing the seeds to imbibe water through the hilar oculus. After dry-heat treatment, on the other hand, blisters formed all over the seed coat, especially near the hilar region and they functioned as the water-gap. This indicates that water-gap formation can differ depending on the PY-breaking treatment. However, seeds collected in the spring of 2012 under the trees (possibly dispersed the previous autumn) on the campus of University of Kentucky had dislodged hilar plugs, indicating that hilar oculus acts as the water-gap under natural conditions (Gama-Arachchige, personal observations). The water-gap region of the *K. paniculata* is similar in several ways to the chalazal oculus of Bixaceae, Cistaceae, Malvaceae, Sarcolaenaceae and Sphaerosepalaceae. In seeds of all of these species, the water-gap is occluded by a plug-like structure formed by water impermeable sclerenchyma cells. During PY-break, this plug is pushed slightly into the seed and forms a circular opening (oculus) through which the seeds imbibe water.

In the family Surianaceae only the Genus *Stylobasium* that has been shown to contain a water impermeable endocarp (Baskin *et al*., 2006). The water-gap of *Stylobasium* is rather different from all the other water-gap regions. Unlike water-gap regions in other taxa, a suture (Endocarp circumlinear suture) is formed all around the endocarp as PY is broken. Moreover, compared to other species the water-gap complex of *Stylobasium* is morpho-anatomically simple.

Ohga (1926) studied the pericarp anatomy of *N. nucifera* and reported a specialized structure known as the protuberance organ near the stylar end in the fruit. In the present study, dye tracking experiments confirmed that the initial imbibition of water takes place through the protuberance organ after the palisade cells at the mouth of the protuberance are dislodged. Moreover, one of the vascular bundles ends at the protuberance organ and thus it can be assumed that this organ may be involved in water flow regulation in young fruits and also during maturation drying.

The hilar slits of *S. angulatus* and *C. americanus* act as water-gaps in the families Cucubitaceae and Rhamnaceae, respectively. Even though the water-gaps of these two families are morphologically similar, their anatomy shows several variations. Moreover, the mechanism of opening of the water-gap differs in the two species. In seeds of *C. americanus*, the hilar slit splits at the narrow ends, while in *S. angulatus* it expands with the PY-breaking treatment.

The order Malvales includes six families with species whose seeds are water impermeable, i.e. Malvaceae, Bixaceae, Cistaceae, Sarcolaenaceae, Sphaerosepalaceae and Dipterocarpaceae. In the present study, the chalazal oculus was confirmed as the water-gap in Bixaceae and Cistaceae. However, due to the unavailability of seeds of Sarcolaenaceae, Sphaerosepalaceae and Dipterocarpaceae, water-gaps of PY species of these families could not be studied. In the family Malvaceae, water-gap complexes of subfamilies Bombacoideae (*A. digitata*) and Brownlowidoideae (*B. cordifolia*) are described for the first time as the chalazal oculus and chalazal cleft, respectively. Water-gaps of these subfamilies are similar to those previously reported in other closely related subfamilies of Malvaceae. Based on

the current data, there are six different kinds of water-gap regions associated with the chalaza, present in those six aforementioned families (Table 6.2).

*Water-gap complex*

Gama-Arachchige *et al* (2011) introduced the term 'water-gap complex' to describe the water-gap region of *G. carolinianum* (Geraniaceae). In general, the water-gap complex in all species with PY is a morpho-anatomically complex structure and is composed of (1) an opening formed after PY-break, (2) specialized structures that occlude the gap and (3) associated specialized tissues. Therefore, the term 'water-gap complex' is an appropriate term to define the whole water-gap region and the term 'water-gap' is suitable when referring to the actual opening. Based on the morphology of the opening and the anatomy of the occluding structures, the water-gap complexes can be divided into three types: Type-I, Type-II and Type-III (Fig.6.12). Type-I water-gap complexes are the water-gaps with narrow-linear openings usually occluded by modified elongated palisade cells. Type-II water-gap complexes have circular or broad openings occluded by lid-like structures formed by the palisade cells and Type-III water-gap complexes are either narrow-linear or circular openings occluded by plug-like structures usually formed by water impermeable sclerenchyma cells.

In some species, more than one opening is involved in the early stages of imbibition after PY is broken (Table 6.2). In genera such as *Canna*, *Cercis*, *Geranium*, *Kosteletzkya*, *Rhus, Sida* and *Stylobasium*, the water-gap along with other closely located structures such as micropyle, chalaza, hilum, carpellary hilar slit or carpellary micropyle are involved in initial water uptake. In the case of *Ipomoea* spp., two

identical water-gap openings are involved in early imbibition. Therefore water-gap complexes can be divided into two groups based on the number of openings involved in early imbibition: (1) simple water-gap complex, where only one opening is involved in initial water imbibition; and (2) compound water-gap complex, where two or more openings are involved in initial imbibition.

The current study is the most recent and most detailed analysis of different water-gap complexes in seeds and fruits of PY/CD species. In this study, for the first time: (1) ten new water-gaps were morpho-anatomically characterized in seven families with PY/CD; and (2) a scheme was proposed for classifying water-gap complexes in 16 of the 18 angiosperm families known to have PY. The classification recognizes three basic types (I, II and III) which are further subdivided into simple and compound water-gap complexes based on the number of openings involved in the initial water uptake. Moreover, the outcomes of this study provide a basis for developing an identification key for different kinds of water-gap complexes in PY seeds/fruits.

Table 6.1. Habit, life form, dormancy class, PY-breaking conditions and seed source of the study species

| Species | Family | Habit | Life form | Seed source | Dormancy class | PY-breaking treatment |
|---|---|---|---|---|---|---|
| *Bixa orellana* | Bixaceae | Shrub | P | B and T World Seeds Company, Paguingan, FR (July, 2012) | PY | 100 $^{o}$C wet for 2 min |
| *Helianthemum apenninum* | Cistaceae | Shrub | P | B and T World Seeds Company, Paguingan, FR (July, 2012) | PY | 100 $^{o}$C wet for 10 min |
| *Sicyos angulatus* | Cucubitaceae | vine | SA | University of Kentucky, Lexington, KY, USA (October, 2010) | CD | Open flame for 30 sec |
| *Cercis canadensis* | Fabaceae | Tree | P | University of Kentucky, Lexington, KY, USA (September, 2011) | CD | 100 $^{o}$C wet for 10 sec |
| *Cladrastis kentukea* | Fabaceae | Tree | P | University Kentucky, Lexington, KY, USA (July, 2012) | PY | 100 $^{o}$C wet for 2 min |
| *Bauhinia acuminata* | Fabaceae | Tree | P | B and T World Seeds Company, Paguingan, FR (July, 2012) | PY | 100 $^{o}$C wet for 30 sec |

Table 6.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| *Adansonia digitata* | Malvaceae | Tree | P | Whatcom Seed Company, OR, USA (May, 2012) | PY | 100 $^{o}$C wet for 2 min |
| *Berrya cordifolia* | Malvaceae | Tree | P | Kenting, Pingtung County, TW (December, 2007) | PY | 100 $^{o}$C wet for 2 min |
| *Guazuma ulmifolia* | Malvaceae | Shrub/ tree | P | Trade Winds Fruit Company, CA, USA (August, 2012) | PY | 100 $^{o}$C wet for 1 min |
| *Nelumbo nucifera* | Nelumbonaceae | Aquatic herb | P | Taipei Botanical Garden, Taipei, TW (October, 2011) ; XTBG, Yunnan Province, CN (April, 2012) | PY | 100 $^{o}$C wet for 5 min and dry 40 $^{o}$C for 24 h (10 cycles) |
| *Ceanothus americanus* | Rhamnaceae | Shrub | P | Pairie Moon Nursery, MN, USA (April, 2010) | CD | 100 $^{o}$C wet for 1 min |
| *Cardiospermum halicacabum* | Sapindaceae | Vine | A | Montgomery County, TN, USA (October, 2000) | PY | 60 $^{o}$C wet for 7 days |
| *Koelreuteria paniculata* | Sapindaceae | Tree | P | University of Kentucky, Lexington, KY, USA (October, 2011) | CD | 60 $^{o}$C wet for 7 days |
| *Sapindus saponaria* | Sapindaceae | Tree | P | University of Hawaii, HI, USA (July, 2010) | PY | 100 $^{o}$C wet for 20 min |

Table 6.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| *Stylobasium spathulatum* | Surianaceae | Shrub | P | Kalabari, WA, AUS (2002) | PY | 100 $^{o}$C wet for 5 min |

A, annual, P, perennial; SA, summer annual

CD, Combinational Dormancy; PY, Physical Dormancy

Table 6. 2. Water-gaps and secondary opening(s) involved in early imbibition of seeds or fruits with physical or combinational dormancy in 18 angiosperm families.

| | Taxa | Species | Water-gap | Secondary opening(s) | Reference |
|---|---|---|---|---|---|
| 1 | Anacardiaceae | | | | |
| | Subfam. Anacardioideae | | | | |
| | Tribe. Rhoeae | *Rhus glabra* | Carpellary blister gap[1] | Carpellary micropyle | Li *et al*., 1999 |
| 2 | Bixaceae | *Bixa orellana* | Chalazal oculus[§‡2] | | Nandi, 1998 |
| 3 | Biebersteiniaceae | *Biebersteinia heterostemon* | ? | | |
| 4 | Cannaceae | *Canna indica* | Gap adjacent to hilum[3] | Hilum and micropyle | Graven *et al*., 1997 |
| 5 | Cistaceae | *Helianthemum apenninum*[Ψ] | Chalazal oculus[§‡4] | | Nandi, 1998 |
| 6 | Convovulaceae | | | | |
| | Clade Convolvuloideae | *Ipomoea lacunosa* | Bulge gap adjacent to hilum[5] | | Jayasuriya *et al*., 2007 |

Table 6.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| | Clade Dicranostyloideae | *Jacquemontia ovalifolia* | Bulge gap adjacent to hilum[5] | | Jayasuriya *et al*., 2009 |
| | Tribe Cardiochalamyeae | *Cardiochlamys madagascariensis* | Bulge gap adjacent to hilum[5] | | Jayasuriya *et al*., 2009 |
| | Tribe Cuscuteae | *Cuscuta australis* | Hilar slit [6] | | Jayasuriya *et al*., 2008b |
| 7 | Cucurbitaceae | | | | |
| | Subfam. Cucurbitoideae | | | | |
| | Tribe Sicyeae: | | | | |
| | Subtribe Sicyinae | *Sicyos angulatus* | Hilar slit[7] | | This study |
| 8 | Dipterocarpaceae | | | | |
| | Subfam. Monotoideae | *Monotes kerstingii* | Chalazal oculus[§4] | | Nandi, 1998 |
| | Subfam. Pakaraimoideae | *Pakaraimaea dipterocarpacea* | Chalazal oculus[§4] | | Nandi, 1998 |
| 9 | Fabaceae | | | | |
| | Subfam. Caesalpinioideae | | | | |
| | Tribe Cercideae | *Cercis canadensis* | Pseudolens gap[8] | Micropyle and hilar slit | This study |

Table 6.2 (continued)

| | | | | |
|---|---|---|---|---|
| | *Bauhinia acuminata* | Pseudolens gap[9] | | This study |
| Tribe. Cassieae | | | | |
| Subtribe Dialiinae | *Dialium guianesis* | Lens gap[§10] | | Lersten *et al*., 1992 |
| Subtribe Cassiinae | *Senna macranthera* | Lens gap[10] | | De Paula *et al*., 2012 |
| Tribe Detarieae *s.l.* | *Sindora supa* | Lens gap[§10] | | Lersten *et al*., 1992 |
| Tribe Caesalpinieae | *Gleditsia triacanthos* | Lens gap[10] | | Geneve, 2009 |
| Subfam. Mimosoideae | | | | |
| Tribe Acacieae | *Acacia kempeana* | Lens gap[10] | | Hanna, 1984 |
| Tribe Mimoseae | *Leucaena leucocephala* | Lens gap[10] | | Serrato-Valenti *et al*., 1995 |
| Tribe Ingeae | *Albizia lophantha* | Lens gap[10] | | Dell, 1980 |
| Subfam. Papilinoideae | | | | |
| Clade Cladrastis | *Cladrastis kentukea* | Lens slit[11] | | This study |
| Clade Genistoids *s.l.* | *Sophora alopecuroides* | Hilar slit[12] | Lens slit | Hu *et al*., 2009 |
| Clade Dalbergioids *s.l.* | *Stylosanthes hamata* | Lens slit[11] | | Mott, 1979 |

Table 6.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| | Clade Millettioids *s.l.* | *Derris scandens* | Lens slit[11] | | Jayasuriya *et al*., 2012 |
| | Clade Robinioids | *Robinia pseudoacacia* | Lens slit[11] | | Karaki *et al*., 2011 |
| | Clade IRLC[Φ] | *Trifolium subterraneum* | Lens slit[11] | | Hagon and Ballard, 1970 |
| | Clade Mirbelioid | *Daviesia alata* | Lens slit[11] | | Morrison *et al*., 1998 |
| 10 | Geraniaceae | *Geranium carolinianum* | Hinged valve gap[13] | Micropyle | Gama-Arachchige *et al*., 2010 |
| 11 | Lauraceae | *Cassytha pubescens* | ? | | |
| 12 | Malvaceae | | | | |
| | Subfam. Bombacoideae | *Adansonia digitata* | Chalazal oculus[14] | | This study |
| | Subfam. Malvoideae | | | | |
| | Tribe Gossypieae | *Gossypium hirsutum* | Chalazal oculus[14] | | Christiansen and Moore, 1959 |
| | Tribe Hibisceae | *Kosteletzkya virginica* | Chalazal slit [15] | Chalazal blister gap | Poljakoff-Mayber *et al*., 1994 |
| | Tribe Malveae | *Sida spinosa* | Chalazal blister gap[16] | Chalazal cleft | Egley & Paul,1981 |
| | Subfam. Tilioideae | *Tilia platyphyllos* | Chalazal oculus[§14] | | Nandi, 1998 |

Table 6.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| | Subfam. Brownlowioideae | *Berrya cordifolia* | Chalazal slit[17] | | This study |
| | Subfam. Grewioideae | *Apeiba tibourbou* | Chalazal oculus[§14] | | Daws *et al*., 2006 |
| | Subfam. Bythnerioideae | *Guazama ulmifolia* | Chalazal oculus[14] | | This study |
| 13 | Nelumbonaceae | *Nelumbo nucifera* | Protuberance organ[18] | | This study |
| 14 | Rhamnaceae | | | | |
| | Clade Ziziphoid | *Ceanothus americanus* | Hilar slit[19] | | This study |
| 15 | Sapindaceae | | | | |
| | Subfam. Dodonaeoideae | | | | |
| | Tribe Dodonaeeae | *Dodonaea viscosa* | Gap adjacent to hilum[20] | | Turner *et al*., 2009 |
| | Subfam. Sapindoideae | | | | |
| | Tribe Koelreuterieae | *Koelreuteria paniculata* | Hilar oculus[21] | micropyle | This study |
| | Tribe Sapindeae | *Sapindus saponaria* | Hilar slit[22] | | This study |
| | Tribe Paullinieae | *Cardiiospermum halicacabum* | Micropylar slit[23] | | This study |

Table 6.2 (continued)

| | | | | | |
|---|---|---|---|---|---|
| 16 | Sarcolaenaceae | *Leptolaena pauciflora* | Chalazal oculus[§4] | | Nandi, 1998 |

| | | | | | |
|---|---|---|---|---|---|
| 17 | Sphaerosepalaceae | *Dialyceras parvifolium* | Chalazal oculus[§2] | | Horn, 2004 |
| 18 | Surianaceae | *Stylobasium spathulatum* | Circumlinear endocarp suture[24] | Carpellary hilar slit | This study |

Same superscript number on each water-gap indicates similar water-gap types
§ Water-gaps inferred based on seed coat anatomy
‡ Inferred water-gaps confirmed by dye tracking in this study
Φ Inverted-repeat-lacking clade
Ψ Seeds of *Helianthemum apenninum* were investigated in the present study and *Helianthemum nummularium* in the study by Nandi
The classification of families into subfamilies, clades, tribes and subtribes is based on Alverson *et al* (1999); Nandi (1998); Yi *et al* (2004); Wojciechowski *et al* (2004); Richardson *et al* (2004); Harrington *et al* (2005); APG III (2009); Jayasuriya *et al* (2009).

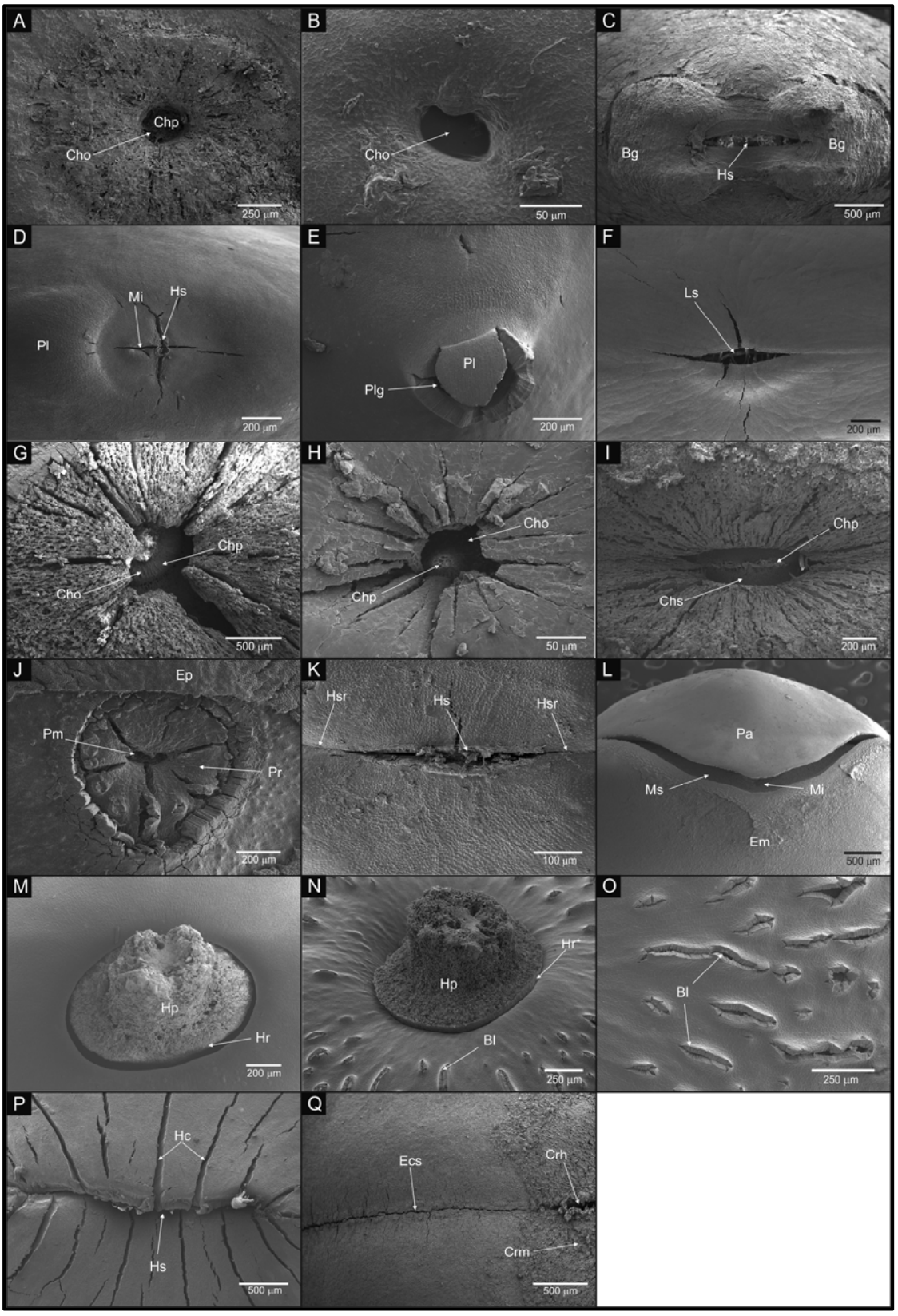
A
Chp
Cho
250 µm
B
Cho
50 µm
C
Bg
Bg
Hs
500 µm
D
Mi
Hs
Pl
200 µm
E
Pl
Plg
200 µm
F
Ls
200 µm
G
Chp
Cho
500 µm
H
Cho
Chp
50 µm
I
Chp
Chs
200 µm
J
Ep
Pm
Pr
200 µm
K
Hsr
Hs
Hsr
100 µm
L
Pa
Ms
Mi
Em
500 µm
M
Hp
Hr
200 µm
N
Hr
Hp
Bl
250 µm
O
Bl
250 µm
P
Hc
Hs
500 µm
Q
Ecs
Crh
Crm
500 µm

Figure 6.1. Scanning electron micrographs of water-gap regions of non-dormant (heat treated) seeds or fruits: (A) Chalazal region of *Bixa orellana* without outer permeable cell layers; (B) Chalazal region of seeds of *Helianthemum apenninum* without outer permeable cell layers; (C) Hilar region of *Sicyos angulatus*; (D) Hilar region of *Cercis canadensis*; (E) Pseudolens (open) of *Bauhinia acuminata*; (F) Lens (open) of *Cladrastis kentuckea*; (G) Chalazal region of *Adansonia digitata* without outer permeable cell layers; (H) Chalazal region of *Guazuma ulmifolia* without outer permeable cell layers; (I) Chalazal region of *Berrya cordifolia* without outer permeable cell layers; (J) Protuberance (open) of fruit of *Nelumbo nucifera* without outer permeable cell layers; (K) Hilar region of *Ceanothus americanus*; (L) Micropylar region of *Cardiospermum halicacabum*; (M) Hilar region of wet-heat treated *Koelreuteria paniculata*; (N) Hilar region of dry-heat treated *Koelreuteria paniculata*; (O) Seed coat away from the hilar region of dry-heat treated *Koelreuteria paniculata*; (P) Hilar region of *Sapindus saponaria*; (Q) Endocarp circumlinear suture of *Stylobasium spathulatum* without outer permeable meso and endocarp cell layers. Ar, aril; Bg, bulges of the hilar region; Bl, blisters formed in the palisade layer; Cho, chalazal oculus; Chp, chalazal plug; Chs, chalazal slit; Crh, carpellary hilar slit; Crm, crystalline cells of the mesocarp; Ecs, endocarp circumlinear suture; Ep, epidermal cell layers; Hc, hilar cracks; Hp, hilar plug; Hs, hilar slit, Hsr, ruptured hilar region; Ls, Lens slit; Mi, micropyle; Ms, micropylar slit; Pl, pseudolens; Plg, pseudolens gap; Pm, mouth of protuberence; Pr, protuberence gap.

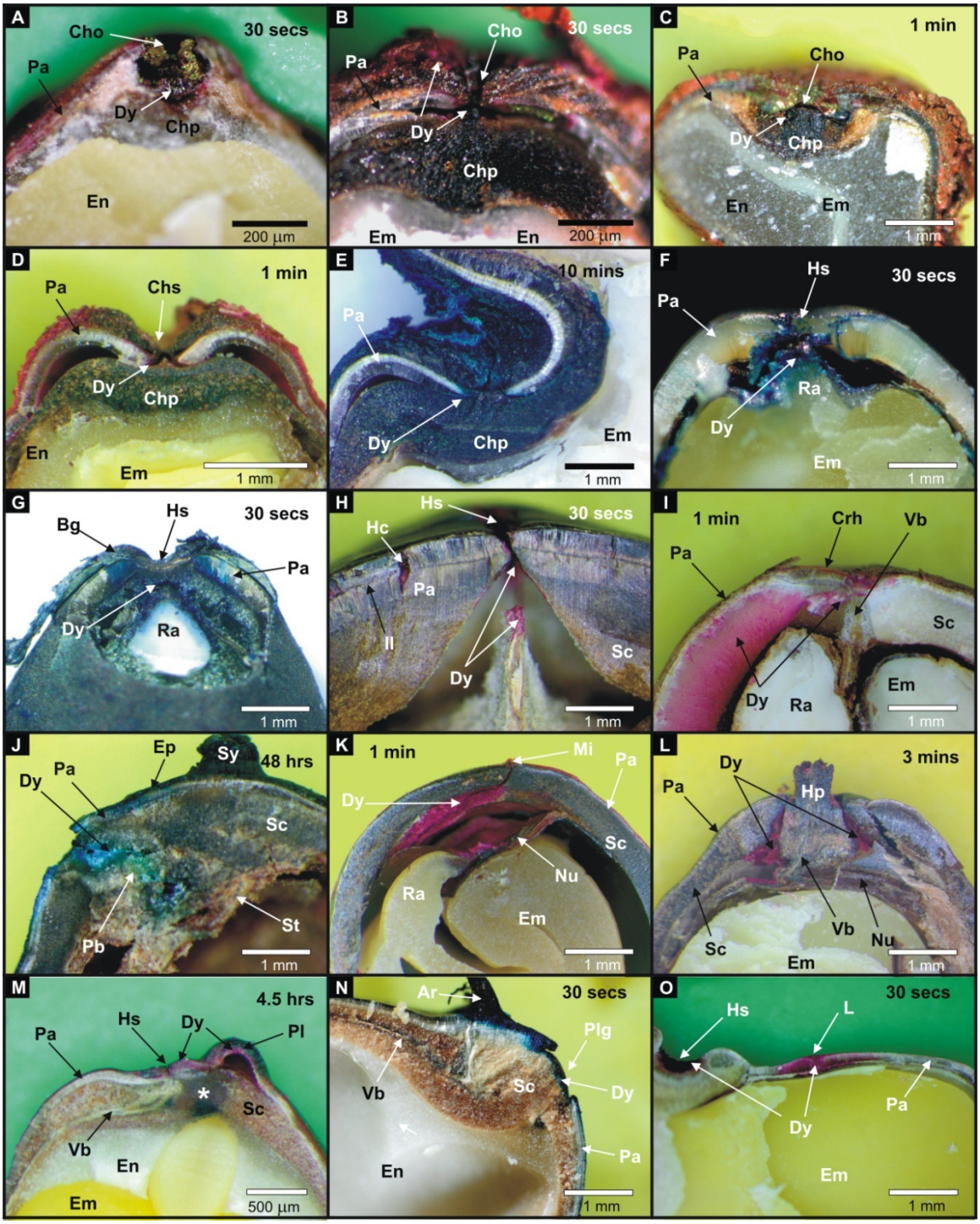
A
Cho
30 secs
Pa
Dy
Chp
En
200 µm
B
Cho
30 secs
Pa
Dy
Chp
Em
En
200 µm
C
1 min
Pa
Cho
Dy
Chp
En
Em
1 mm
D
1 min
Pa
Chs
Dy
Chp
En
Em
1 mm
E
10 mins
Pa
Dy
Chp
Em
1 mm
F
Hs
30 secs
Pa
Ra
Dy
Em
1 mm
G
Bg
Hs
30 secs
Pa
Dy
Ra
1 mm
H
Hs
30 secs
Hc
Pa
Il
Dy
Sc
1 mm
I
1 min
Crh
Vb
Pa
Sc
Dy
Ra
Em
1 mm
J
Ep
Sy
48 hrs
Dy
Pa
Sc
St
Pb
1 mm
K
1 min
Mi
Pa
Dy
Sc
Nu
Ra
Em
1 mm
L
Dy
3 mins
Pa
Hp
Vb
Sc
Nu
Em
1 mm
M
4.5 hrs
Hs
Dy
Pl
Pa
*
Sc
Vb
En
Em
500 µm
N
Ar
30 secs
Plg
Vb
Sc
Dy
Pa
En
1 mm
O
Hs
L
30 secs
Dy
Pa
Em
1 mm

Figure 6.2. Light micrographs of longitudinal sections of water-gap regions of seeds or fruits imbibed in acid fuchsin/methylene blue for different periods of time: (A), (B), (C), (D) and (E) chalazal region of *Helianthemum apenninum* , *Guazuma ulmifolia*, *Bixa orellana*, *Berrya cordifolia* and *Adansonia digitata*, respectively; (F), (G) and (H) Hilar region of *Ceanothus americanus*, *Sicyos angulatus* and *Sapindus saponaria*, respectively; (I) Carpellary hilar region of the endocarp of *Stylobasium spathulatum*; (J) Protuberance region of fruit of *Nelumbo nucifera*; (K) Aril-micropylar region of *Cardiospermum halicacabum*; (L) Hilar region of *Koelreuteria paniculata*; (M) and (N) Hilar region of *Cercis canadensis* and *Bauhinia acuminata*; (O) Hilar-lens region of *Cladrastis kentuckea*. Ar. Aril; Bg, Bulges of the hilar region; Cho, chalazal oculus; Chp, chalazal plug; Chs, chalazal slit; Crh, carpellary hilar slit; Crm, crystalline cells of the mesocarp; Dy, dye; Em, embryo; En, endosperm; Ep, epidermal cell layers; Hc, Hilar cracks; Hp, hilar plug; Hs, hilar slit; L, lens; ll, light line; Mi, micropyle; Pa, palisade cells; Plg, pseudolens gap; Ra, radicle; Sc, sclerenchyma; Sy, style; Vb, vascular bundle. Time given in each figure is the duration of imbibition in the dye.

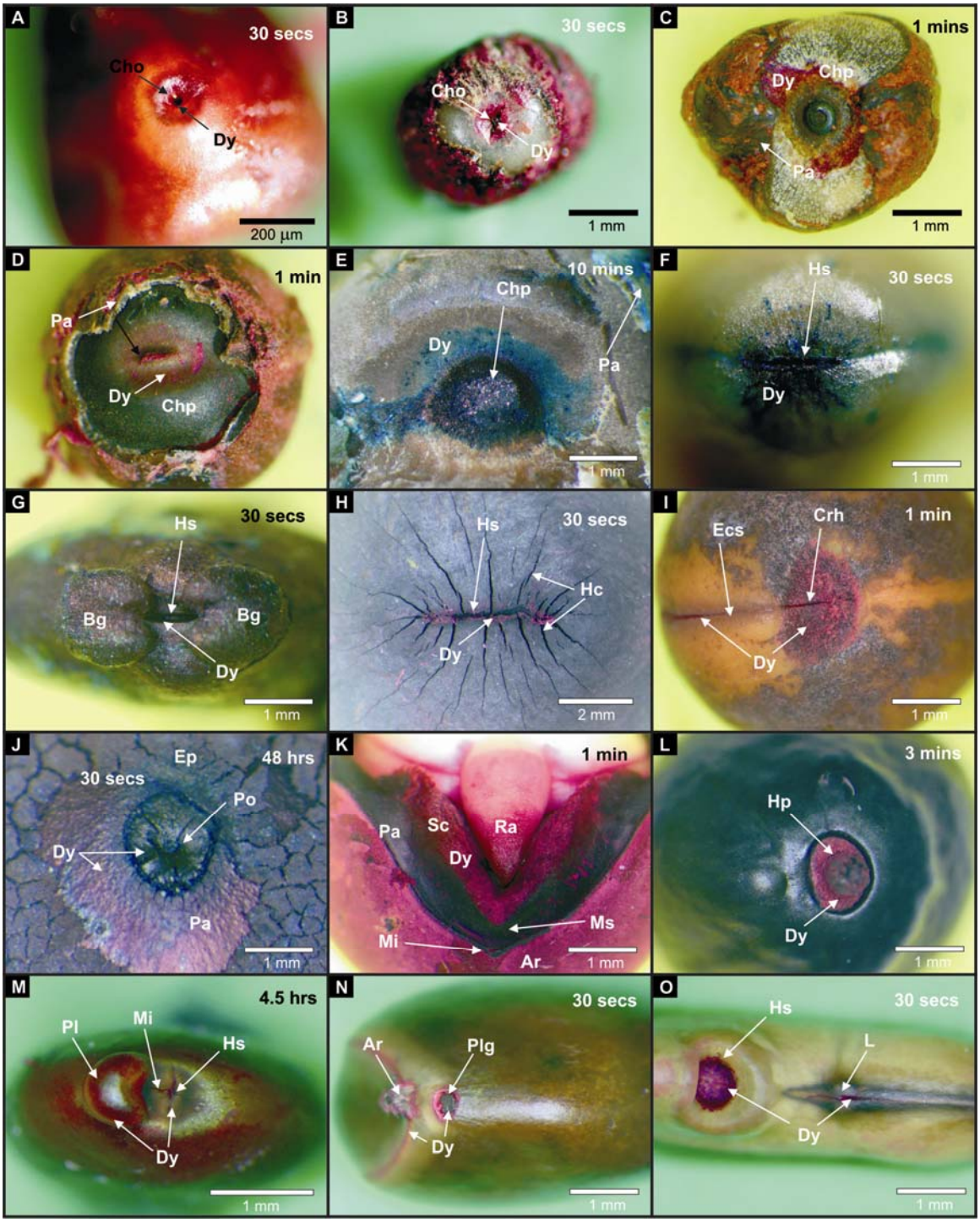
A
30 secs
Cho
Dy
200 µm
B
30 secs
Cho
Dy
1 mm
C
1 mins
Dy
Chp
Pa
1 mm
D
1 min
Pa
Dy
Chp
E
10 mins
Chp
Dy
Pa
1 mm
F
Hs
30 secs
Dy
1 mm
G
Hs
30 secs
Bg
Bg
Dy
1 mm
H
Hs
30 secs
Hc
Dy
2 mm
I
Ecs
Crh
1 min
Dy
1 mm
J
Ep
48 hrs
30 secs
Po
Dy
Pa
1 mm
K
1 min
Pa
Sc
Ra
Dy
Ms
Mi
Ar
1 mm
L
3 mins
Hp
Dy
1 mm
M
4.5 hrs
Pl
Mi
Hs
Dy
1 mm
N
30 secs
Ar
Plg
Dy
1 mm
O
Hs
30 secs
L
Dy
1 mm

Figure 6.3. Light micrographs of the surface view of water-gap regions of seeds or fruits imbibed in acid fuchsin/methylene blue for different periods of time: (A) and (B) chalazal regions of *Helianthemum apenninum* and *Guazuma ulmifolia*, respectively, with outer permeable layers removed to expose the chalaza; (C), (D) and (E) chalazal regions of *Bixa orellana*, *Berrya cordifolia* and *Adansonia digitata*, respectively of which the outer permeable and impermeable palisade layers removed to expose chalazal plug; (F), (G) and (H) Hilar regions of *Ceanothus americanus*, *Sicyos angulatus* and *Sapindus saponaria*, respectively; (I) Carpellary hilar region of the endocarp of *Stylobasium spathulatum*; (J) Protuberance region of fruit of *Nelumbo nucifera*; (K) Aril-micropylar region of *Cardiospermum halicacabum*, impermeable palisade layer removed to expose radicle; (L) Hilar region of *Koelreuteria paniculata*; (M) and (N) Hilar region of *Cercis Canadensis* and *Bauhinia acuminata*; (O) Hilar-lens region of *Cladrastis kentuckea*. Ar. Aril; Bg, Bulges of hilar region; Cho, chalazal oculus; Chp, chalazal plug; Chs, chalazal slit; Crh, carpellary hilar slit; Crm, crystalline cells of the mesocarp; Dy, dye; Ep, epidermal cell layers; Hc, Hilar cracks; Hp, hilar plug; Hs, hilar slit; L, lens; ll, light line; Mi, micropyle; Pa, palisade cells; Plg, pseudolens gap; Ra, radicle. Time mentioned in each figure is the duration of imbibition in the dye.

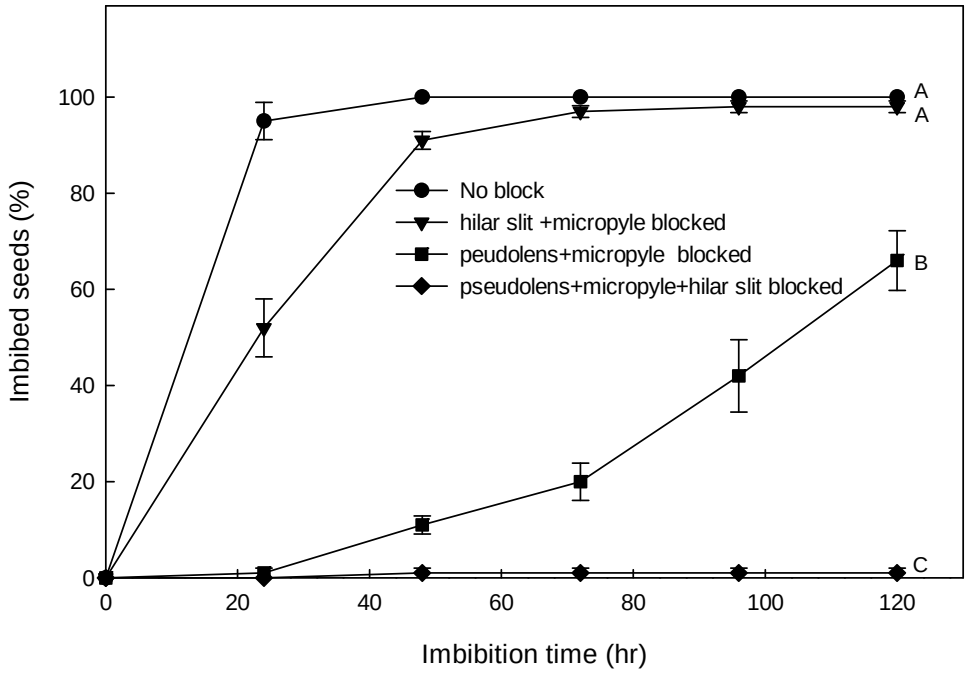


Figure 6.4. Percentages (mean± s.e.) of *Cercis canadensis* seeds imbibed during 120 hr of incubation at ambient room temperature with hilar slit+micropyle blocked, pseudolens+micropyle blocked, pseudolens+micropyle+hilar slit blocked and no structures blocked. Different letters indicate significant differences between treatments ($P<0.05$)

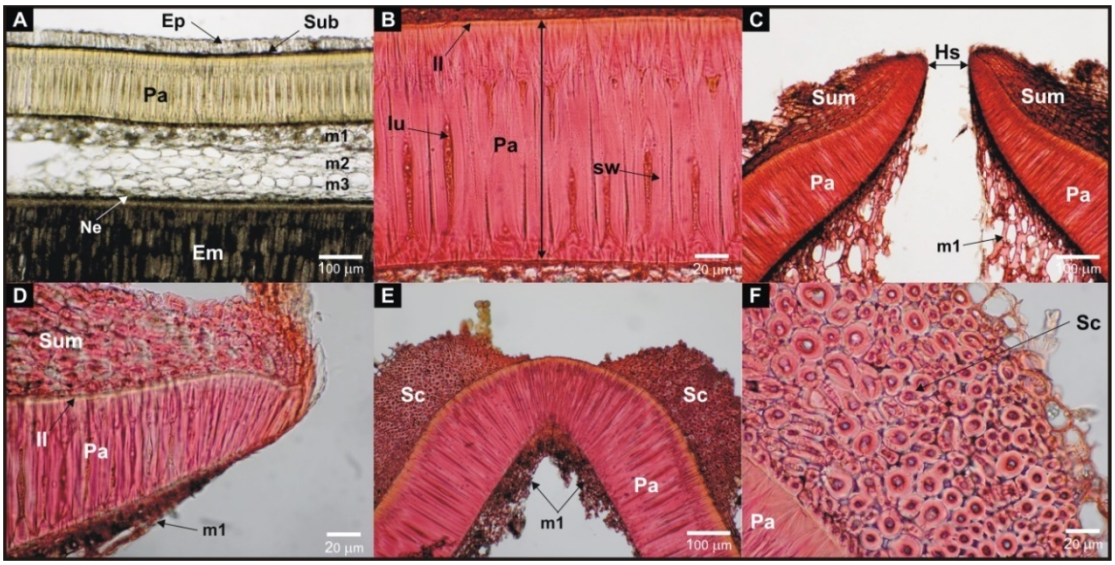


Figure 6.5. Longitudinal sections of hilar and non-hilar regions of seed coat of *Sicyos angulatus*: (A) Seed coat of non-hilar region; (B) Close-up of palisade cells of seed coat; (C) Hilar region; (D) Close-up of hilar region (one side); (E) Seed coat of bulges adjacent to hilar region; (F) Close-up of sclerenchyma cells of bulge. Em, Embryo; Ep, epidermal mesophyll cells; Hs, hilar slit; ll, light line; Lu, lumen of palisade cell; m1, m2 and m3, mesophyll layers of seed coat; Pa, palisade cells; Sc, sclerenchyma; Sub, subepidermal mesophyll cells; Sum, multiple layers of subepidermal mesophyll cells; Sw, palisade cell wall.

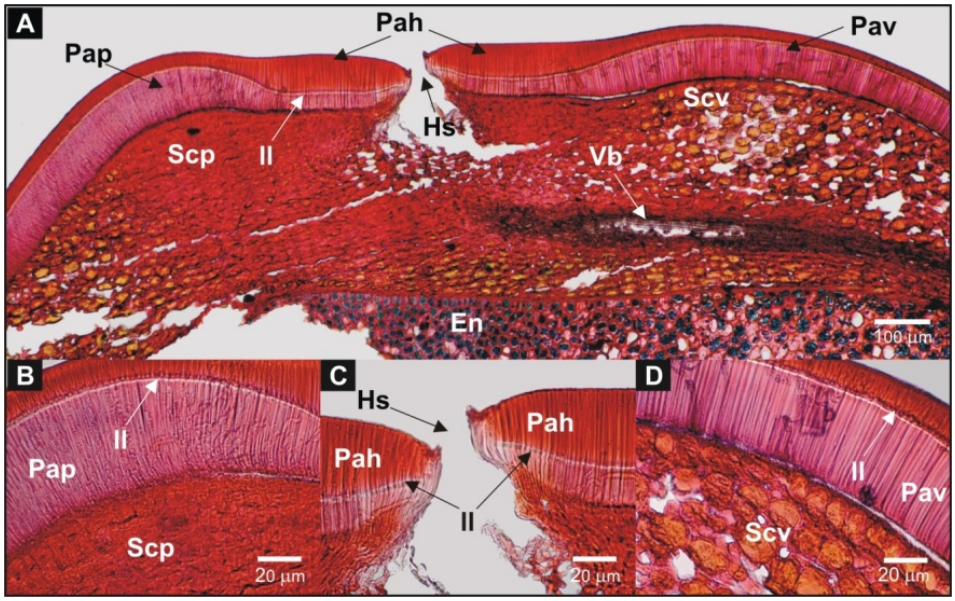


Figure 6.6. Longitudinal sections of hilar and non-hilar regions of seed coat of *Cercis canadensis*: (A) Hilar region; (B) Close-up of palisade and sclerenchyma cells of pseudolens; (C) Close-up of hilar slit; (D) Close-up of palisade and sclerenchyma cells of vascular bundle region. En, endosperm; Hs, hilar slit; ll, light line; Pah, palisade cells of hilar slit; Pap, palisade cells of pseudolens; Pav, palisade cells of the vascular bundle side; Scp, sclerenchyma cells of pseudolens; Scv sclerenchyma cells of vascular bundle side; Vb, vascular bundle.

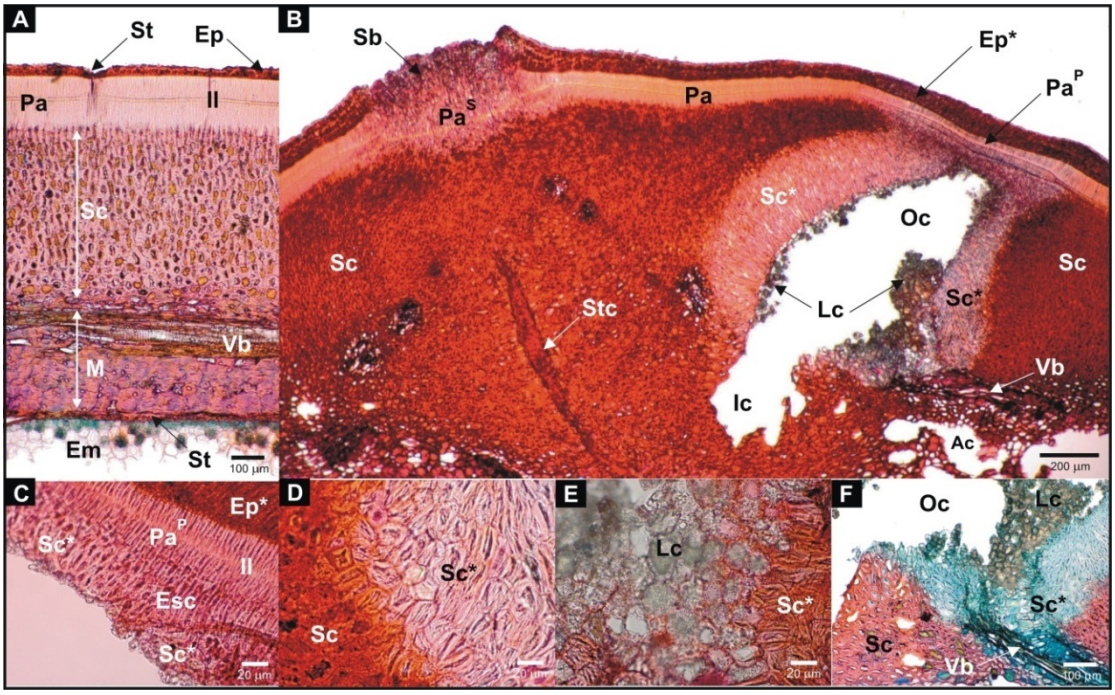


Figure 6.7. Longitudinal sections of the stylar and non-stylar regions of the pericarp of *Nelumbo nucifera*: (A) pericarp of non-stylar region; (B) pericarp of stylar region including protuberence organ; (C) Close-up of palisade cells of protuberence mouth; (D) Close-up of sclerenchyma cells of wall of the protuberence organ; (E) Close-up of crystalliferous cells of wall of protuberence organ; (F) Close-up of vascular connection to protuberence organ. Ac, aerenchyma cells; Em, embryo; Ep, epidermal mesophyll cells; Ep*, multiple layers of epidermal mesophyll cells; Esc, elongated sclerenchyma cells of protuberence mouth; Ic, inner cavity of protuberence organ; Lc, crystalliferous cells of protuberence organ; ll, light line; M, mesophyll cells of endocarp; Oc, outer cavity of protuberence organ; Pa, palisade cells; $Pa^p$, palisade cells of pseudolens mouth; $Pa^s$, palisade cells of stylar base; Sb, base of style; Sc, sclerenchyma cells of pericarp; Sc* sclerenchyma cells of wall of protuberence organ; Stc, stylar canal; Vb, vascular bundle.

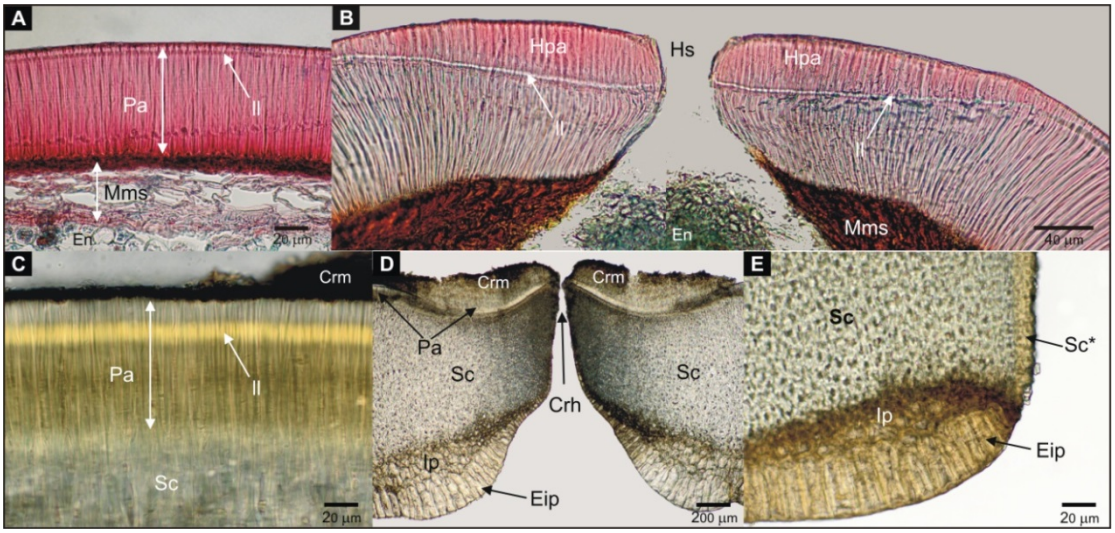


Figure 6.8. Longitudinal sections of hilar and non-hilar regions of the seed coat of *Ceanothus americanus* and endocarp of *Stylobasium spathulatum*: (A) Seed coat of non-hilar region of *C. americanus*; (B) Hilar region of *C. americanus*; (C) Endocarp of non-carpellary hilar region of *S. spathulatum*; (D) Carpellary hilar region of *S. spathulatum*; (E) Inner part of endocarp of non-carpellary hilar region of *S. spathulatum*. Crm, crystalliferous cells of mesocarp; Crh, carpellary hilar slit; Eip, epidermal cells of inner wall of endocarp: En, endosperm; Hpa, hilar palisade cells, Hs, hilar slit; ll, light line; Mms, multiple layers of mesophyll cells of seeds coat; Pa, palisade cells; Sc, sclerenchyma; Sc*, yellowish sclerenchyma cells of endocarp.

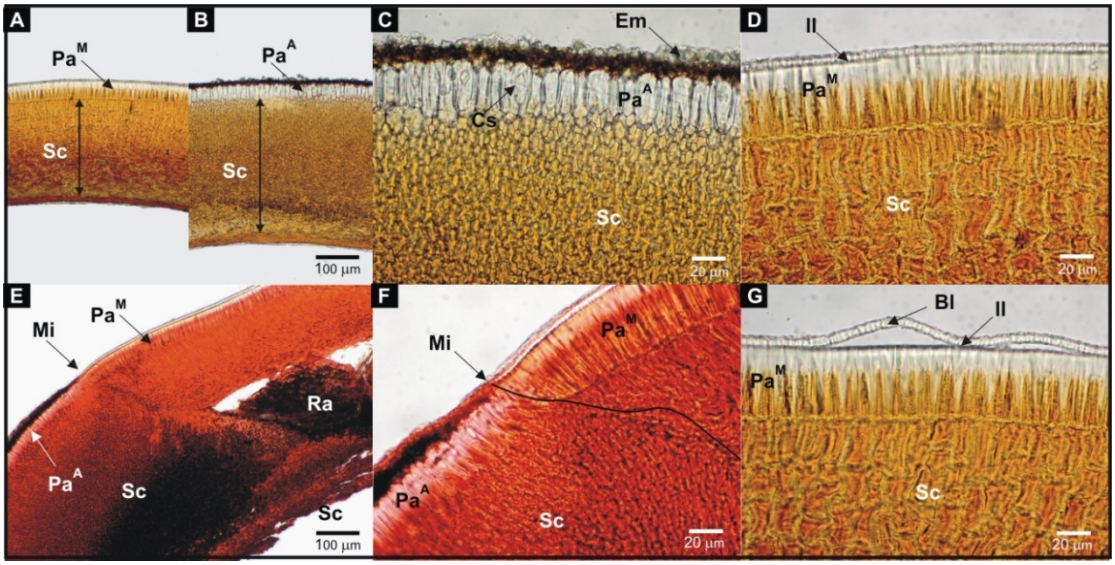


Figure 6.9. Longitudinal sections of the micropylar and non- micropylar regions of seed coat of *Cardiospermum halicacabum*: (A) Seed coat of micropylar region; (B) Seed coat of aril region; (C) Close-up of seed coat of aril region; (D) Close-up of seed coat of micropylar region; (E) Seed coat of micropylar region; (F) Close-up of micropylar region; (G) Seed coat of imbibing seeds with blisters. Bl, blisters; Cs, crystals; Em, epidermal mesophylls; ll, light line; Mi, micropyle; $Pa^A$, palisade cells of aril region; $Pa^M$, palisade cells of micropylar region; Sc, sclerenchyma.

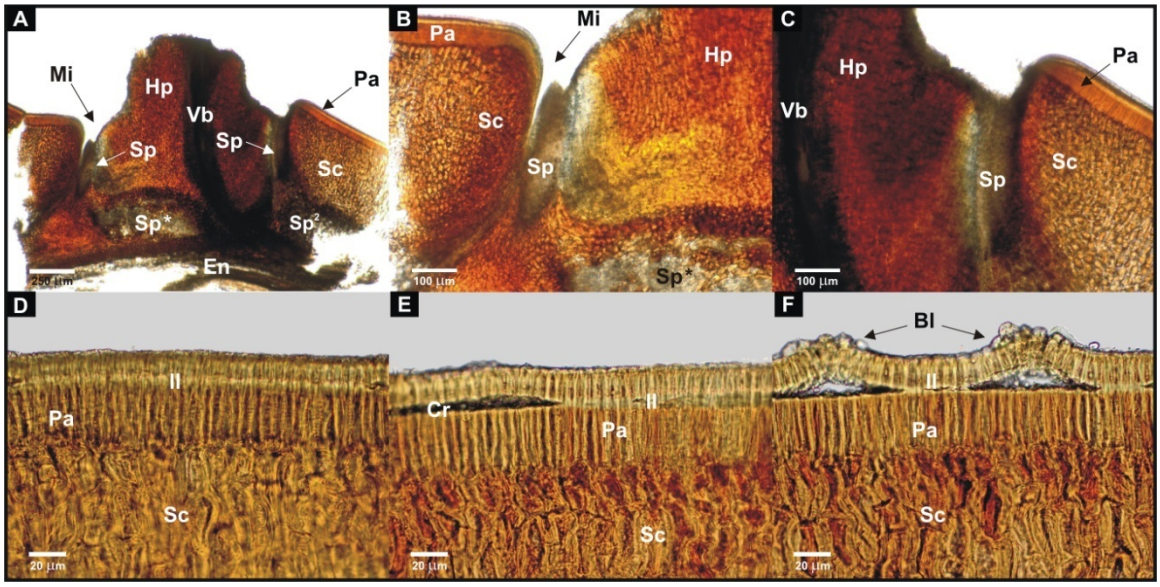


Figure 6.10. Longitudinal sections of hilar and non-hilar regions of seed coat of *Koelreuteria paniculata*: (A) Hilar region; (B) Close-up of micropylar side of hilar region; (C) Close-up of vascular bundle side of hilar region; (D) Seed coat (dry heat-treated) of non-hilar region without blisters; (E) Initial stage of blister formation of palisade cells of seed coat (dry heat-treated) of non-hilar region; (F) Seed coat (dry heat-treated) of the non-hilar region with blisters. Bl, blisters; Cr, cracks of palisade cells at light line; En, endosperm; Hp, hilar plug; ll, light line; Mi, micropyle; Pa, palisade cells; Sc, sclerenchyma; Sp, spongy mesophyll cells; Sp*, spongy mesophyll cell mass of hilar plug; Vb, vascular bundle.

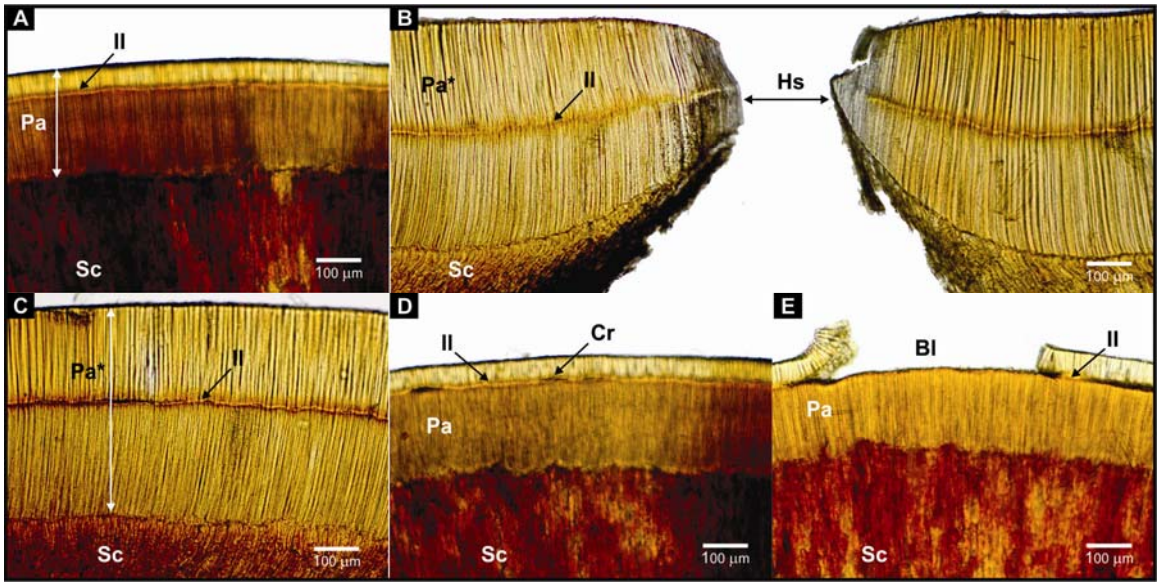


Figure 6.11. Longitudinal sections of hilar and non-hilar regions of the seed coat of *Sapindus saponaria*: (A) Seed coat of non-hilar region; (B) Hilar region; (C) Palisade cells of hilar region; (D) Initial stage of blister formation of palisade cells of non-hilar region; (E) Seed coat of non-hilar region with blisters. Bl, blisters; Cr, cracks of palisade cells at light line; Hs, hilar slit; ll, light line; Pa, palisade cells; Pa*, elongated palisade cells of hilar region; Sc, sclerenchyma.

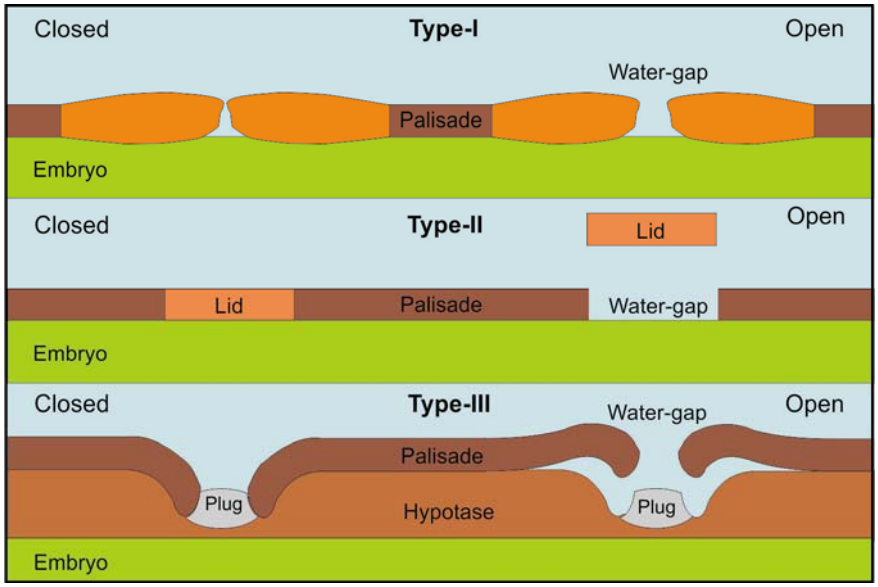


Figure 6.12. Schematic diagrams of the three basic water-gap types. Type-I, narrow-linear opening margined by modified palisade cells; Type-II, circular opening occluded by a lid-like structure formed by modified palisade cells; Type-III, narrow-linear or circular water-gap opening margined by palisade cells and opening occluded by a plug like structure formed by sclerenchyma cells.

# CHAPTER 7

## Summaries and general conclusions

The results of this dissertation expand the understanding of the role of temperature in PY-breaking and in morphology, anatomy and development of the water-gap complex of the family Geraniaceae and its relation to the other water-gap complexes in 15 other families with PY. The dissertation presents the first-ever report of (1) the introduction of the term water-gap complex to describe the water-gap region, (2) a classification system for water-gap complexes in PY seeds/fruits of 16 angiosperm families, (3) the water-gap complex in PY seeds of Geraniaceae and seven other families and (4) a thermal-time model that quantifies the thermal requirement for sensitivity induction in seeds of *Geranium carolinianum*. The summary of each chapters two to six and general conclusions are briefly discussed below.

## CHAPTER SUMMARIES

### Chapter 2: Identification and characterization of the water-gap in physically dormant seeds of Geraniaceae, with special reference to *Geranium carolinianum* L.

A detailed morpho-anatomical characterization of the water-gap region in Geraniaceae was carried out on seeds of *G. carolinanum,* and water-gap regions were compared in 30 species of Geraniaceae (Genera *California*, *Erodium, Geranium, Hypseocharis, Monsonia* and *Pelargonium*). Location, anatomy and morphology of the water-gap of seeds of *G. carolinanum* were characterized using free-hand and

microtome tissue sectioning, light microscopy, scanning electron microscopy, dye tracking, blocking and seed-burial experiments. After dry heat treatment, a colour change was observed near the micropylar region of seeds of *G. carolinianum,* and blocking of this region and dye tracking experiments confirmed the location of the water-gap in this region. Anatomical studies revealed that the micropylar region is more anatomically complex than the rest of the seed coat.

Scanning electron micrographs of the micropylar region of imbibing seeds of *G. carolinianum* revealed a small circular opening connected to the micropyle. This opening is occluded by a lid-like structure hinged at the micropylar side, formed by modified palisade cells. Based on the morpho-anatomical features, the water-gap of *G. carolinianum* was given the name 'hinged valve gap'. The water-gaps of *California, Erodium, Monsonia* and *Pelargonium* species were similar to that of *Geranium,* and slight variations in morphology were observed within and between genera. Moreover, due to the lack of enough seeds, the water-gap of *Hypseocharis* could not be identified.

**Chapter 3: Acquisition of physical dormancy and ontogeny of the micropyle-water-gap complex in developing seeds of *Geranium carolinianum* L. (Geraniaceae)**

Seeds of *G. carolinianum* were studied from the ovule stage until dispersal. The developmental stages of acquisition of germinability, physiological maturity and PY were determined by seed measurement, germination and imbibition experiments using

intact seeds and isolated embryos of both fresh and slow-dried seeds. Ontogeny of the seed coat and water gap was studied using light microscopy.

Developing seeds achieved germinability, physiological maturity and PY on days 9, 14 and 20 days after pollination (DAP), respectively. The critical moisture content of seeds on acquisition of PY was 11 %. Slow-drying caused the stage of acquisition of PY to shift from 20 to 13 DAP. Greater extent of cell division and differentiation at the micropyle, water gap and chalaza than at the rest of the seed coat resulted in particular anatomical features. Various forms of palisade and subpalisade cells develop in these sites. Ontogeny of the micropyle and water-gap regions takes place simultaneously and the micropyle and water-gap cannot be considered as two separate entities, thus forming an anatomically complex structure. Therefore, the term 'water-gap complex' was introduced to describe the water gap-region. In Geraniaceae the water-gap region was named the 'micropyle-water-gap complex'.

**Chapter 4: Timing of physical dormancy-break in two winter annual species of Geraniaceae by a stepwise process**

The primary aims of the research reported in this chapter were to determine whether a temperature-controlled stepwise PY-breaking process occurs in seeds of the winter annuals *G. carolinianum* and *G. dissectum* and to study the roles of temperature and moisture in synchronization of PY-break and germination with the onset of autumn. Seeds of *G. carolinianum* and *G. dissectum* were stored under different temperature regimes to test the effect of storage temperature on PY break. The roles of temperature and moisture regimes in regulating PY break were investigated by

treatments simulating natural conditions. Greenhouse (non-heated) experiments on seed germination and burial experiments (outdoors) were carried out to determine the PY-breaking behaviour in the natural habitat. Irrespective of moisture conditions, sensitivity to the PY-breaking step in seeds of *G. carolinianum* was induced at temperatures ≥20 °C, and exposure to temperatures ≤20 °C made the sensitive seeds permeable. Sensitivity of seeds increased with time. In *G. dissectum*, PY break occurred at temperatures ≥20 °C in a single step under constant wet or dry conditions and in two steps under alternate wet–dry conditions if seeds were initially kept wet.

**Chapter 5: Quantitative analysis of the thermal requirements for stepwise physical dormancy-break in seeds of the winter annual *Geranium carolinianum* L. (Geraniaceae)**

A thermal time model was developed to quantify the thermal requirement for sensitivity induction, and a mechanism was proposed for stepwise PY-breaking in *Geranium carolinianum*. Seeds of *G. carolinianum* were stored under dry condition at different constant and alternating temperatures to induce sensitivity (step-I). Sensitivity induction was analyzed based on the thermal time approach using Gompertz function. Effect of temperature on step-II was studied by incubating sensitive seeds at cool temperatures. Scanning electron microscopy, penetrometer techniques and different humidies and temperatures were used to explain the mechanism of stepwise PY-break.

The base temperature ($T_b$) for sensitivity induction was 17.2 $^oC$ and constant for all seed fractions of the population. Thermal time for sensitivity induction during step-I in the PY-breaking process agreed with the three-parameter Gompertz model. Step-II (PY-break) did not agree with the thermal time concept. $Q_{10}$ values for the rate of sensitivity induction and PY-break were between 2.0 - 3.5 and 0.02 - 0.1, respectively. The force required to separate the water-gap palisade layer from the sub-palisade layer was significantly reduced after sensitivity induction.

**Chapter 6: Identification and characterization of 10 new water-gaps in seeds and fruits with physical dormancy and classification of water-gap complexes**

The primary aims of this study were to identify the water-gaps of Cucurbitaceae, clade Cladrastis of Fabaceae; subfamilies Bombacoideae, Brownlowioideae and Bythnerioideae of Malvaceae; Nelumbonaceae; subfamily Sapindoideae of Sapindaceae; Rhamnaceae; and Surianaceae and to classify all the known water-gap regions based on their morpho-anatomical features.

Breaking of PY in 15 species was done by exposing seeds or fruits to wet or dry heat under laboratory conditions. Identification and characterization of water-gap regions were done using microtome sectioning of seeds and fruits, light microscopy, scanning electron microscopy, dye tracking and blocking experiments.

Ten new water-gap regions were identified in eight different families and two previously hypothesized ones were confirmed. Water-gap complexes consist of (1) an opening that forms after PY is broken, (2) a specialized structure that occludes the gap

and (3) associated specialized tissues. In some species, more than one opening is involved in the initial imbibition of water.

## GENERAL CONCLUSIONS

Acquisition of PY in seeds of *G. carolinianum* occurs after physiological maturity and is triggered by maturation drying. The micropyle and water-gap region cannot be considered as two separate entities, and thus it is more appropriate to consider them together as a 'micropyle–water-gap complex'. Dislodgment of swollen 'hinged valve' palisade cells adjacent to the micropyle caused the water gap to open in physically dormant seeds of *G. carolinianum,* and it was clear that initial water uptake takes place through this gap and not via the chalazal opening as previously reported. The water-gap complex of Geraniaceae ('hinged valve gap') differs from those previously described for other families in morphology, anatomy and location in the seed coat.

Timing of seed germination with the onset of autumn can be explained by a PY-breaking processes involving (*a*) two temperature-dependent steps in *G. carolinianum* and (*b*) one or two moisture-dependent step(s) along with the inability to germinate under high temperatures in *G. dissectum*. Geraniaceae is the third of 18 families with PY in which a two-step PY-breaking process has been documented. Step-I and step-II in PY-breaking of *G. carolinianum* are controlled by chemical and physical processes, respectively. This study indicates the feasibility of applying the developed thermal time model to predict or manipulate sensitivity induction in seeds with two-step PY-breaking processes. The model is the first and most detailed one yet developed for sensitivity induction in PY-break. Moreover, a mechanism was proposed for PY-

break in seeds of *G. carolinianum*, based on differential thermal contraction of the palisade layer in the water-gap region.

Based on the morpho-anatomical features, three basic water-gap complexes (types I, II and III) were identified in species with PY in 16 families. Depending on the number of openings involved in initial imbibition, the water-gap complexes were subdivided as simple and compound. The proposed classification system enables understanding of the relationships between the water-gap complexes of taxonomically unrelated species with PY.

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# VITA

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## PROFESSIONAL POSITIONS

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Teaching assistant, Department of Biology, University of Kentucky, Lexington, USA.

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Temporary Demonstrator, Department of Botany, University of Peradeniya, Sri Lanka.

## PROFESSIONAL PUBLICATIONS

### Peer reviewed publications

Gama-Arachchige NS, Baskin JM, Geneve RL, Baskin CC. 2010. Identification and characterization of the water gap in physically dormant seeds of Geraniaceae, with special reference to *Geranium carolinianum*. *Annals of Botany* 105 977-990.

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2013