THESIS

SEED DORMANCY IN *CALLIRHOE INVOLUCRATA* (TORR. & GRAY) GRAY, (MALVACEAE)

Submitted by

Dianne Skogerboe

Department of Horticulture and Landscape Architecture

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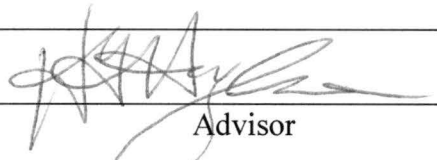
WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY DIANNE MICHELE OAKLEY SKOGERBOE ENTITLED, " SEED DORMANCY IN *CALLIRHOE INVOLUCRATA* (TORR. & GRAY) GRAY, (MALVACEAE) " BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work



Advisor

Department Head

ABSTRACT OF THESIS

SEED DORMANCY IN *CALLIRHOE INVOLUCRATA* (TORR. & GRAY) GRAY, (MALVACEAE)

Propagation of *Callirhoe involucrata* (Torr. & Gray) Gray, for use as a landscape ornamental has been encumbered by a lack of understanding of the seed dormancy and the development of a practical technique for overcoming it. In the populations tested, hard seed accounted for 49 % of an average sample of viable seed. Three disjunct populations of seed, representing two different collection years, were used to investigate practical methods of overcoming impermeability due to hard seed. Methods tested were moist pre-chilling, hot water, leaching, gibberellic acid, hydrogen peroxide, citric acid, potassium nitrate, mechanical and chemical scarification. Standard germination tests were conducted at three-month intervals for 15 months. In addition to hard seed, high percentages of seedless fruit, aborted and unfilled seed were identified. Hand scarification was the most effective means for improving germination in *Callirhoe involucrata*, resulting in germination as high as 98 %. Scarifying seed with concentrated sulfuric acid was highly effective when a double 60-minute exposure was used, generating germination percentages between 75 - 90 %. Single timed exposure treatments to sulfuric acid stimulated germination in some fractions and caused embryonic damage in others suggesting a variation in seed coat thickness. Similar results were obtained

using a pressurized air scarifier; the hard seed coat of some seed fractions were precisely scarified while others were physically damaged using the same treatment. Soaking the fruit in boiling water (100°C) proved to be the most practical means of improving germination (over control) with increases from 12, 22, 30 % to 48, 58 and 91 %, in the three populations respectively. Leaching for 24 and 48 hours in cold (18°C) aerated water showed no improvement over control. A significant increase in germination was established for one lot when leaching occurred for 24 hours in warm (40°C) aerated water. Prechilling at 5°C did not improve germination in unscarified seed. Gibberellic acid, citric acid, and potassium nitrate did not significantly improve germination in scarified seed. Similarly, soaking seeds for 24 hours in 3 % hydrogen peroxide did not increase germination and at a 30 % concentration germination was reduced. Embryo dormancy did not appear to be a factor although some question remains about the presence of chemical inhibitors. The considerable variation in seed dormancy expression may be a function of differences in environmental factors during development. Success was obtained using vegetative stem cuttings dipped in 1.0 % IBA powder and placed under mist. Rooting reached 86 % in some trials.

Dianne Michele Skogerboe
Department of Horticulture
and Landscape Architecture
Colorado State University
Fall 2001

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The journey has just begun ....

Dianne Michele Oakley Skogerboe
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INTRODUCTION

A. Statement of Problem

As a hardy, drought tolerant perennial with striking claret-colored flowers and attractive foliage, *Callirhoe involucrata* is an obvious choice for use in landscapes. In 1999, *Callirhoe involucrata* was selected and promoted by the Plant Select®[1] plant introduction and promotion program. As a native, *C. involucrata* has evolved characteristics that make it especially suitable for the challenges of growing in the prairies, high plains and intermountain regions of Colorado, the primary market of the Plant Select® introduction program. Unfortunately, it has proven difficult to propagate in quantities that satisfy commercial demand. There is a paucity of information that details the propagation requirements or dormancy inducing conditions associated with any species of the *Callirhoe* genera. Therefore, the objective of this research was to (1) determine the nature of the germination inhibiting factor(s) and (2) establish a propagation protocol suitable for commercial production of *Callirhoe involucrata.*

One element known to contribute to a reduced germination capacity is poor seed fill, a condition associated with a variety of factors including premature harvesting and environmental stresses. Unfilled seed comprised an average of 26 - 40 % of those

Callirhoe samples tested, depending on the seed lot origin and year of collection. Variation in response to treatments was shown to occur by geographic location and from year to year within the same source population. The considerable variation in seed dormancy expression may be a function of differences in environmental factors during development.

To determine effective procedures for overcoming dormancy, wild collected seeds, from Kansas, Missouri and Nebraska, were subjected to various chilling, scarification and chemical treatments. Despite demonstrating high viability in preliminary studies, seed of *Callirhoe involucrata* exhibited a low germination capacity and rate. As with many members of the Malvaceae family, dormancy in *C. involucrata* is predominantly due to a water-impermeable seed coat. Coat-imposed dormancy is a condition that has likely developed to delay germination until environmental conditions are propitious for seedling establishment.

Vegetatively, *Callirhoe* can be multiplied by division of its thickened taproot and by cuttings, both methods historically producing too few progeny to be useful for large-scale production purposes.

[1] *The Plant Select® plant introduction program is a cooperative effort administered by Denver Botanic Gardens, Colorado State University and green industry professionals.*

Chapter II:
LITERATURE REVIEW

A. *Callirhoe involucrata*

1. Description

Callirhoe involucrata is a decumbent to ascending herbaceous perennial reaching 30 - 60 cm. in height. The vine-like stems, 10 - 60 cm long, arise from napiform to fusiform taproots which may reach 3 dm. Stellate hairs occur on the stem, leaves, calyces and peduncles. Dark green, stipulate leaves are palmately lobed and variously incised enhancing the overall appearance. The solitary flowers, which may be perfect and strongly protandrous or male sterile (geitonogamous), are 4 - 6 cm in diameter and occur in a raceme. A corolla-size dimorphism is exhibited with perfect flowers being nearly twice as long and twice as wide as those of male-sterile flowers. The corolla is generally claret colored with a white eye but may also be white or pale lavender with white margins. An involucel of prominent bracts subtends the calyx. Nectaries are located between each of the five petals, on the adaxial surface, at the juncture of the calyx and corolla. The fruit, a shizocarp, consists of 9 - 23 keeled and beaked, one-seeded, indehiscent mericarps. Seeds are black to brown and reniform (Waterfall, 1951; Zomlefer, 1994). The carpel and seed dehisce as a single unit. The carpel wall, when mature, is reticulate and strigose (Dorr, 1990). A mature seed is exotegmic and small in

size (2.3 mm x 1.5 mm) (Corner, 1976). The ovule is campylotropus. The oil and protein rich endosperm is persistent, often thick and congruent about the embryo and in areas between folds of the cotyledons. Outside the endosperm exists a thin layer of remnant nucellus and occasionally perisperm (Reeves, 1936a).

2. Taxonomic background

Callirhoe is a small North American genus composed of herbaceous annual, biennial and perennial plants belonging to the Malvaceae family, Malveae tribe and Malvinae subtribe. *Abutilon, Althaea, Lavatera, Malva, Sida* and *Sidalcea* are some of the more commonly encountered closely related genera (Corner, 1976). Nine species of *Callirhoe* are generally recognized: *C. alcaeoides, C. bushii, C. digitata, C. involucrata, C. leiocarpa, C. papaver, C. pedata, C. scabriuscula, C. triangulata* (Dorr, 1990). First described in 1827, Nuttall ex Torrey, *Callirhoe involucrata* was initially termed *Nuttalia involucrata. Nuttalia* after the author and *involucrata* referring to the characteristic whorl (involucre) of bracts that subtend each flower. In 1938, the name was changed to *Malva involucrata* (Torr. & Gray), which, in turn, was replaced with *Callirhoe involucrata* (Torr. & Gray) Gray in 1943. The etymology of the genus appellation is not clear but is thought to be derived from Greek mythology; Callirhoe was the mythological daughter of King Oeneus and his wife Althaea. (Dorr, 1990; Waterfall, 1951). *Involucrata, lineariloba,* and *tenuissima* are recognized as three varieties of *C. involucrata.* Geographic distribution and morphology serve as the basis for varietal distinction (Bates,

et. al, 1989). Common names for *Callirhoe involucrata* include purple poppy mallow, low poppy mallow, buffalo rose, winecup (Dorr, 1990) and prairie winecup.

3. Distribution

Distribution in the United States occurs throughout the north central and plains states, extending into southern Texas, east to northwestern Missouri, west to eastern New Mexico, Colorado and Wyoming and distinctly in the Sierra Madre of northern Mexico. It is adventive in Florida, Pennsylvania, eastern Iowa, Illinois, eastern Oklahoma and Oregon. The three varieties of *C. involucrata* are located in distinct geographic regions with very little overlap occurring. The variety *involucrata* is most strongly represented in Kansas, Oklahoma and North Dakota; *lineariloba* in Texas and *tenuissima* in Mexico. Habitats include high plains, prairies, dry open grasslands, dry streambeds and roadsides in sandy, clay or calcareous soils (Dorr, 1990).

4. Reproductive strategies

Dicliny is said to occur where not all genets of a population exist as hermaphrodites. Gynodioecy, a type of dicliny, occurs as a population of perfect (bisexual) and male sterile (female) genets (Richards, 1997). Individual plants of *Callirhoe involucrata* may produce either geitonogamous (male sterile) or strongly protandrous, hermaphoritic flowers (Dorr, 1990). Gynodioecy has also been reported in *Callirhoe leiocarpa* and *C. alcaeoides* but is apparently rare in other genera of the Malvaceae family (Dorr, 1990).

In gynodioecious populations, femaleness is commonly linked to smaller corolla size and increased seed set (relative to bisexual flowers) (Bates, 1992). Dissertation work conducted by Amos, 1981, using *C. involucrata*, confirmed that geitonogamous crosses (pollination between different flowers on the same plant) result in greater fecundity than autogamous crosses (within-flower pollination) (50.7 % v. 8.3 %). Male sterility can be controlled genetically or environmentally induced. In gynodioecious populations flowers produced exceptionally early or late in the season tend to be male sterile (Richards, 1997). The significance of this condition is that (1) it compliments protandry by encouraging outcrossing, hence deterring inbreeding depression and (2) has implications for the timing of harvesting events.

Experiments conducted by Neff, *et. al*, 1982, demonstrated that despite being fully self-compatible *C. involucrata* is rarely autogamous purportedly the result of pollen senescence. In other words, pollen present on an individual androgynous flower possessed low viability by the time the stigma was receptive. Amos (1981), on the other hand, speculated that the lack of successful autogamous crosses could be attributed to a physiological barrier derived from an evolutionary shift in breeding systems (from autogamy to xenogamy).

Plants, such as *Callirhoe* spp., which produce a high percentage of unfilled, rudimentary and aborted seed may do so as a function of indeterminate flowering habit, resource competition (between flowers) or limitation, unsuccessful crosses, or exposure to

unfavorable temperatures. Hot weather during late anthesis has been documented to reduce pollination and subsequent seed set in many plants (Baskin & Baskin, 1998; Majerus, 1999). Water availability may also be a factor in limiting fruit production (Stephenson, 1981; Willson and Price, 1980). In one study, using *Sidalcea malvaeflora* ssp. *elegans* (Malvaceae), plants receiving supplemental water during fruit development produced more than four times the number of viable seeds per fruit (schizocarp) than the control (Dimling, 1992).

Indeterminate flowering leads to the presence of seed in all stages of maturity at any given time. This means that unless collection efforts are strictly monitored a percentage of the seed harvested will always be underdeveloped. Furthermore, proximal fruit on a raceme, like that produced by *Callirhoe involucrata*, generally develops earlier and grows larger than the younger distal fruit. Older fruits repress the development of younger fruits through the production of IAA (Indole-acetic acid). Often, the result of this inhibitory action is seed abortion (Taiz and Zeiger, 1998). It is also known that resources are not allocated equally to all seeds (Richards, 1997) and that different positions on the mother plant may not have the same dormancy-breaking or germination requirements (Baskin & Baskin, 1998). Another potential factor is lack of pollinators. In the *Callirhoe involucrata* populations studied by Neff *et. al*, 1982, a variety of insects were identified but only one was a regular pollen-collecting insect, *Diadasia afflicta*. The majority of floral visitors were only observed nectaring. It is noted by Neff , *et. al,* 1982, that species in the genus *Callirhoe* have developed specialized (oligolectic) relationships with solitary bees. While

the social honeybees do not appear to visit *Callirhoe*, the oligoleges are dependent upon the pollen to supply their nests. Should conditions be unfavorable for the oligogene population, flowers could end up largely unpollinated. Gorchov (1988) points out that aborted seeds should be characterized by their function. Abortion of unfertilized ovules is a function of pollination limitation while the abortion of fertilized ovules is largely a consequence of resource limitation. The production of large numbers of empty fruit (including aborted fertilized and unfertilized seed) has been reported in other malvaceous species: *Sphaeralcea grossulariaefolia* (Page *et. al*, 1966) and *Sidalcea malvaeflora* ssp. *elegans* (Dimling, 1992).

B. Dormancy and the development of hard seed

Failure of a viable seed to germinate has two basic causes: first, the absence of favorable environmental conditions. In this circumstance the seed is considered quiescent and will remain so until provisions suitable for germination have been met. The second cause is dormancy. A seed is considered dormant when there is a failure to germinate in the presence of environmental conditions suitable for germination. The cause of dormancy may be physical and/or physiological in nature. The biological significance of dormancy is to control timing such that germination occurs in the season most propitious for seedling survival. Dormancy may also function to maintain a persistent seed bank, through nonsynchronous germination, permitting carryover of seeds between years. In

other species, commonly annuals, dormancy may act in a converse manner by working to synchronize germination (Grime, 1989; Baskin & Baskin, 1998).

Nikolaeva, 1969 (English translation), was the first to publish a seed dormancy classification system. It consisted of two main categories (endogenous and exogenous) and six sub-categories. Endogenous dormancy, as defined by Nikolaeva, is caused by a condition that originates in the embryo. Exogenous dormancy, however, is a condition that originates in structures external to the embryo (Nikolaeva, 1969). Many dormancy classification schemes have been proposed since 1969 leading to confusion and inconsistency in published works. Today, the most commonly accepted classification system uses two broad categories of dormancy; primary (or innate) and secondary (or induced) (Richards and Beardsell, 1987). Seeds that develop a block to germination while still attached to the mother plant are said to have primary dormancy. This contrasts with secondary dormancy, which develops in response to unfavorable conditions after dispersal from the mother plant (Bewley & Black, 1994; Mayer & Poljakoff-Mayber, 1989).

Some seeds have "coat-imposed dormancy"; a type of primary dormancy whereby the tissues enclosing the embryo, generally the testa, are impermeable to water and this can delay germination for years. Seeds of this type (termed 'hard') are prevalent in certain plant families; Fabaceae, Convolvulaceae, Geraniaceae and Malvaceae among them

(Baskin & Baskin, 1998; Crocker & Barton, 1957). Coat-imposed dormancy is the condition this paper will focus on.

The mechanisms by which the seed coat imposes dormancy include: (a) interference with water uptake; (b) interference with gas exchange; (c) mechanical restraint to embryo enlargement and radicle protrusion; (d) prevention of the diffusion of inhibitors from the embryo; (e) supply a source of inhibitors (Bewley & Black, 1994); or (f) act as a filter/barrier to light (Ellis, *et al.*, 1985b). Prevention of germination due to coat-imposed dormancy may be the result of one or more of these conditions.

1. Environmental and genetic effects on the development of hardseed

Development of water-impermeability in seed has both a genetic and environmental basis (Quinlivan, 1971; Rolston, 1978; Bewley & Black, 1994). Lee, 1975, determined that two genes were responsible for hardseed in cotton and that dry conditions during maturation enhanced the expression of these genes. Genes controlling the expression of hardseededness have also been identified in *Lens culinaris* (Ladizinsky, 1985), *Phaseolus vulgaris* (Dickison & Boettger, 1982), and *Glycine max* (Tulley *et al.,* 1981).

Of the three basic seed structures, embryo, endosperm, and seed coat, only the seed coat is derived exclusively from diploid maternal cells. It is not surprising then, to find that there is frequently a strong correlation between the ecological conditions that the mother plant is exposed to and the environmental cues for germination required by the seed it produces

(Mayer and Poljakoff-Mayber, 1989; Antonovics and Schmitt, 1986; Lacey, 1991; Kelly *et al.*, 1992; Meyer, 1992; Meyer and Kitchen, 1994; Platenkamp, *et al.*, 1993). In this same way, environmental conditions imposed upon the parent plant during seed maturation can modify the expression of permeability (Rolston, 1978). Photoperiodic effects (Karssen, 1970) and the rate and degree of drying have been shown to contribute to the development of hard coats in some seeds (Quinlivan, 1968). Seeds with low permeability and a thickened cuticle developed in *Ononsis sicula* when the mother plant was exposed to long days during the final eight days of seed ripening (Evanari, *et al.,* 1966). Using *Lupinus varius*, Quinlivan (1968) demonstrated that seed dried to a moisture content below the critical level of 8.5 % developed "absolutely hard" seed that could be permeated only when the testa was fractured. In contrast, seed dried to a moisture content above 10 % were "conditionally hard", softening slowly when exposed to moist environmental conditions. Drought, thermoperiod and nutrient status of the mother plant have also been cited for their effects on the development of hardseed (Egley, 1989; Quinlivan, 1966; Rolston, 1978). Furthermore, different populations of the same species have been shown to have adaptations in their germination requirements that are in accordance with the localized environmental factors prevailing with their distribution (Meyer, 1992; Meyer and Kitchen, 1994; Vickery, 1967). The fact that abiotic factors, in particular weather, are not in stasis helps to explain why seed of a given species can vary in the degree of permeability by locality and from year to year (Fenner, 1985).

2. Seed coat structure: Anatomy and physiology of hard seed

The water-impermeable condition imposed by the testa may be conferred by a waxy cuticle, suberized sub-cuticle, osteosclerid layer, palisade layer when composed of lignified sclerid cells (Bewley & Black, 1994) and/or by inhibitory substances such as quiniones and other phenols which impregnate cell walls (Stuart and Loy, 1983). The deposition of callose (*Sesbania punicea*), lipids and suberin (*Meliotus alba*) into the cell walls, of the palisade layer, also has been shown to confer impermeability (Kelly, *et al.*, 1992).

Mechanical restriction of the embryo occurs when cells known as Malphigian or macrosclerid, of the testal palisade layer, shrink and become tightly oppressed. (Egley, 1979; Egley, *et al.,* 1983; Egley and Paul, 1993). In addition, histochemical analysis of the seed coat revealed that the conversion of hydroxyphenols into insoluble lignin-like polymers could be correlated with the onset of impermeability in *Sida spinosa*. These polymers are synthesized in the nascent seed, while still attached to the mother plant, by the peroxidase-catalyzed oxidation of phenolics (Egley, *et al.*, 1983; 1986b, 1993). These studies agree with reports for other seeds (Marbach and Mayer, 1974; Rangaswamy and Nandakumar, 1985; Serrato-Valenti, *et al.,* 1992).

Histochemical analysis of seeds of *Callirhoe involucrata* revealed that the cell walls of the outermost layer of the testa are composed predominately of cellulose with tannin present in minor amounts (Reeves, 1936a). The outer integument consists of two to three

layers of cells, except in the chalazal region where it is thicker. The mechanical layer exists as a palisade layer of the outer epidermis of the inner tegmen (Corner, 1976). Tightly packed, prismatic parenchyma macrosclerid cells make up the water-impermeable palisade layer. Lignin, tannins, cellulose and traces of cork make up the parenchyma cells. A distinct light line can be observed in the parenchymatous cells. Below the light line exists a pigmented layer containing suberin and tannin (Reeves, 1936a).

In many members of the Malvaceae family, it has been observed that the palisade layer is not continuous in the chalazal region. The chalaza is the area on an attached seed where the integuments fuse with the funiculus. The discontinuity, described as a "chalazal slit" in *Abutilon theophrasti* (Winter, 1960) and *Sida spinosa* (Egley and Paul, 1981; 1982); a "chalazal cleft" in *Kosteletskya virginica* (Poljakoff-Mayber, *et. al,* 1992) and a "chalazal aperture" in cotton (*Gossypium hirsutum*) (Christiansen and Moore, 1959), is plugged with tightly packed prismatic parenchyma cells (Rolston, 1978; Winter, 1960; Werker, 1980/81). In hard seed cotton this plug, composed of maternal tissue, is described as a chalazal cap that acts as an impervious seal to the palisade layer (Christiansen and Moore, 1959; Serrato-Valenti, *et. al*, 1992). Under permissive conditions water enters the chalazal opening moving laterally between the upper palisade layer and the underlying subpalisade layer. A 'blister' forms in the region of the chalaza as the two palisade layers separate. Specific to this site are cells that appear to act hygroscopically initiating the hydration process and aiding in the apoplastic water flow in the seed. With the continued absorption of water, the blister breaks creating a portal for unrestricted imbibition (Egley

and Paul, 1981; Egley, *et. al*, 1986b). The Prussian blue (Serrato-Valenti, *et. al,* 1992) and Fast Green (FCF) tests (Kelly and Van Staden, 1987) have been used to confirm the path of water entry. This phenomenon has been documented in many malvaceous species: *Gossypium hirsutum* (Christiansen and Moore, 1959), *Abutilon theophrasti* (La Croix and Staniforth, 1964), *Abelmoschus esculentus* (Serrato-Valenti, *et. al,* 1992), *Anoda cristata* (Egley, *et. al*, 1985), and *Kosteletzkya virginica* (Poljakoff-Mayber, *et. al*, 1992). It has been proposed that by functioning as a moderator of water diffusion the chalaza minimizes the risk of imbibitional injury to the embryo (Woodstock, 1988).

Genetics, atmospheric moisture and the stage of seed development when the drying starts act to determine the rate and degree of impermeability in seeds (Baskin and Baskin, 1998). Ontogeny studies indicate that water impermeability occurs during the latter stages of seed development (Egley, 1976). In seed of *Sida spinosa* (Malvaceae) the testa was demonstrated to be "conditionally hard" at a moisture level between 15 - 20 % during which time the chalazal slit remained opened to water movement. Only after the level dropped below 15 % did the testa become "absolutely hard"; at this time the chalazal slit was impermeable to water diffusion (Egley, 1976).

Research conducted on *Abelmoschus esculentus,* described as having a seed coat anatomically similar to other members of the Malvaceae (Reeves, 1936b; Corner, 1976), has revealed some of the intimate histochemical and structural changes that occur during

imbibition. The palisade cells of the seed coat are composed of three distinct parts: (a) the upper 'prismatic part', columnar in shape with longitudinal wall thickenings, a light line and polygonal lower ends, (b) the 'transition part' and (c) the 'twisted part' with twisted columnar thickenings. During imbibition these parts were observed to be differentially responsive. The hydrophilic prismatic parts of the parenchyma swelled readily while the lignified 'twisted' parts remained unchanged. In response, the upper palisade layer physically separated from the subpalisade layer creating a pathway for water movement. This was followed by the oft-observed kidney-shaped 'blister' forming at the chalaza. The water entry point, the chalaza, was found to be abundant in a hydrophilic "strongly acidic polysaccharide material" (Serrato-Valenti, *et. al*, 1992).

C. Propagation by seed

The presence of an impermeable testa, while ecologically advantageous to the species producing the seed, can be confounding to a plant propagator. Hardseed requires pre-treatment before being sown. Popular commercial methods of inducing imbibition in hard seeds are by mechanical abrasion and treatment with sulfuric acid (Richards and Beardsall, 1987). These treatments scarify the seed coat rendering it permeable to water, gases and light; reduce mechanical restraint of the seed coat; and possibly reduces or destroys the effect of chemical inhibitors (Mayer and Poljakoff-Mayber, 1989; Bewley and Black, 1994). Other methods used to a lesser extent are percussion, dry storage, heating, freezing, high humidity, extreme temperature fluctuations, organic solvents,

boiling water, and stratification/prechilling. There is little evidence that confirms how seeds lose their impermeability in nature. However, high relative humidity, diurnal fluctuations in temperature, microbial digestion, fire, ingestion by birds and non-masticating animals, and mechanical abrasion have been offered as plausible mechanisms (Rolston, 1979; Kelly, *et. al*, 1992; Egley, 1989). No formal speculation has been made on how the hard seed condition in *Callirhoe involucrata* is reduced in nature. Nonetheless, due to its prairie habitat, ingestion by birds, microbial digestion and possibly fire seem to be the most probable sources of hard seed disruption.

1. Pre-chilling of scarified seed

Pre-chilling has been shown to be effective in breaking dormancy in some seeds with coat-imposed dormancy (Nikolaeva, 1969; Bewley and Black, 1994; Webb and Wareing, 1972). Alterations of membrane permeability at low temperatures could contribute to the effect of pre-chilling on breaking dormancy in some hardseeds (Bewley and Black, 1994). Cold-moist treatment can also contribute to after-ripening in some seeds, producing growth and metabolic change within the embryo (Mayer and Poljakoff-Mayber, 1989). The membrane must be permeable for metabolic processes to take place.

Dormancy in the majority of temperate grassland forbs is physiological and is broken by prechilling. Spring simulated temperatures promote high germination in many grassland forbs (Baskin and Baskin, 1998). Some members of the Malvaceae family are suspected (*Sphaeralcea grossulariaefolia*) or known to have (e.g., *Malva parviflora*) physiological

dormancy (Sumner and Cobbs, 1967; Page, *et. al,* 1966). Similar results have been obtained with other Malvaceae members: *Sphaeralcea grossulariaefolia* (Page, *et. al,* 1966), *Abutilon theophrasti* (Winter, 1960), *Sida spinosa* (La Croix & Staniforth, 1964; Egley, 1976) and *Kosteletskya virginica* (Poljakoff-Mayber, *et. al*, 1992). One author reported enhanced germination by pre-chilling seed of *C. involucrata* for three months prior to sowing (Phillips, 1995). Voight, 1977, demonstrated that a 60-day prechill treatment produced 71 % germination (vs. 30 % for control) in *Callirhoe triangulata*, "presumably due to softening of the hard seed coat".

For many species, it is the amplitude of temperature fluctuation that determines the effectiveness of the treatment. Studies conducted with *Rumex* sp. (Totterdale and Roberts, 1980), *Sphaeralcea grossulariaefolia* (Page, *et. al,* 1966) and *Triflolium subterraneum* (Quinlivan, 1966) demonstrated the apparent ability of fluctuating temperatures to break seed coat-imposed dormancy.

2. Gibberellic acid

The classic concept of (embryo) dormancy attributes the induction and subsequent release to be regulated by ratios of promotive and inhibitory hormones. Specifically, the levels of abscisic acid (ABA), gibberellic acid (GA), indole-acetic acid (IAA) and cytokinins have been implicated in the explanation of varying depths of dormancy and degrees of germination (Khan and Waters, 1969; Khan, 1977). However, evidence that is more

recent also supports the role of changes in tissue sensitivity to hormones as being important in modulating dormancy and germination (Rock and Quatrano, 1995).

The requirement of exogenous GA for germination in the gibberellic acid deficient mutant (*ga1*) of *Arabidopsis* conclusively establishes a role for the hormone in seed development. Furthermore, the suppression of exogenously applied GA in the *ga1/aba1* double mutant supports the view that a balance of ABA and GA rather than absolute levels of either regulate seed development in *Arabidopsis* (McCarty, 1995). ABA, an antagonist of gibberellin action in many plant systems, is inhibitory to the synthesis of alpha-amylase and other hydrolytic enzymes essential for the breakdown of storage reserves, such as aleurone, in seeds. In this way, the developmental effects of GA can be held in check until insensitivity to ABA develops late in maturation, hormone ratios shift or dormancy is induced (Black, 1991).

It has often been observed that the exogenous application of GA correlates with the termination of developmental arrest (Foley, 1996). The specific mode of action that gibberellins have on the promotion of germination is largely unknown. Baskin and Baskin (1971) proposed that GA , in *Ruellia humilis*, acts to increase the growth potential and expansive force of the embryo thereby allowing it to overcome the mechanical restriction imposed by the seed coat. Watkins and Cantlife (1983) demonstrated that exogenously applied gibberellins reduced endosperm resistance to radicle emergence in *Capsicum annuum.* Gibberellins, they speculated, regulate seed

germination by direct control of radicle elongation and/or the enzymatic degradation of the endosperm. It has in fact been shown that GA stimulates a number of hydrolases needed to mobilize storage reserves from the endosperm to the growing embryo in some seeds (Jones, 2000). The activity of the hydrolases results in cell wall loosening, thereby, weakening the seed coat sufficiently to allow germination to proceed (Chen and Thimann, 1964). While GA will not break coat-imposed dormancy it will enhance germination potential of scarified seed.

There is research evidence that demonstrates that there is a rise in GA accumulation when pre-chilled seed of certain species is subsequently exposed to warmer temperatures (Bewley and Black, 1994). The stimulation of GA biosynthesis is known to increase radicle thrust and hydrolases that degrade the endosperm. This may permit the radicle to penetrate the hard seed coat (Juntilla, 1973). Alternatively, physiological changes that occur during chilling may reduce sensitivity to GA-antagonist ABA or shift the ABA:GA ratio such that germination may proceed. Furthermore, at certain low temperatures a phase change of cell membranes occurs that can affect seed permeability. The temperature in which the phase change occurs varies by species but is often in the range of 15/20°C; whereas the optimal temperature for pre-chilling is generally around 5°C. Gibberellins play an important role in mediating the effects that environmental stimuli, such as photoperiod and temperature, can have on seed development. Exogenous applications of GA have been successfully used to overcome the dormancy-breaking light, chilling, and after-ripening requirements of some seeds (Taiz and Zeiger, 1998;

Walck, *et. al*, 1999). Photoperiod is known to be one of the most important factors regulating gibberellin metabolism, as is temperature in regulating the biosynthesis of GA. It has been shown that these environmental factors alter the levels of active GA by altering specific steps in the biosynthetic pathway (Taiz and Zeiger, 1998), thereby shifting hormone ratios in favor of GA. It is thought that by enhancing cell elongation, the ability for radicle protrusion is increased (Bewley and Black, 1994).

In species where pre-chilling breaks dormancy, a notable increase in the amount of growth promoting hormones, especially the gibberellins, have been identified (Khan, 1977). Of the more than 110 known gibberellins (Taiz and Zeiger, 1998), GA_3, GA_4, and GA_7 are the most commonly used for stimulating seed germination (Bewley and Black, 1994).

3. Other exogenously applied chemicals

A wide range of chemicals have been applied to seeds in an effort to break dormancy or accelerate germination: hydrogen peroxide, ethyl alcohol, sodium hypochlorite, thiourea, cyanide, gibberellins, cytokinin, and ethylene are some examples. The method of chemical application is called *pre-applied* if the seed is exposed to the chemical treatment prior to the germination test and called *co-applied* if the chemical treatment is applied to the substratum used during the germination test period (Ellis, *et. al*, 1985a). Like GA, none of the chemicals mentioned here have the ability to break coat-imposed dormancy; however, they may act to increase the germination rate and potential.

a. Potassium nitrate (KNO_3). Certain nitrogenous compounds (nitrate, nitrite, thiourea) have long been known to break dormancy and stimulate germination in certain species (Bewley and Black, 1982). There is circumstantial evidence that the sensitivity and biosynthesis of GA is incited by the combined action of light and nitrate in some seeds (Bewley and Black, 1994). A synergistic effect appears to occur when potassium nitrate is used in conjunction with gibberellic acid (Young and Young, 1986). AOSA (1993) recommends using KNO_3 as a substitute for illumination (co-applied) in light-requiring seeds. Although conclusive evidence does not exist to support this, it has been proposed that KNO_3 is physiologically active at the molecular level, perhaps by activating the oxidative pentose pathway, thereby increasing oxygen diffusion (Roberts and Smith, 1977). Further, the acidification of intracellular pH, incurred by KNO_3, may also act to decrease ABA levels permitting germination to proceed (Footitt and Cohn, 2001).

b. Hydrogen peroxide (H_2O_2). Hydrogen peroxide is an active oxygen source that can diffuse into plant tissues. In relation to seeds it can be used as a disinfectant or as a stimulant to seed germination. Its specific mode of action is not fully understood but it has been hypothesized that hydrogen peroxide acts to promote germination by (1) lowering the pH (see citric acid) or (2) increase the oxygenating environment immediately surrounding the seed (Mayer and Poljakoff-Mayber, 1989). Hydrogen peroxide has been classified as a compound that may have effects on ABA degradation (Wang, *et. al*, 1998). Neill and Horgan

(1987) concluded that increased oxygen levels, as caused by the addition of H_2O_2, accelerated the degradation of ABA. Germination of lettuce seeds was improved from 79 % to 96 % when the oxygen content of the germinating environment was increased from 15 % to 20 % (Harel and Mayer, 1963).

c. Citric acid ($C_6H_8O_7$). Weak acids, such as citric, have been shown to terminate seed dormancy, in some species, in a pH-dependent manner (Footitt and Cohn, 2001). Studies using Jeruselum artichoke tuber buds (Genraud and Lafleuriel, 1983), red rice embryos (Footitt and Cohn, 1992) and barley aleurone cells (Van Beckum, *et. al*, 1993) have shown that intracellular pH is higher in dormant than in non-dormant tissues. Increased cellular pH and the expression of dormancy-associated genes are induced by the germination protagonist ABA (Gehring, *et. al*, 1990). In contrast, certain weak acids lower cytosolic pH and inhibit ABA-induced gene expression (Van der Veen, et al., 1992). Current research points to this inverse relationship when suggesting the possible role of cellular pH in the induction/maintenance of seed dormancy. It is therefore possible that citric acid enhances germination by modulating cellular pH which, in turn, effects developmental signals (Footitt and Cohn, 2001).

4. Hot water

Hot to boiling water can effectively promote seed germination in several ways: (1) leach chemical inhibitors found in the seed coat, (2) soften the hard seed coat reducing

resistance to radicle protrusion, (3) promoting imbibition by activating natural sites of water entry (e.g., chalaza, hilum, lens) or (4) disrupt the palisade layer causing separation from the underlying mesophyll and thus promoting water uptake in non-localized areas of the seed coat (Baskin and Baskin, 1998). In hardseeded malvaceous species hot water acts to create pressure changes, which are thought to break cell walls at weak sites rupturing the chalazal cap, thereby allowing water to imbibe freely (Rolston, 1978; Egley, 1989). Seed permeability of velvetleaf (*Abutilon theophrasti)* was increased to 87 % when submerged in hot water (70°C / 60 sec.) and 88 % (85°C / 30 sec.) when compared to the control (30 %) (Le Croix & Staniforth, 1964). In another study, hard seed of velvetleaf was reduced from 99 % to 15 % after 1 hour in hot water (Young and Young, 1986). Hot water treatment is commonly recommended for many species of cotton (*Gossypium* sp.), a hardseeded member of the Malvaceae family (Ellis, *et. al*, 1985b). Experiments by Christiansen and Moore (1959) found an exposure time of 1 minute at 80°C to be optimum for *Gossypium hirsutum.*

Submerging hardseed in hot or boiling water can be a highly effective but erratic means of promoting germination. Due to variation in seeds, from year to year and population to population, it is difficult to standardize this treatment (Cavanagh, 1987). Hot water treatment can also be disadvantageous in that it tends to promote greater fungal invasion during germination (AOSA, 1993).

5. After-ripening

After-ripening is the gradual process by which seeds lose their dormancy in the dry (unimbibed) state (Ellis, *et. al*, 1985a). The period required is dependent upon the temperature, moisture content of the seed and oxygen levels surrounding the seed (Richards and Beardsell, 1987). Certainly, not all seeds require after-ripening; however, those that do may require it for a variety of reasons. During storage seeds may undergo embryo maturation, drying, chemical or physical changes (Mayer and Poljakoff-Mayber, 1989). Research with *Kosteletzyka virginica* (Malvaceae) demonstrated that the seed coat became more permeable with extended time of storage. Imbibition and germination were also accelerated with time (Poljakoff-Mayber, *et. al*, 1992). In *Sida spinosa*, a relative of *Callirhoe*, dry storage for 4 months or longer at room temperature was found to break the dormancy of most of the seed tested (Egley, 1976). Imbibition in *Malva parviflora* was improved from <2 % (fresh seed) to 26.3 % (one year-old seed) (Sumner & Cobb, 1967). Dormancy is also reported to be lost in cultivated cotton (*Gossypium* spp.) seed when after-ripened for 1 - 24 months (Ellis, *et. al*, 1985b).

The specific means by which after-ripening releases hard seed from dormancy is still largely unknown. Consistent with the hormonal balance theory, Naylor and Simpson (1961) proposed that after-ripening was controlled through changes in the content of an endogenous inhibitor [now known to be ABA] that antagonizes GA, a germination promoter. Data that substantiates the physical changes of the testa, occurring during extended storage, was presented in research by Vertucci and Leopold (1986). Their work

suggested that polymer swelling, hence structural alteration, might occur in the testa of some seeds at very low hydration levels (8 – 10 %).

In order to determine whether a species has after-ripening requirements, repeated germination tests occurring at regular intervals must be imposed starting with early maturity and continuing for at least 6 months (Young and Young, 1986).

6. Acid scarification

Acid scarification is a method widely used on seeds that develop an impermeable seed coat. Exposure to concentrated sulfuric acid increases permeability through corrosive damage to the cuticle, osteosclerids, hilum and strophile in *Asplanthus linearis* (Kelly and Van Staden, 1987; Kelly, *et. al,* 1985); to the macrosclerid caps in *Coronilla varia* (Brant, *et. al,* 1971) and the strophiole in *Astragalus cicer* (Miklas, *et. al,* 1987). Acid scarification remains a popular method of pretreating hard seed for commercial production. It is, however, a process that should be carefully monitored as prolonged exposure or excessive concentrations can cause damage to embryonic parts resulting in low seed vigor and consequently reduced production or mortality. In *Abutilon theophrasti* (Malvaceae), the duration of treatment for maximum germination is 15 minutes (Steinbauer and Grigsby, 1959); in *Hibiscus trinonum* (Malvaceae) it is 20 minutes (Everson, 1949).

Sulfuric acid produces a lot of heat, driving moisture from the seed, during the scarification process. In addition to general moisture loss the dehydration capacity of sulfuric acid can increase the water potential at damaged sites of the testa creating rapid, uneven imbibition and subsequent embryo damage (Kelly, *et. al,* 1985). The process, called imbibitional injury, can occur naturally in very dry seeds (Ellis, *et. al*, 1985a). The effectiveness of using acid can vary depending on seed coat thickness and composition, exposure time and concentration, skill of the handler, thoroughness of post-washing, moisture content of seed and temperature.

7. Mechanical scarification

Mechanical scarification is the most commonly used method for commercial treatment of hard seed (Rolston, 1978). Mechanical scarification can be accomplished by abrading hard seed against a rough surface to reduce testal wall thickness or cause cellular separation or breakage. The goal is to initiate seed coat permeability, which can also be induced by the piercing or removal of a portion of the seed coat. Studies using *Callirhoe triangulata* showed that scarification (using an emory board) resulted in 90 % germination as compared to 30 % in the nonscarified control (Voight, 1977).

Scarification machines make hard seed coats permeable by blowing or rolling seeds against an abrasive surface such as sandpaper. A drawback to this method is the elevated potential for injury to the embryo; the result of which can be reduced vigor, viability or

even death (Hamly, 1932). Scarification machines are most effective with thin-coated hard seed and less effective with thick-coated hard seed. It is also ineffective for seed produced with variable testa thickness (Baskin and Baskin, 1998).

8. Leaching

Leaching, also called pre-washing, can be used effectively on some species to remove germination inhibitors or reduce mechanical resistance by softening the seed coat or surrounding fruit structures. The water supply must contain enough dissolved oxygen to support imbibing seeds. Very dry seeds are not good candidates for this type of pre-treatment as they may succumb to imbibitional injury (Ellis, *et. al*, 1985a). Solvents such as ethanol or acetic acid may be used when it is suspected that the inhibitor(s) are not water-soluble.

9. Viability

The tetrazolium test is used to estimate seed viability. When used in conjunction with a germination test it may also be used to determine percentage of dormant seed. The tetrazolium test enables the investigator to discriminate between viable and non-viable hydrated tissues of the embryo based on their relative respiration rate. Dehydrogenase enzymes released during respiration reduce the colorless tetrazolium salt solution changing it into an insoluble red pigment, formazan. The red color of formazan acts as a visible indicator of actively respiring tissue by dying those tissues associated with it.

Viability is determined by the extent and location of stained embryo tissue. The most critical areas of an embryo are those where cell division occurs as they represent the embryos potential to germinate. If the areas of cell division are unstained or abnormally stained, the analyst can predict that the seed's germination potential is weakened (Copeland and McDonald, 1995).

10. Relationship between pre-treatment and seedling vigor

Good laboratory performance does not always translate to good field performance. Conditions found outside a laboratory, whether in a production field or greenhouse setting, are often sub-optimal. Therefore, it is important to develop test situations that provide a realistic gauge of seedling performance when exposed to unfavorable conditions. Vigor testing was developed in an effort to use seed quality as a predictive measure of field performance.

D. Propagation by asexual methods

Asexual propagation of *Callirhoe involucrata* has not been well documented although Ryan (1998) states without reference, "3 - 4 inch stem cuttings taken in early summer are said to root quickly". *Callirhoe* has also been multiplied using root sections that include a bud eye or node (Phillips, 1985; Ryan, 1998).

Chapter III:

MATERIALS & METHODS

A. Seed[z] acquisition

The bulk seed collections used were obtained from 3 different sources representing 3 populations and 2 consecutive collection years; one from east central Nebraska (1997), northeast Kansas (1997/1998), and the southeast region of Missouri (1997/1998). Seeds were collected from mid- to late summer prior to fruit shattering. *Callirhoe involucrata* seeds were harvested with fruit intact; one seed per mericarp. Once received, collections were removed of debris using an air density-separator and then stored in manila envelopes under laboratory conditions (20/22°C, 6 - 8 % moisture). One 150 g sample of each lot was stored in a walk-in cooler (-18°C) within 2 months of being received to preserve the integrity for a study on the effect of seed aging. Collections used in this research are referred to as 'lots' and have been assigned an identification code for ease of reference (Table 3.1).

[z] *The word 'seed' presumes the presence of a fruit coat unless otherwise stated.*

Table 3.1. Seed source, lot identification code, collection year and origin for *Callirhoe involucrata* v. *involucrata* seed used in research.

Lot i.d.	Origin	Collection year
(W97)	Kansas, Riley County	1997
(W98)	Kansas, Riley County	1998
(A98)	Nebraska, Grand Island	1998
(H97)	Missouri, Oregon County	1997
(H98)	Missouri, Oregon County	1998

B. Experimental procedures and statistics

Standard germination tests used 200 seeds (4 replications of 50) per lot in accordance with Association of Official Seed Analysts (AOSA) Rules for Testing Seeds (1993). The majority of other tests used 100 seeds (4 replications of 25) per lot. With few exceptions, tests were incubated in a 20/30°C walk-in germination chamber (16 hours of dark at 20°C and 8 hours of illumination at 30°C). All tests were accomplished using a completely randomized design (CRD) and were conducted for 28 + 5 days with observations taken every 7 days. At the conclusion of each experiment, ungerminated seeds were tested for viability using TTC testing methods (see C). All tests were replicated a minimum of 2 times with the exception of preliminary tests which were run only once. Research was conducted at the U.S.D.A.- A.R.S.- National Seed Storage Laboratory (NSSL) in Fort Collins, Colorado.

Experimental data was analyzed using the general linear models (GLM) procedure of Statistical Analysis System software (SAS Inst. Inc., 1988). Analysis of variance (ANOVA) was performed where F-values were significant at .05 and means were compared using Duncan's Multiple Range test.

C. Viability testing

Viability was determined using 2,3,5-triphenyl-tetrazolium chloride (TTC) testing methods (ISTA, 1985b). One hundred randomly selected seeds from each lot were removed from their mericarps, chipped and then hydrated on sterile blotter paper moistened with distilled water (dH_2O) until fully imbibed. Embryos were then carefully removed from their covering structures: the testa, perisperm and endosperm. Embryos that incurred tissue damage during the extraction process were discarded. Intact embryos were placed in a lightproof vial containing a 1.0 % buffered TTC solution for 6 - 8 hours at room temperature until uniform staining had been achieved. Viability determination was based on embryo color and staining pattern using criteria described in the Handbook on Tetrazolium Testing (Table 3.2)(ISTAb, 1985).

Table 3.2. Classification of ungerminated seed remaining at the termination of a testing period.

Hard	Seed that has not imbibed water (remains firm) after the prescribed test period.
Empty	Fruit devoid of seed.
Unfilled	Partially filled seed or a developed testa devoid of an embryo.
Aborted	Highly rudimentary seed.
Dead	Embryo is mushy or flaccid, often gray or yellow. Does not stain during TTC.

D. Developmental germination test

Periodic germination testing was conducted to establish baseline and timeline germination percentages. While specific (AOSA) testing procedures have not been established for any species of *Callirhoe*, they have been established for other members of the Malvaceae family. It was from these tests that the determination for substratum, incubation temperature, test duration and procedures were initially derived. As a reflection of procedures used by commercial propagators, germination tests were conducted using mericarps (fruit with seed intact) in addition to tests using seed alone. Fifty seeds were placed in a clear plastic germination box (11 cm x 3 cm x 11 cm) on a moistened, sterile blotter and then covered with a clear lid. Blotters were moistened with distilled water (dH_2O) and re-moistened as needed. Each box of 50 seeds represented one repetition. Each seed lot utilized 4 repetitions for a total of 200 fruits per lot / per test.

Germination boxes were arranged on trays in germination carts, using a completely randomized design (CRD), and placed in a walk-in germination chamber programmed at 20°/ 30°C (16 hours of dark at 20°C and 8 hours of illumination at 30°C). Seeds were evaluated every 7 days for 28 days. The criteria for germination was radicle protrusion of >2 mm. Germinated seeds were classified (Table 3.3), recorded and removed from the germination box. At the end of 28 days ungerminated seeds were extracted from their fruit covering (carpel) and hand scarified. Scarification was conducted under a light microscope with a razor blade. In this process a small section of the testa was removed in the hypocotyl region. Seeds were then given an additional 5 days after which final germination was recorded. Any seeds remaining ungerminated after the 28 + 5 days were analyzed for viability using the tetrazolium testing procedure. Variations conducted on some germination tests were as follows: 1) true seed used (manually extracted from the fruit prior to testing) and 2) additional time allotted for germination following the scarification procedure, e.g. test was run for 28 +14 days. The standard germination test was replicated 5 times for each seed lot.

Table 3.3. Seedling classification according to AOSA criteria

Normal	Containing an embryo that has " those structures which are indicative of the ability to produce a normal plant under favorable conditions"
Abnormal	"Anything not classified as normal"

Germination percentage was calculated by dividing the total number of seeds germinated by the total number of seeds tested minus any hard, empty or dead seeds (multiplied by 100). Hard seed percentage was calculated by dividing the total number of hard seeds by the total number of seeds tested minus any dead or empty seeds (multiplied by 100).

E. Measuring seed moisture content

Seed moisture was determined by measuring the loss in weight through drying. First, the fresh weight was measured using an analytical scale; seeds were then placed in an oven set at 103°C (220°F). After 24 hours the seeds were removed and re-weighed. Percent moisture content was calculated by subtracting dry weight from fresh weight and multiplying by 100. 'True' seeds (not including fruit) were used. Tests were repeated every 6 months for 18 months. Control seed was stored in a walk-in refrigerator set at -18°C. Each lot was represented by three repetitions of 50 seeds.

F. Seed processing

The removal of seed from fruit was accomplished with a slightly textured rubber-lined rubbing board. Using this method nearly 500 seeds could be extracted from their fruit in 15 minutes. A seed blower or graded screens were then used to separate seeds from chaff and debris.

G. Prechill

Treatments testing for the effect of pre-chilling on germination were as follows:

a) pre-chilling 30d
b) pre-chilling 45d
c) pre-chilling 70d
d) hand scarification + pre-chilling 30d
e) hand scarification + pre-chilling 45d
f) hand scarification + pre-chilling 70d
g) hand scarification + pre-chilling 120d

Four replications of 25 seeds per lot / per treatment were placed into plastic mesh bags and moved to germination boxes filled with moistened (dH_2O), sterile vermiculite. Boxes were then placed in a refrigerator (5°C) for the prescribed duration of time. After pre-chilling, the seeds were removed from the mesh bags and placed on clean, moistened (dH_2O) blotters, in germination boxes, and moved to the 20/30°C germination chamber for the duration of the test. Hand scarification was conducted with a razor blade (see section L). The control remained unchilled.

H. Gibberellic acid and other exogenously applied chemicals

Treatments testing for the effect of gibberellic acid (GA_3) on germination were as follows:

a) hand scarification + GA_3 (400 ppm)
b) hand scarification + GA_3 (500 ppm)
c) hand scarification + GA_3 (1000 ppm)
d) hand scarification + GA_3 (500 ppm) + 30d pre-chill

Scarified seeds were placed on blotter paper, in germination boxes, moistened with either 400, 500 or 1000 ppm GA_3 and allowed to imbibe for 24 hours at room temperature. Boxes were covered with aluminum foil to prevent GA_3 degradation due to light exposure. The unchilled treatments were moved to the 20/30°C germination chamber for the duration of the testing period. Seed used for chilled treatments were placed into plastic mesh bags and moved to germination boxes filled with moistened (dH_2O), sterile vermiculite. Boxes were then placed in a refrigerator (5°C) for 30 days. Only one lot (W97) was used to test the effect of GA (500 ppm) and pre-chilling (30 days) on germination. After pre-chilling, the seeds were removed from the mesh bags and placed on clean, moistened (dH_2O) blotters, in germination boxes, and moved to the 20/30°C germination chamber for the duration of the test. Controls were a) scarified and b) scarified with 30-day pre-chill (5°C), respectively. The GA_3 solutions were prepared by dissolving GA_3 (potassium salt form) in 1000 ml dH_2O, as follows: 0.4 g (400 ppm), 0.5 g (500 ppm) and 1.0 g (1000 ppm).

A 0.2 % potassium nitrate (KNO_3) solution was prepared by dissolving 0.2 g of KNO_3 in 1000 ml of distilled water. Scarified seeds were placed on KNO_3 moistened blotters and allowed to imbibe for 24 hours at room temperature (20°C). Imbibed seeds were then placed on clean, moistened (dH_2O) blotters, in germination boxes, and moved to a 20/30°C germination chamber for the duration of the test. The control used untreated, scarified seed. The same testing procedures were used with citric acid ($C_6H_8O_7$) as for

potassium nitrate. A 0.1 % solution was prepared by dissolving 0.1 g of citric acid with 1000 ml of distilled water. The control used untreated, scarified seed.

I. Hot Water

Hot water soaks for varying times, as follows, were evaluated for their influence on germination. Treatments:

a) hot water (100°C) for 1.5 hour
b) hot water (100°C) for 4.0 hour
c) hot water (100°C) for 24.0 hour
d) hot water (80°C) for 4.0 hour

Seeds, 100 per lot, were placed in individual 500 ml beakers filled with hot water. The water was allowed to cool while the seeds continued to soak for the prescribed duration of time. Once cooled to room temperature the water was periodically stirred to promote aeration. Treatments (a-d) were replicated a minimum of two times each. Treatments were immersed in the water either: (1) in plastic mesh bags or (2) without mesh bags and (3) in fruit or (4) as 'true' seed (extracted from fruit). The control was set up as a standard germination test using either fruit or 'true' seed.

J. After-ripening

After-ripening was evaluated through a series of germination tests taken at 3-month intervals for 15 months. Seed was stored at room temperature in paper sacks. A control sample of each lot was kept in cold storage (-18 C) and tested at the start and finish of the evaluation period.

K. Acid scarification

Seeds were treated at varying lengths of time in concentrated sulfuric acid as follow: 2 min., 5 min., 15 min., 30 min., 60 min., 120 min., control, and an interrupted treatment (60 min-rinse-rub-dry-60 min-rinse)

One hundred seeds (fruit) of each lot were placed in separate vials of concentrated H_2SO_4 (enough to cover twice the volume of seed). The sulfuric acid was stirred periodically to insure even coverage. After the predetermined period of exposure time was completed, seeds were placed in a metal strainer and rinsed under tap water for 5 minutes. Once rinsed thoroughly, seeds were allowed to dry on a paper towel before placing them in germination boxes on moistened blotter paper. Incubation took place in the 20/30°C germinator. The interrupted treatment used the same basic procedure with a few exceptions. In this treatment, following the initial rinse, the seed was rubbed against the mesh strainer until the fruit covering was removed. Seeds were then allowed to dry

completely. They were returned to the sulfuric acid for an additional 60 minutes followed by a thorough rinse and incubation.

L. Mechanical scarification

Mechanical scarification treatments: hand (razor blade, needle), sandpaper (30, 40 grit) or air pressure scarifier. Air scarification treatments were applied as follows:

P.S.I.	Minutes	Grit
20	4, 5, 8	40
25	3, 4, 5, 6, 7, 8	30
25	3, 4, 5	40
30	3, 4, 5	30
30	3, 4, 5	40

One hundred fruits from each lot were placed between two sheets of 40-grit sandpaper and rubbed for 1 minute. Fruit was also placed in a pressurized air scarifier lined with sandpaper and run at various pressure rates (p.s.i.) and exposure times.

Hand chipping seed utilized a light microscope and either a razor to chip off a small portion of the testa (hypocotyl region) or a needle to pierce it. Fifty fruits per lot were used in each treatment. The sandpaper and air pressure scarifier were only used in preliminary tests due to unpromising results. Fifty fruits per lot per treatment were used.

M. Leaching

Leaching treatments were applied to fruits as follows: 18°C/24h, 18°C/48h and 40°C/24h. One hundred fruits of each lot were placed in individual plastic mesh bags and then sealed. Bags were put into separate beakers filled with water. Water was aerated and maintained at a specific temperature by dripping temperature-regulated water for the predetermined period of time. Fruits were then removed and placed on moistened (dH_20) blotters and incubated.

N. Cold exposure

Twenty-five fruits from each lot were placed in separate paper envelopes within the storage container of a portable cryotank. The container was situated such that the seed was above the liquid nitrogen (-160°C), in the vapor, and not directly submerged. After 24 hours, the standard exposure time (Stanwood, 1984), the seed was removed and tested for viability using TZ testing methods.

Bulk quantities of *Callirhoe* seed, contained within brown paper bags, were placed in a cold vault (-18°C) in November of 1999 and removed in November of 2000. Moisture tests were taken for each seed lot prior to exposure. One hundred fruits from each lot were run through a standard germination test prior to and following cold exposure. Seed

remaining at the end of the test period were examined for viability using TZ testing methods. The control was seed stored at room temperature for one year.

O. Asexual propagation

Ten plants representing lot A98 and ten representing lot W97 served as the source of cutting material. These plants were grown from seed in the greenhouse. Between one and four cuttings were taken per plant for a total of 30 cuttings per lot in trial one. Cuttings were taken in mid-April from non-flowering, new growth on one year-old plants. Stem sections were 2 - 4" in length with at least two nodes per cutting. The detachment cut was made roughly ¼" below the proximal node. Stem cuttings were dipped, to the depth of the first node, in 0.1 % IBA and inserted into individual 2 ¼" plastic pots containing 30:70 (peat to perlite) media. Trays with cuttings were placed on a mist bench until rooting began. Mist controls were set to activate every 6 minutes for 7 seconds from dawn to dusk and were manually adjusted to accommodate weather fluctuations. Cuttings with 10 or more rootlets were removed from the mist and transferred to pots containing Fafard® #2 growing media. A dilute concentration of ZeroTol® (1:50) was poured over the rooting media at the first sign of tissue deterioration. Trials two and three followed the same methods but used 15 cuttings per plant. Trials were started at five-week intervals and rooting data was collected every seven days for six weeks.

Chapter IV

RESULTS AND DISCUSSION

Preliminary testing established that the primary cause of low germination in *Callirhoe involucrata* was due to the existence of a hard seed coat. Evidence came from hand-scarified seed that produced a dramatic increase in mean germination (98 %) over the nonscarified control (35 %) (Figures 4.1.a – 4.1.c).

Figure 4.1.a. Comparison of various treatments on seed germination of *Callirhoe involucrata* (W97).

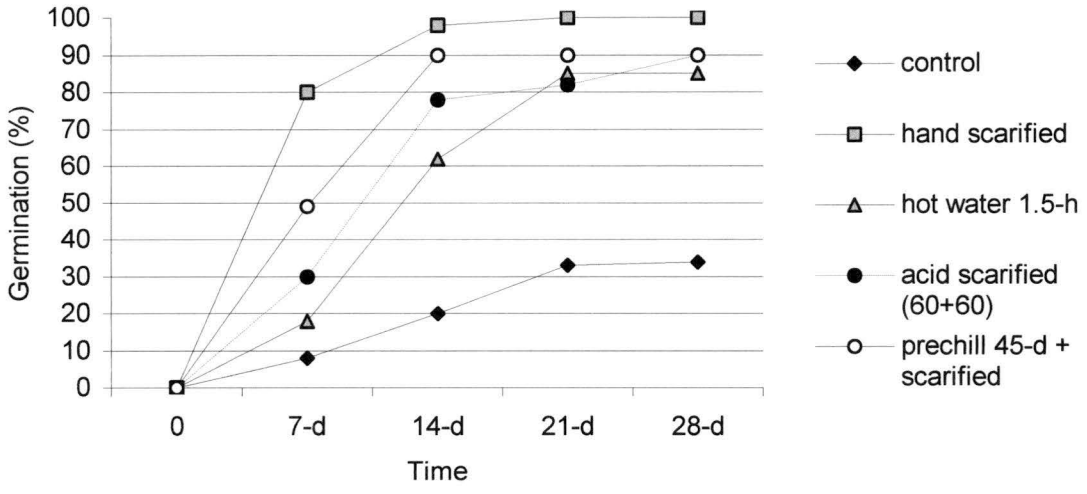


Figure 4.1.b. Comparison of various treatments on seed germination of *Callirhoe involucrata* (A98).

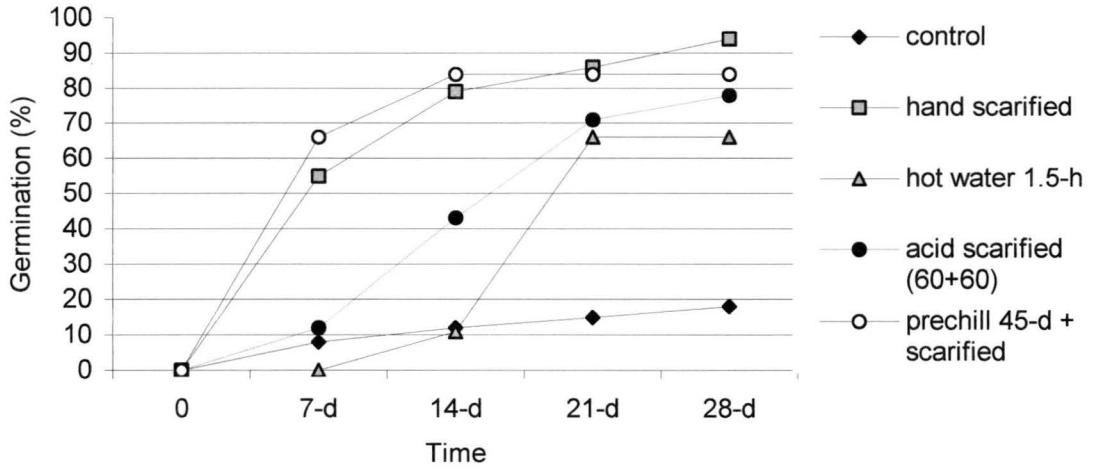
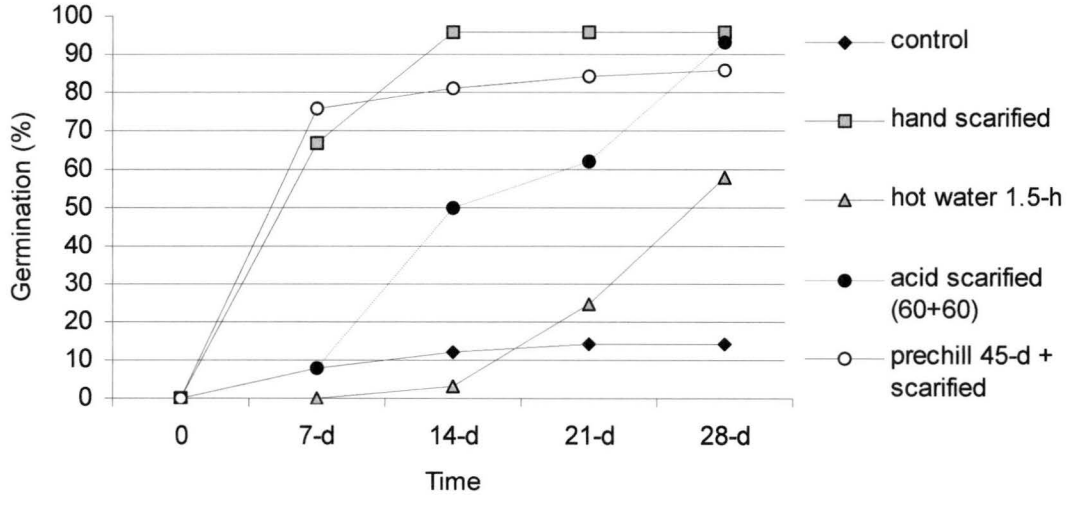
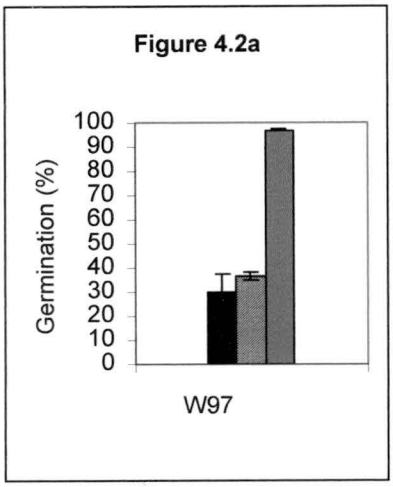


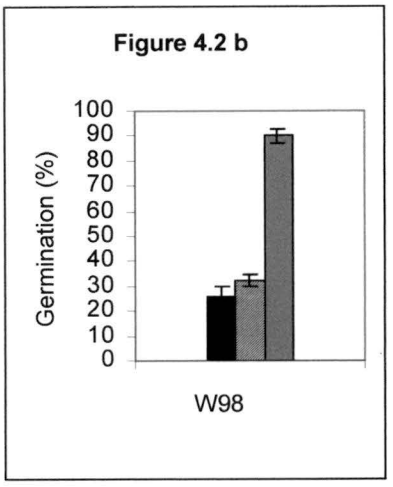
Figure 4.1.c. Comparison of various treatments on seed germination of *Callirhoe involucrata* (H98).

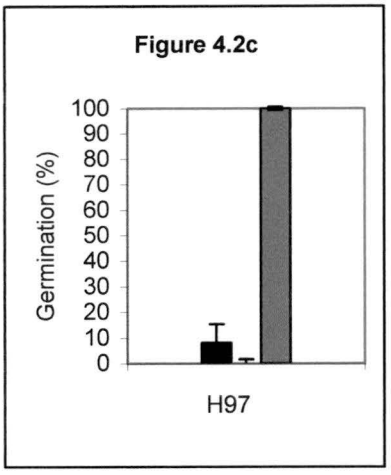


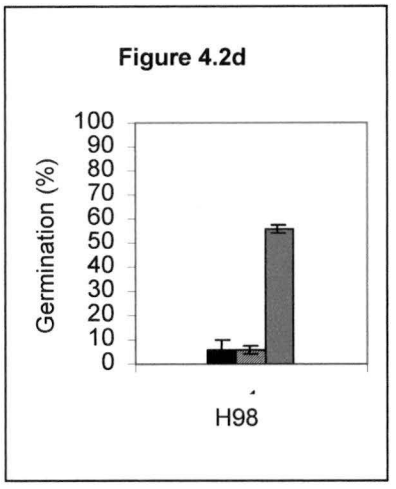
The influence of the fruit coat, a potential source of chemical inhibitors and mechanical restraint, was not significant, as determined by a series of germination tests comparing fruit, unscarified seed without fruit and scarified seed (Figures 4.2.a – 4.2.e). These tests further supported hard seed as the basis for limited germination.

The physical similarity of *Callirhoe involucrata* seed to seed of other members of the Malvaceae family suggests that the chalazal region is the critical zone for water uptake. Observations made during testing supported this; it was consistently noted that hard, unimbibed seed had a closed chalazal slit while imbibed seed developed a blister at the site of the chalaza and a clear separation of testal layers, data not shown.









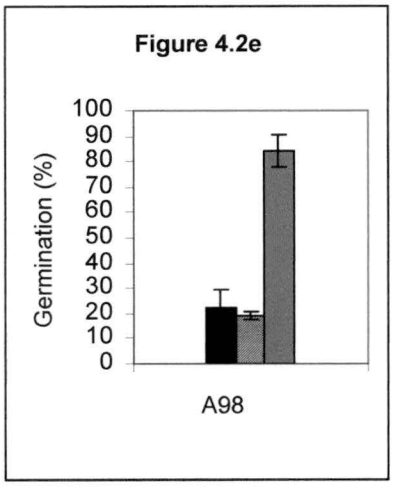


Figure 4.2 (a-e). Relative germination of 5 seed lots of *Callirhoe involucrata* that represent 3 collection sites (W, H, A) and 2 collection years (1997 & 1998).

Fruit ■ Unscarified seed ▨ Scarified seed ▩

Storage and after-ripening

Linear regression analyses demonstrated no differences in germination of dry stored seed that was tested at regular intervals over a 15-month period (Figure 4.3). R^2-values were .4843 (W97), .7557 (H98) and .6466 (A98).

In April of 1999, standard germination tests (for all lots) of nonscarified seed revealed a combined mean of 19.3 % versus 25.3 % in June of 2000. Individually, lot A98 started and ended with the same germination percentage (24 %) while lot W97 improved minimally, 6 %, for a high of 34 % after 15 months of storage. Seed lot H98 tripled its germination from 6 to 18 %; yet the improvement was not statistically significant. This data suggests that dry storage produced a negligible increase in testa permeability, in contradiction to what was anticipated. *Callirhoe involucrata* seed may require more storage time for the affects of after-ripening to be significant. It is also possible that seed of *Callirhoe involucrata* has no after-ripening requirement. However, the slight but linear increases in germinability seen in this storage experiment (Figure 4.3) suggests that these tests should be repeated using newly matured seed observed over a period longer than 15 months.

Exposure tests subjected seed of *Callirhoe involucrata* to LN_2 vapor (-160°C for 24h) and conventional cold storage (-18°C for 1 year). After freezing, samples were tested for viability using standard germination testing procedures. Germination of LN_2-exposed seeds was comparable to the untreated control (Table 4.1). These results established that the LN_2 method of storage could be used for seed of *Callirhoe involucrata.*

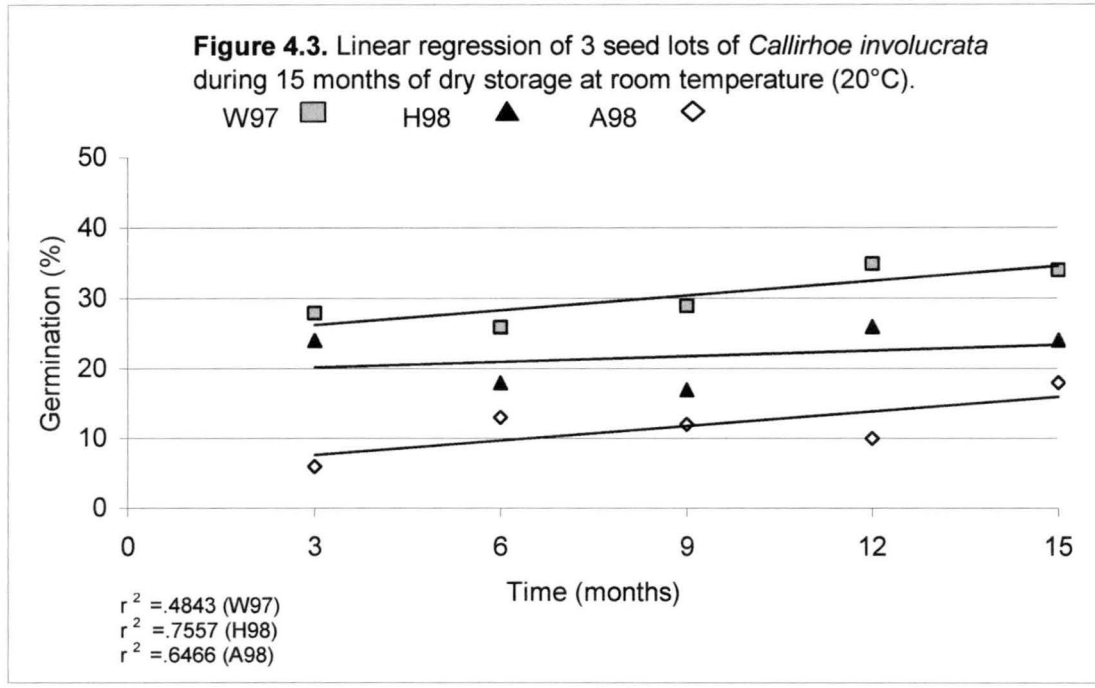


Figure 4.3. Linear regression of 3 seed lots of *Callirhoe involucrata* during 15 months of dry storage at room temperature (20°C).

The conventional (-18°C) method of cold storage did not diminish the germinability of seed tested. A98 seeds subjected to -18°C produced germination percentages above that of the control (24% v. 16% control). W97 resulted in germination percentages of 28% and 34% and H98 produced 20% and 23% when compared to the room temperature (control) and cold-stored seed, respectively (Table 4.1).

These results were expected since the association between plant ecology and seed storage behavior has been established through research (Roberts and King, 1980). In accordance with this concept, seeds that originate from places that experience seasonal droughts and freezing temperatures store well in dry, cold environments and generally fall into the seed storage category of orthodox. The seeds used in this research were collected in regions of

the United States that experience subfreezing winter low temperatures and seasonal dry periods.

Table 4.1. Percent germination of unscarified fruit from three lots of *Callirhoe involucrata* subjected to LN_2 vapor (-160°C for 24h) and conventional cold storage (–18°C for 1 year).

	Germination (%)		
Lot	Control [z]	After LN_2 [y]	After cold storage [z]
W97	28	32	34
H98	20	20	23
A98	16	12	24

[z] *Based on 100 seeds per lot.*
[y] *Based on 25 seeds per lot.*

Viability

Viability for 'true' seed was high in two lots (W97, H98), both with a mean of 97 %. The combined (adjusted) mean for all lots combined was 90 %; the unadjusted mean for all lots was 13 % (Table 4.2). Twenty-six percent of an average sample of *fruit* was either empty or harbored aborted or unfilled (embryoless) seed envelopes (Table 4.2). Seed lot A98 averaged the greatest percentage of seedless fruit, 28 %, reaching as high as 55 % in a single 100-seed sample. That a large percentage of fruit does not contain seed is significant because it may explain, in part, the low germination capacity reported by nursery growers.

Differences in the quantity of filled seed, between the lots, may be a reflection of the varied quality of seed cleaning each received. The specific reason for low rates of seed production is unknown; fruit position on the raceme, indeterminate blooming, low pollinator populations or efficiency, environmental stress and harvesting methods could all contribute to the problem (Richards, 1997). Further study examining this phenomenon could lead to a better understanding of the reproductive biology of *Callirhoe involucrata* as well as other members of the Malvaceae family with which this characteristic is shared.

Table 4.2. Comparison of seed quality characteristics of 3 lots of unscarified *Callirhoe involucrata* seed. Germination percentages [z] reflect ± error of the means.

Lot	Normal Seedlings	Hard	Unfilled [y]	Dead
W97	21±8	49±6	26±5	3±3
H98	9±5	61±4	25±3	3±1
A98	10±9	37±3	28±9	23±6

[z] *Values are not adjusted.*
[y] *Includes empty, poorly filled & aborted seed.*

Rubbing board and seed blowing

On the practical side, skilled use of a seed blower proved to be an effective means of removing fruit containing underdeveloped, empty or aborted seed. Precision seed blowing creates density-segregated fractions, whereby fruit containing germinable seed can be separated from fruit that is either empty or has lighter non-germinable seed.

Removal of the fruit coat using a slightly textured (rubber-lined) rubbing board improved seed blower accuracy. Trials showed that in 15 minutes 500 seeds could be removed from their fruit coat and cleaned of debris using an electric seed blower and rubbing board. With practice this number could be increased. In the absence of a blower, graded mesh screens could be used. Use of these techniques in separating quality seed from trash benefits the grower by allowing them to more accurately predict the amount of seed required to meet production goals.

Pre-chilling

Despite the fact that "dormancy in the majority of temperate grassland forbs is physiological and is broken by stratification" (Baskin and Baskin, 1998), moist-chilling of scarified *Callirhoe* seed produced contrary results. Scarified seed exposed to chilling periods between 30 - 70 days produced germination rates that ranged from 50 - 86 % (Table 4.3). However, the scarified, unchilled control averaged 97 % germination (Table 4.3; Table 4.4). Chilling of scarified seed produced diminished germination when compared to unchilled scarified seed (Figures 4.1.a – 4.1.c) and is therefore, not recommended as a pre-treatment.

The primary function of moist pre-chilling or stratification is to satisfy a physiological need, hormonal shift, embryonic developmental period or other requirement, which is normally met in seed of temperate latitude forbs by winter conditions (Mayer and Poljakoff-Mayber, 1989; Fenner, 1985). In tests conducted, the maximum increase in

Table 4.3. Germination of scarified *Callirhoe involucrata* seed, for 3 lots, using various prechill (5°C) treatments. Time taken to reach 50 % germination also recorded.

Lot	Prechill time (d)	Germination (%)[z]	Time until G50[y] reached (d)
W97	0	98 *a*	< 7
	30	78 *c*	< 7
	45	80 *bc*	< 7
	70	80 *bc*	-[x]
H98	0	98 *a*	< 7
	30	80 *c*	14
	45	85 *bc*	14
	70	50 *d*	-
A98	0	94 *a*	< 7
	30	58 *c*	14
	45	86 *b*	14
	70	50 *d*	-

[z] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's Multiple Range test (P< 0.05).*
[y] *G50 is the amount of time, in days, it takes to reach 50 % germination*
[x] *(-) data was not collected.*

germination of chilled seed was achieved at 45 days (Table 4.3). Longer periods of stratification led to an increase in premature germination and a decrease in overall germination due to seed death. In preliminary tests seed coat permeability (as measured by germination) was not improved with chilling for unscarified samples (Table 4.4). Freshly collected seed should be studied before the cold requirements of *Callirhoe involucrata* can be conclusively determined.

Table 4.4. Comparison of percent germination[z] of scarified and unscarified seed of 3 lots of *Callirhoe involucrata*. Germination percentages reflect ± error of the means.

Lot	Unscarified (no prechill)	Unscarified + 30d prechill	Scarified (no prechill)
W97	30±6	20±2	98±2
H98	12±5	4±3	98±3
A98	22±9	12±3	94±2

[z] *Germination percentages based on filled seed.*

Mechanical scarification

The effect of mechanical scarification on the seed coat of *Callirhoe involucrata* was varied, ranging from slightly and well scarified to highly damaged. Individual fruit coats seemed to exert differential resistance to air blasting producing unpredictable results. Adjusting the p.s.i., exposure time and grit of sandpaper, in the air-scarifier, did not improve the outcome to a reasonable extent (Table 4.5). Similarly, abrading fruit between two pieces of sandpaper had equally poor results. This method produced a high mean percentage of seed damage (23 %) and low mean germination (15 %) (Table 4.6). Pressurized air scarification was not attempted on 'true' seed but is recommended for future testing. As previously mentioned the highest germination resulted from hand-scarified seed (98 % v. 35 % control). While suitable for a limited quantity of seed, hand scarification is an impractical method for large-scale production.

Table 4.5. Germination of *Callirhoe involucrata* seed following various pressurized air scarification treatments. All values for pressurized air treatments represent 5-minute exposure times (not all treatments tested are represented).

	Germination (%)[z]		
P.S.I. / sandpaper grit	**W97**	**H98**	**A98**
20 / 30	10	0	20
20 / 40	0	0	10
30 / 30	17	8	6
30 / 40	16	12	8
Control	35	26	10

[z] *Values are not adjusted for dead or empty seed.*

Table 4.6. Germination and damage percentages of 3 lots of *Callirhoe involucrata* seed following scarification using sandpaper [z].

	%	
	Germination[yx]	Damaged seed
W97	19	29
control	29	-[w]
H98	18	24
control	21	-
A98	9	15
control	17	-

[z] *40 grit.*
[y] *Based on 100 seeds per treatment.*
[x] *Values are not adjusted for dead or empty seed.*
[w] *(-) not applicable.*

Acid scarification

Acid scarification demonstrated the durability of the hard seed coat of the *Callirhoe* seed tested (Table 4.7). Some seeds remained hard after 120 minutes of continuous exposure to concentrated sulfuric acid. On the other hand, the same treatment caused considerable damage to other seeds from the same lot. Variation in seed coat thickness or structural integrity may account for the level of reaction. Testa thickness is largely attributed to maternal response toward environmental conditions during seed development (Mayer and Poljakoff-Mayber, 1989). Unfortunately, little is known about the specific climatic conditions of the maternal populations of the seed used for this research.

Preliminary acid scarification tests using treatment times of 2 minutes and 5 minutes proved ineffective at enhancing germination when compared to the control. Other treatment times (15 min., 30 min., 60 min, 120 min., and 60 min. + 60 min.) produced germination results that were improved over the control (Table 4.7). In lot H98 a trend of improvement in germination can be correlated with increased treatment time; 120-minutes of exposure produced the best response at 83 %. However, the double 60-minute exposure to sulfuric acid resulted in a statistically significant increase in germination percentage, over other treatments, in lot A98 but not in W97 or H98. In the double 60-minute exposure technique the first 60-minute treatment was used to degrade the fruit coat of the treated seed. The acid-exposed fruit was then rubbed against a screen while rinsing to remove remaining fruit coat particles. The second 60-minute treatment in acid scarified the testa of the 'true' seed.

Table 4.7. Comparison of percentage germination of *Callirhoe involucrata* as affected by various acid scarification treatment times.

Lot	Scarification time (min.)	Germination (%)[zy]	Number of seeds germinated
W97	0	23 *d*	18
	15	31 *cd*	26
	30	64 *b*	33
	60	40 *c*	35
	120	82 *a*	42
	60 + 60	90 *a*	66
H98	0	10 *d*	7
	15	12 *d*	8
	30	33 *b*	22
	60	24 *c*	19
	120	83 *a*	39
	60 + 60	75 *a*	60
A98	0	27 *d*	16
	15	62 *b*	36
	30	52 *c*	25
	60	52 *c*	34
	120	63 *b*	40
	60 + 60	78 *a*	40

[z] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's Multiple Range test (P< 0.05).*

[y] *Means represent adjusted values.*

Sulfuric acid is widely used for scarification purposes in the nursery industry. Generally, however, recommendations call for a single exposure to acid. The presence of the fruit coat renders the single acid exposure method less effectual. In support of this, the death rate of the double 60-minute treatment was considerably less than the single 120-minute treatment (data not shown). At termination, both tests had hard seeds remaining but the 120-minute treatment resulted in 1.5 to 16 times more dead and/or damaged seed than did the double 60-minute exposure.

Removal of the fruit coat, in the double treatment, allows for even exposure of seed surfaces to sulfuric acid thus facilitating the uniformity of the treatment. This may help explain why the double acid treatment worked better in some cases than the single 120-minute treatment in stimulating germination. However, if seed coat thickness varies within or between lots then the difference in the outcome of these two tests is more difficult to explain. The double-60 minute method of scarification, while successful, retains some undesirable characteristics. Acid scarification requires the use and disposal of a caustic substance, safety training and equipment, close monitoring and potential for seed damage.

Gibberellic acid and other exogenously applied chemicals

Scarified seed treated with gibberellic acid (400 ppm, 500 ppm, 1000 ppm), citric acid (1000 ppm) or potassium nitrate (2000 ppm) resulted in high germination percentages comparable to the untreated scarified control (Table 4.8; Table 4.9; Figure 4.4).

Lot W97 and H98 responded well to all treatments. A98 also performed well except when treated with the highest concentration of GA. At 1000 ppm, 77 % of A98 seeds germinated; a statistically significant value that was 17 % below that of the control. The lower germination of A98 at 1000 ppm (GA) may be due to differences in its ability to metabolize the excess hormones as compared to the other seed lots (Derkx and Karssen, 1994).

Table 4.8. Comparison of the effect of various concentrations of GA_3 on percent germination using scarified seed of *Callirhoe involucrata*.

	GA_3			Control
Lot	400 ppm	500 ppm	1000 ppm	
W97	96 *a*[zy]	92 *a*	95 *a*	98 *a*
H98	100 *a*	100 *a*	93 *b*	98 *a*
A98	98 *a*	97 *a*	77 *b*	94 *a*

[z] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's Multiple Range test (P< 0.05).*
[y] *Means represent adjusted values.*

Pre-chilling of scarified seed, on GA-imbibed blotters, resulted in 100 % germination in the W97 seed lot (other lots were not tested). The scarified pre-chilled control, for W97, had 78 % germination (data not shown). The low control value was not surprising as germination results for the pre-chilling experiments (Figure 4.4), previously discussed, were similar; pre-chilled, scarified treatments had germination rates that were 18 - 20 %

lower than the non-chilled, scarified control. That the GA/prechill treatments performed as well as GA alone may be due to the ability of GA to counter the negative effects of extensive pre-chilling.

Table 4.9. Comparison of the effect of citric acid and KNO_3 on percent germination using scarified seed of *Callirhoe involucrata*.

	Citric Acid	KNO_3	Control
Lot	1000 ppm	0.2 %	
W97	100a[z]	100a	98a
H98	100a	96a	98a
A98	92a	64b	94a

[z] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's Multiple Range test (P< 0.05).*

One hundred seedlings derived from GA treated seed were grown to maturity to observe the long-term effect of GA, if any, on growth. Control plants were stouter than those treated with GA and slower to reach maturity. However, by the end of one year treated plants were indistinguishable from control plants. Furthermore, seedling survival was similar (93 % v. 97 % control), data not shown.

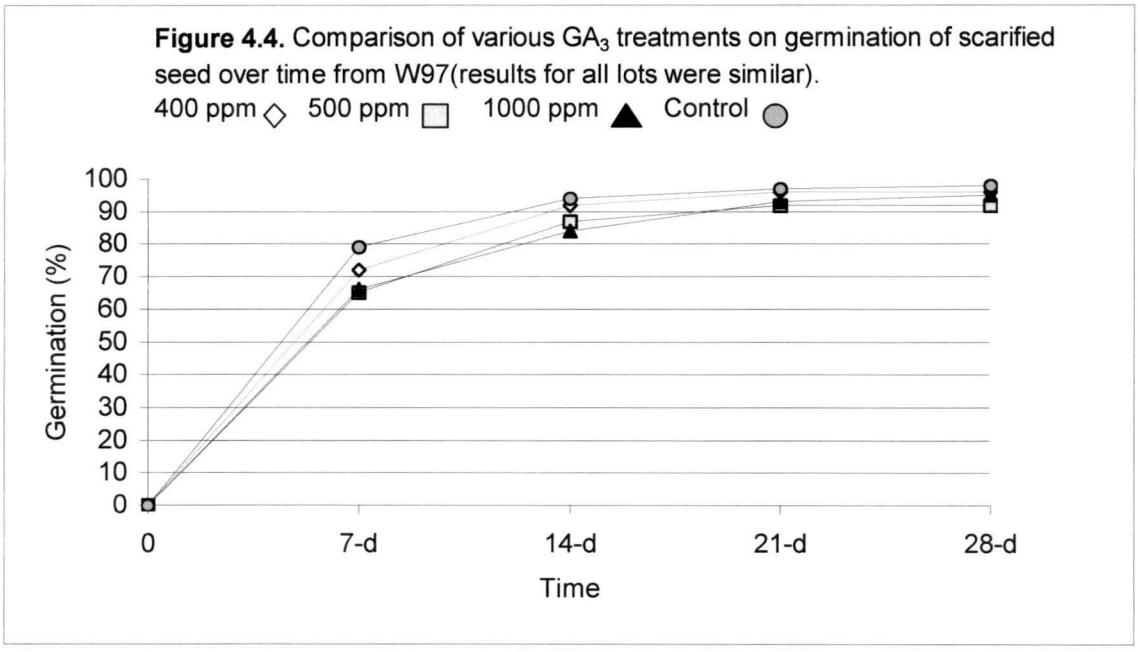


Figure 4.4. Comparison of various GA_3 treatments on germination of scarified seed over time from W97(results for all lots were similar).

Potassium nitrate had a negative affect on A98, lowering germination by 30 % when compared to the control; a response not seen in the other seed lots, Table 4.9. Germination in lots W97 and H98 reached 100 % when treated with citric acid, a value comparable to the 98 % germination reached with the control. The response, by all lots, to the chemical treatments used in these experiments was minimal, even negative in the case of A98, when compared to the control. Hydrogen peroxide did not improve germination of *Callirhoe* seed (data not shown). In fact, 30 % H_2O_2 (24 hour exposure) killed most of the treated seed. A 3.0 % concentration of H_2O_2 (24 hour exposure) showed no improvement, for any lot, over the control (data not shown). As with pre-chilling, the chemicals tested are not recommended as a pre-treatment given that untreated scarified seed produced similar results.

Leaching and hot water

Leaching of seed in water was used to test for the presence of chemical inhibitors. There was no significant improvement of germination in any lot, as compared to the control, when seeds were leached at 18°C. Leaching at 40°C for 24 hours resulted in a significant improvement in germination, for lot W97, when compared to all other treatments (Table 4.10). That lots A98 and H98 did not also show improvement at 40°C advances the probability that chemicals are not active in inhibiting germination in seeds of *Callirhoe involucrata.* If chemical inhibitors were present a consistent response in all lots to the 40°C leaching treatment would be expected. As this is not the case, it is more likely that the warm leaching treatment acted to soften the testa in W97 seeds, thereby, improving germination. As previously noted, hardseededness is a function of the maternal environment and would therefore be expected to vary in seeds collected from different populations.

Hot water acts by softening the seed coat allowing for imbibition to occur and/or gaseous exchange to take place (Bewley and Black, 1982). Hot water treatments, of 100°C, provided consistently high germination percentages in one seed lot, W97 (87 % on average). The other two lots demonstrated improvement over the control but, with one exception, did not exceed 50 % germination (Figure 4.5.a - 4.5.c).

Lot A98 showed only a slight difference between treatments ranging from 44 - 48 % germination. Lot H98 fared slightly better reaching 58 % germination in response to a

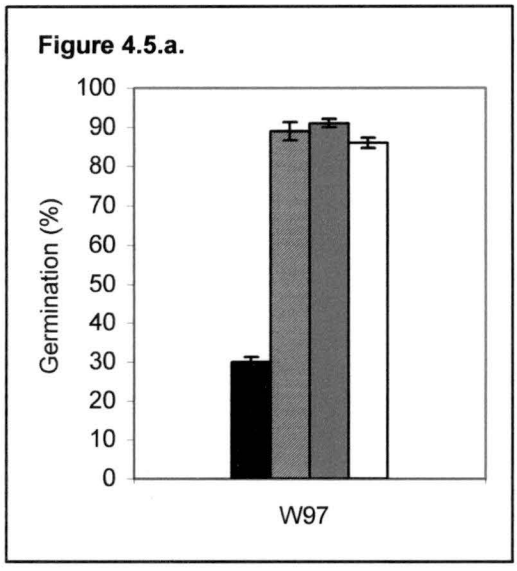
Table 4.10. Germination following leaching of *Callirhoe involucrata* seed in water maintained at various temperatures for selected exposure times.

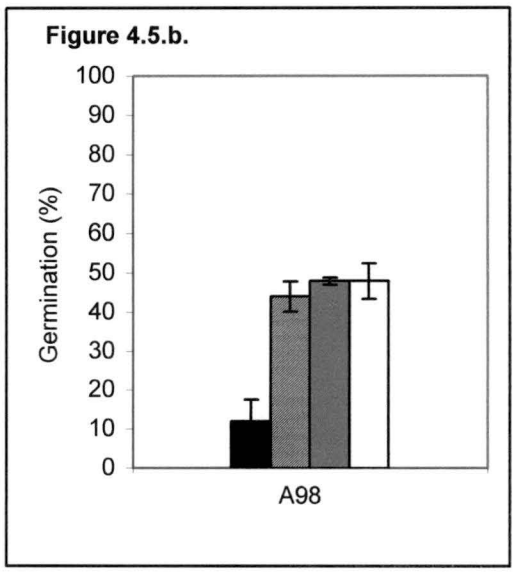
	Germination[zy] (%)		
Lot / Exposure time	18°C	40°C	Control
W97	-[w]	-	25 *b*[v]
24h	20 *b*	41 *a*	-
48h	26 *b*	-	-
H98	-	-	13 *a*
24h	6 *b*	12 *a*	-
48h	3 *b*	-	-
A98	-	-	19 *a*
24h	6 *b*	7 *b*	-
48h	8 *b*	-	-

[z] *Based on 100 seeds per treatment.*
[y] *Germination percentages based on filled seed.*
[w] *(-) not applicable.*
[v] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's Multiple Range test (P< 0.05).*

1.5 hour hot water soak. These results suggest that there are variations in seed coat thickness or structural integrity, *i.e.* how tightly the parenchyma cells are packed.

It would be beneficial to determine a water temperature that would disrupt the chalazal cap. Furthermore, it would also be advantageous if treated seed could be dried down after the hot water treatment but remain readily germinable. Support for this procedure comes from studies on hard seed cotton (Christiansen and Moore, 1959) in which dormancy did not reoccur upon drying.





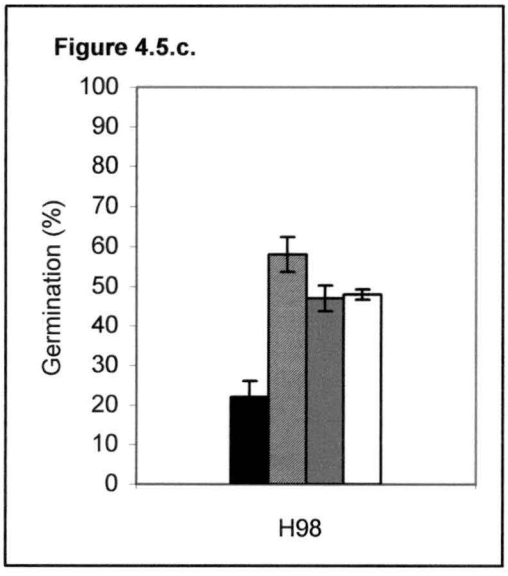


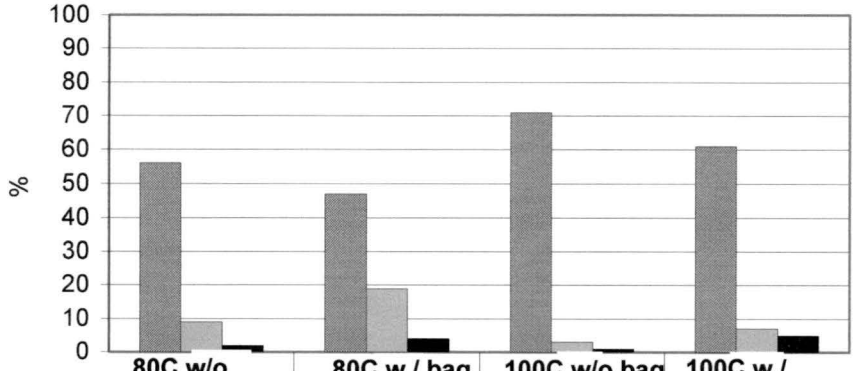
Figure 4.5 (a-c). Comparison of hot water treatment (100°C) on germination in 3 seed lots of *Callirhoe involucrata* as compared to a control[zy].

Control ■ 1.5h ▒ 4h ▒ 24h □

[z] *Comparisons are made within lots.*
[y] *Germination percentages based on filled seed.*

Different temperature and submergence techniques were used to test their effect on germination in one seed lot (W97). Germination percentages were lower for lot W97, whether submerged within or without a mesh bag, when treated with 80°C water (Figure 4.6). The higher percentage of hard seed, remaining after treatment, explains why 80°C was a less effective temperature; at 100°C the average amount of hard seed remaining after treatment was 5 % v. 14 % at 80°C (Figure 4.6). Fruit allowed to free-float (no bag) in the hot water germinated better at both temperatures. Seed of *Callirhoe* probably responded less favorably to treatment in a bag because of the protection from direct exposure, to the heated water, the bag provided.

Figure 4.6. Comparison of various hot water treatments on percent germination, hard and dead seed using *Callirhoe involucrata*, lot W97[z].



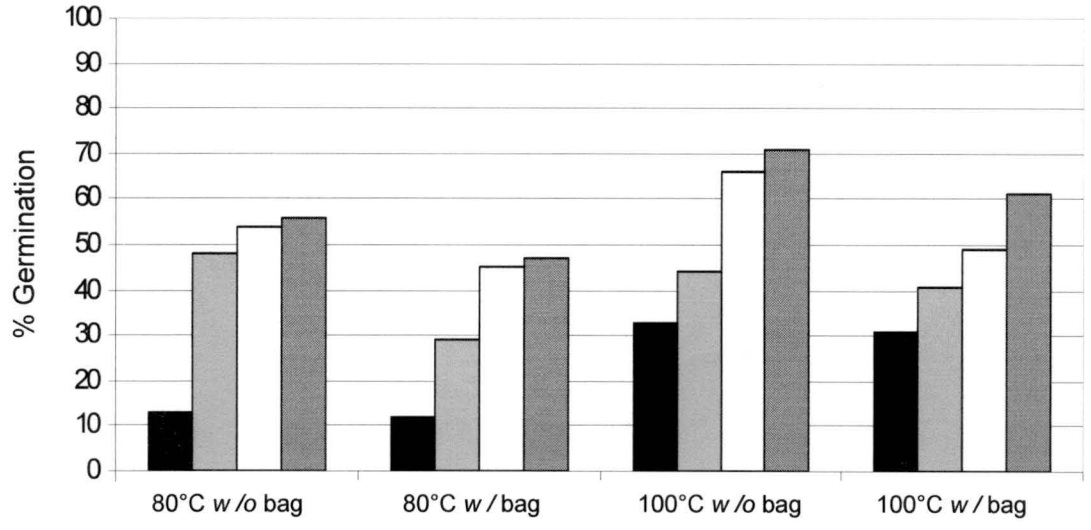
[yx] %	80C w/o	80C w / bag	100C w/o bag	100C w /
germination	56	47	71	61
hard seed %	9	19	3	7
dead %	2	4	1	5

[z] *All treatments represent a 4h exposure time.*
[y] *Based on 100 seeds per treatment.*
[x] *Germination percentages are not adjusted for dead or empty seed.*

Rate of germination increased with time for all treatments (Figure 4.7). In this study percentage hard seed and water temperature have an inverse relationship suggesting that an increase in water temperature (above 100°C) may produce more favorable results.

It is unlikely that the seed envelopes of *Callirhoe involucrata* are a source of chemical inhibitors. However, these results do not eliminate the possibility that impermeability to gases may play a role. Both hot water and scarification increase gas diffusion between the embryo and the external environment.

Figure 4.7. Comparison of various hot water treatments on rate of germination of *Callirhoe involucrata* seed, lot W9 [zy].



[z] *All treatments represent a 4h exposure time.*
[y] *Germination percentages are not adjusted for dead or empty seed.*

Asexual propagation

Success was obtained using vegetative stem cuttings dipped in 1.0 % indole-butyric acid (IBA) powder and placed under mist. Thirty cuttings of new growth were taken on April 16, 2000 from one-year old greenhouse specimens of *Callirhoe involucrata*. In the first trial, 47 % of the cuttings (W97) were fully rooted within 30 days and 83 % after 45 days; 12 % died (Table 4.11). Within 3 days, 17 % had begun to root during the second trial and full rooting was achieved by 80 % (W97) of the cuttings by day 52; 7 % died or never rooted. In trial three, 33 % (W97) rooted fully while 44 % produced only callus or a limited number of roots within 6 weeks while the remainder died. Results for the three trials were similar for A98 (Table 4.11). Following a gradual increase in success from trial one to trial two there was a drastic decrease in rooting in trial three. An observational trial conducted mid-summer, 1999, produced 0 % rooting. It appears that timing is important when attempting to root stem cuttings of *Callirhoe*. With success as high as 93 %, cutting propagation could supplement seed as a means for plant production. Advantages of cutting propagation include a shorter time until maturity and potential for clonal selection. Disadvantages are the maintenance of stock plants; limited window for cutting collection, and a limited number of cuttings that can be produced per plant.

Table 4.11. Rooting[z] of vegetative stem cuttings, of *Callirhoe involucrata*, under mist in a series of three 6-week trials.

Source of stem cuttings	Rooting (%) after 6 weeks		
	Trial 1[yx]	Trial 2	Trial 3
W97	83 *a*[w]	80 *a*	33 *b*
A98	80 *a*	86 *a*	40 *b*

[z] *Full rooting was defined as having 10 or more roots with a minimum length of 1 cm.*

[y] *Trials were started at 5 week intervals.*

[x] *Trial one used 30 cuttings per source and trials two and three used 15* cuttings per source.

[w] *Means followed by the same letter are not significantly different (within lots) as determined by Duncan's multiple range test (P< 0.05).*

Chapter V

CONCLUSIONS

There is a paucity of information on the propagation of any species from the genus *Callirhoe*. That which does exist is highly generalized and often inaccurate. Therefore, the purpose of this research was to establish a propagation protocol that could be adopted by nursery growers for commercial production and determine the nature of the germination inhibiting factor(s) in seed of *Callirhoe involucrata*.

The greatest factor inhibiting germination in *Callirhoe involucrata* is the presence of a hard seed coat. This fact was made clear when comparing germination percentages of unscarified and hand scarified seed. Because hand scarification is not practical for use with large quantities of seed other methods of scarification were explored.

Freezing seed had no effect on germination, seed coat permeability or viability. Mechanical scarification using pressurized air proved highly unpredictable making it a poor choice for use with seed of *Callirhoe involucrata*. Moist prechilling did not alter the permeability of unscarified seed and actually lowered germination of scarified seed in some lots. Hot water is a highly desirable method of seed pretreatment because it does not require special tools or training and does not involve dangerous chemicals. However,

it does appear to require adjustment based on the particular characteristics of the seed being treated. The author believes that further experimentation with temperature and treatment times would have made hot water a more successful treatment for *all* the lots used in this research. As with all treatments, the propagator needs to be aware of response differences between years and among populations, testing small quantities of seed first.

The most successful pretreatment for seed of *Callirhoe involucrata* was the double 60-minute acid scarification method. It is the recommended protocol having produced exceptional germination results for all lots tested. It should be used with knowledge, training and close monitoring, as sulfuric acid can be hazardous to both the seeds and the propagator.

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