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Time Requirement from Pollination to Seed Maturity in Waterhemp (*Amaranthus tuberculatus*)

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Experiments were conducted to determine the amount of time required for waterhemp to produce mature seeds after pollination. Female waterhemp plants were pollinated over a 24-h time period and then isolated from males. Two branches, each containing at least 500 flowers, were harvested from each female at the time of the initial pollination, designated as 0 d after pollination (DAP), as well as at multiple other times after pollination up to 62 DAP. One branch from each harvest was stored at 30 C for 48 h, while the other branch was stored at −20 C for 48 h. Branches were then stored at room temperature until all harvests were complete, at which time seeds from each branch at each time after pollination were collected, weighed, and stratified. Germination tests were then conducted to determine the time at which seeds become viable after pollination. Seeds that had not germinated by the end of the germination tests were subjected to tetrazolium testing for viability. Germination tests were also conducted on nonstratified seeds to investigate changes in seed dormancy that were expected to occur over the amount of time the seeds were allowed to remain on the plants. Seeds stored initially at 30 C postharvest became viable 7 to 9 DAP, whereas seeds stored initially at −20 C postharvest did not become mature until 11 DAP. Seed coat color was white soon after pollination and became dark brown to nearly black by 12 DAP, and seed weight increased until 12 DAP. Tetrazolium tests for seed viability correlated well with the germination tests. Germination tests on nonstratified seeds indicated that dormancy level was initially high in the population used, but began to decrease between 15 and 30 DAP. Results of this study have implications both for waterhemp management and research.
Nomenclature: Waterhemp, *Amaranthus tuberculatus* (Moq.) Sauer AMATU.
Key words: Dormancy, germination, pollination, seed set, seed weight.

Waterhemp is a dioecious summer annual weed indigenous to the midwestern United States. It is a C_4 plant with a rapid growth rate, which can produce many seeds under ideal growing conditions. However, it has only recently become a prevalent weed throughout much of the midwestern corn (*Zea mays* L.) and soybean [*Glycine max* (L.) Merr.] production areas (Hager et al. 1997). Waterhemp has become a major problem weed for several reasons. First of all, it is a small-seeded broadleaf plant. Thus this weed has found its niche in agronomic cropping systems with the widespread adoption of no-tillage practices. A lack of tillage allows the seeds to remain on the soil surface where they can easily grow after germination, whereas burial of waterhemp seeds by tillage can greatly reduce germination and emergence (Hager et al. 1997). Another factor contributing to the weediness of this species is that its seeds germinate throughout much of the growing season, making season-long control with a single herbicide application difficult (Hager et al. 1997).

The multiple herbicide applications required for season-long control of waterhemp have facilitated the appearance of yet another characteristic that contributes to the weediness of this species—the rapid evolution of herbicide resistance (Jasieniuk et al. 1996). In fact, waterhemp has evolved resistance to herbicides with four different modes of action—namely triazines (Anderson et al. 1996; Foes et al. 1998), acetolactate synthase (ALS) inhibitors (Horak and Peterson 1995), protoporphyrinogen oxidase inhibitors (Shoup et al. 2003), and most recently glyphosate (Legleiter and Bradley 2008). All four of these resistances may be nuclear-inherited and thus can be spread through pollen flow (M. S. Bell and P. J. Tranel, unpublished data; Heap 2009; Patzoldt et al. 2003; Shoup et al. 2008; Tranel and Wright 2002). Waterhemp is a prolific species, as a single female plant may produce up to 1×10^6 seeds (Sellers et al. 2003). Consequently, if the ovules on a female plant are fertilized by pollen containing herbicide-resistance alleles and those ovules develop into mature seeds, eradication of herbicide-resistant waterhemp plants from a field will become very difficult. Thus, an important focus of any weed management strategy involving waterhemp control should be the prevention of seed production, which entails having some knowledge of the seed maturation biology of this species.

The primary objective of this study was to determine the amount of time required for female waterhemp plants to produce viable seeds after being pollinated. Secondary objectives included determination of the length of the seed-filling period based on seed weight, as well as an attempt to determine whether the seeds become dormant during or after the maturation process. To address these objectives, the basic strategy was to fertilize ovules during a controlled pollination event and then to subsequently harvest flowers from the female plants at select times after the controlled pollination event. For the purposes of this paper, a mature seed is defined as a seed that is viable. Maturation will be used to refer to the set of processes that occur from the time of pollination until a seed becomes viable. And finally, dormancy will be defined as the failure of a mature seed to germinate under ideal conditions.

Materials and Methods

Preparation of Plants. The waterhemp population used in this study, designated as ACR, was described previously (Patzoldt et al. 2005). This population was chosen because it tends to have a lower level of dormancy than most of the other populations that were immediately available for use in this study—an important aspect in an experiment in which seed viability is determined by performing germination tests.

Multiple females were required to perform this experiment. However, plants could not be identified as females until flowering began. Thus, 20 plants were grown for this study with the hope that at least five of them would be female. Seeds

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were sown in a 12-cm by 12-cm by 5-cm container in a medium consisting of a 3 : 1 : 1 : 1 mixture of commercial potting mix[1] to soil to peat to sand. When the seedlings exhibited two true leaves, they were transplanted into 7.6-L pots containing commercial potting mix.[1] Plants were fertilized as needed using a slow-release complete fertilizer,[2] and the plants were grown in the greenhouse under mercury halide and sodium vapor lamps that provided a minimum photon flux of 800 μmol m^{-2} s^{-1} at the plant canopy in addition to the light incident from the sun. The lamps were programmed for a 16-h photoperiod, and the greenhouse was maintained at temperatures of 22/28 C night/day.

In addition to multiple females being required, numerous flower branches from each of the females were required to allow for germination tests at multiple times after pollination. Therefore, when the plants reached approximately 40 cm in height, the apical meristems were removed. This released the axillary buds from apical dominance, allowing these buds to produce new growth, which ensured that numerous branches were present on each plant at the onset of flowering.

Besides the requirement that each plant contain multiple branches, numerous nonpollinated flowers were needed on each of these branches in order to perform the germination tests. To ensure that each branch contained a sufficient number of nonpollinated flowers, female plants were isolated from the males by moving them to a separate greenhouse room as soon as they were identified as females. Once isolated, the female plants were allowed to continue to grow in the greenhouse until abundant stigmas were visible on each branch, at which time the plants were pollinated.

Pollination. On the morning of pollination, the females were reintroduced to the room containing the male plants. Male plants were then selected one at a time, held above the female plants and shaken to release pollen. This was done in the morning, because that is the time at which many species, including some in Amaranthaceae, produce the most pollen (Rodríguez et al. 2000; Singh and Babu 1980). We assumed that the same would hold true for waterhemp, although Singh and Babu (1980) also suggest that some species in Amaranthaceae may actually produce the majority of their pollen in the afternoon.

After each of the male plants was used to pollinate the females, the female plants were kept among the males for 24 h, after which the males were once again used to pollinate the female plants. Immediately following the second pollination, the females were again isolated from the males in order to prevent any later uncontrolled pollination from occurring. Also at this time the harvest phase of the experiment began.

Included as a test to check for apomixis and uncontrolled pollination were two additional female plants. These plants also were isolated from the males as soon as they were identified as female plants. However, these additional two females were never reintroduced to the room containing the male waterhemp plants. Thus, any seed collected from these two females resulted from one of the following: apomixis, uncontrolled pollination that occurred prior to isolation of the females, or uncontrolled pollination due to the presence of foreign pollen in the female isolation room.

Harvests. In the first run of the experiment, branches were harvested from six female plants at 0, 3, 5, 7, 9, 12, 14, and 16 d after pollination (DAP). In the second run of the experiment, branches were harvested from five female plants at 0, 3, 5, 7, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 24, 30, and 62 DAP. The harvests consisted of collecting two branches, each containing an estimated number of at least 500 flowers, from each female at each harvest time. One of each pair of branches was incubated 48 h at −20 C while the other was incubated 48 h at 30 C. These two postharvest treatments were chosen as different attempts to achieve our goal of abruptly stopping seed maturation without damaging seeds that may not be fully developed (and therefore fragile). Further, these two postharvest treatments simulated, respectively, a frost event or the fate of seeds in a harvested field. After the initial treatment at −20 C or 30 C, branches were stored at room temperature until all harvests were completed, at which time seeds were prepared for germination testing.

Germination Tests. Seeds were manually harvested from each branch. A digital camera[3] connected to a microscope[4] was used along with computer software[5] to take a picture of a random assortment of seeds from each branch. In each random assortment of seeds, a range of maturity levels was observed from infancy up to a maturity level that the majority of the observed seeds had reached, designated as the most prevalent maturity level (MPML). Next, seeds at the MPML from each treatment (10 seeds/repetition in the first run, and 20 seeds/rep in the second run) were randomly selected. One such seed from each treatment and rep was photographed in order to record seed color and to compare sizes and shapes of seeds at different times after pollination. Each of the seeds was then surface-sterilized by soaking for 10 min in a 1 : 1 bleach : water solution. The seeds were then stratified in sterile deionized water at 4 C for at least 10 d, after which time they were placed on moist filter paper in petri dishes. The petri dishes were kept in an incubator at temperatures of 35/30 C day/night, as it has previously been shown that alternating temperatures improve waterhemp seed germination (Leon et al. 2006; Steckel et al. 2004). After 1 wk, the fraction of seeds germinating out of the total number of seeds initially present in each plate was recorded and a germination percentage calculated.

For the second run of the experiment, 20-seed samples were weighed prior to stratification. Additionally, extra 20-seed samples were collected from the branches harvested at 10, 15, 30, and 62 DAP, which had been subjected to the 30 C postharvest treatment. Germination tests were conducted on these samples without stratification to investigate possible changes in the level of dormancy over time in this population.

Tetrazolium (TZ) Tests. After the 7-d germination test in the second run of the experiment, the viability of the stratified seeds that had not yet germinated was examined using a TZ test. Harvest times investigated were 7, 10, 13, and 30 DAP, for both of the postharvest treatments. Ungerminated seeds from each of the female plants were pooled for each of these eight treatments, and six seeds were then randomly selected from each treatment for TZ testing. To perform the TZ tests, the seeds were placed in a line on damp filter paper in a petri dish. The seeds were held with forceps while they were cut longitudinally with a scalpel to expose the embryos. After being sliced, the seeds were placed face down on the filter paper, keeping the two halves of each seed in close proximity

to one another. Approximately 1 ml of 1% TZ (wt/v) solution was applied to the filter paper in each petri dish, after which the dishes were covered and placed in the dark for 24 h. After 24 h of exposure to TZ, the seed halves were turned over and investigated for embryo staining. Due to the small size of these seeds and the ease with which such small embryos can be damaged during the slicing process, seeds were scored as viable if any purple staining was apparent on an intact embryo or an embryo fragment. The overall procedure was modeled after that of Peters (2002).

In addition to testing the ungerminated seeds remaining in the petri dishes after the germination tests, 20-seed samples from each of the five 13 DAP/30 C postharvest replicates were combined to make a 100-seed pool. The same was done separately from the 13 DAP harvests that had received the −20 C postharvest treatment. Ten seeds were randomly selected from each of the 100-seed pools and subjected to TZ testing, as was described above.

Results and Discussion

Seed Collection. For several of the harvests, including some that were collected as early as 0 DAP, mature-looking seeds were found. However, this amounted to a maximum of two seeds in a particular treatment, or less than 1% of all the seeds or flowers observed in each treatment. These mature-looking seeds were dark brown or nearly black in color. The presence of these seeds at such early harvest times is likely due to pollination that occurred before the plants were identified as females and subsequently isolated from the male plants. However, because the frequency of these mature-looking seeds was extremely low—amounting to less than 1% of all of the seeds analyzed from a particular treatment—such seeds were ignored. What remained then was a large number of seeds that all appeared to be at the same maturity level, designated as the MPML. Reference to the MPML becomes important when investigating seeds at later harvest times, as at nearly every harvest time a range of seed maturity levels were represented from infancy up to the MPML of the given harvest time. This is likely due to embryo abortion, as the nonpollinated females produced very few seeds even at the later harvest times.

Although mature-looking seeds were also collected from the nonpollinated plants at all harvest times, these seeds were present in fewer than 1% of the flowers observed, and thus any possible effects of early pollination, foreign pollen entering the female isolation room, or apomixis were considered insignificant sources of error in this study.

Seed Appearance. At 0 DAP, ignoring the one or two mature-looking seeds that were present in some of the treatments, nothing was found that bore any resemblance to a seed after observation of approximately 500 flowers per treatment. In fact, nearly every flower appeared empty at this harvest time. By 3 DAP ovules were visible (Figure 1). They appeared as thin, translucent discs, which were light tan in color. Some darkening occurred over the next 2 d, and by 5 DAP the seeds receiving the 30 C postharvest treatment (oven-treated) were a matte tan-brown color, while those seeds receiving the −20 C postharvest treatment (cold-treated) were a glossy light tan color. Both oven- and cold-treated seeds were still relatively thin at this point. By 7 DAP, seeds had continued to darken, reaching a reddish-brown color for both oven- and cold-treatments. A notable difference between these two treatments at this stage was that the cold-treated seeds were rounded, appearing to already be nearly filled, while the oven-treated seeds still appeared to be relatively thin. Seeds from both postharvest treatments displayed glossy seed coats by this time. At 9 DAP, seed color had darkened even more, and by 12 DAP, all seeds appeared to finish darkening, reaching a terminal color of dark brown to black when viewed under a dissecting microscope. As for visual filling, the cold-treated seeds appeared nearly full by 7 DAP, and totally full by 14 DAP, while the oven-treated seeds showed a more gradual increase in width that also appeared to terminate at approximately 14 DAP.

Seed Weight. Analysis of the 20-seed weights painted a slightly different picture of the seed filling process when compared with the visual observations, as the weights of both the oven-treated and the cold-treated seeds increased at the same rate when compared at multiple times after pollination (Figure 2). Seed weight appeared to increase approximately linearly with time after pollination between 7 and 12 DAP, at which time the seeds attained their maximum weight. The 20-seed weights remained constant from 12 DAP until the conclusion of the experiment at 62 DAP. The fact that the same pattern of weight increase occurred for both postharvest treatments is rather interesting as the cold-treated seeds appeared fuller than the oven-treated seeds as early as 7 DAP when viewed under a dissecting microscope. Why these cold-treated seeds appeared fuller than the oven-treated seeds while sharing the same weight is an interesting question that was not addressed in this study.

Germination Tests. In the first run of the experiment, six females were pollinated using 10 males, and samples of 10 seeds at the MPML were collected from each harvest. The one or two mature-looking seeds present at some of the earlier harvest times were included in these germination tests, which explains the apparent nonzero germination percentages at 5 and 7 DAP (Figure 3A). In fact, the only germination that occurred from these harvest times was due to those seeds that already displayed a dark brown or purple color. However, no seeds at the MPML began germination until 9 DAP, at which time germination of the oven-treated seeds reached approximately 12%. Germination of the oven-treated seeds reached a maximum of approximately 78% by 12 DAP, after which point it leveled off and remained at or near 75% for the remainder of the harvests collected.

The germination profile of the cold-treated seeds was slightly different, in that germination did not begin until 12 DAP, at which time it reached about 25%. By 14 DAP the germination percentage reached a maximum of 49%, after which it dropped slightly to 31% by 16 DAP. Based on the results of this run, we designed the second run of the experiment to provide more germination data between 9 and 12 DAP, as well as to provide more data at later time points. In addition, we chose to perform the germination tests only on seeds at least as old as 7 DAP, as it was assumed that once again no germination would occur in the MPML seeds prior to that time point.

Analysis of the second run, in which six females were pollinated using eight males, shows a story similar to what was previously described for the first run. The oven-treated seeds

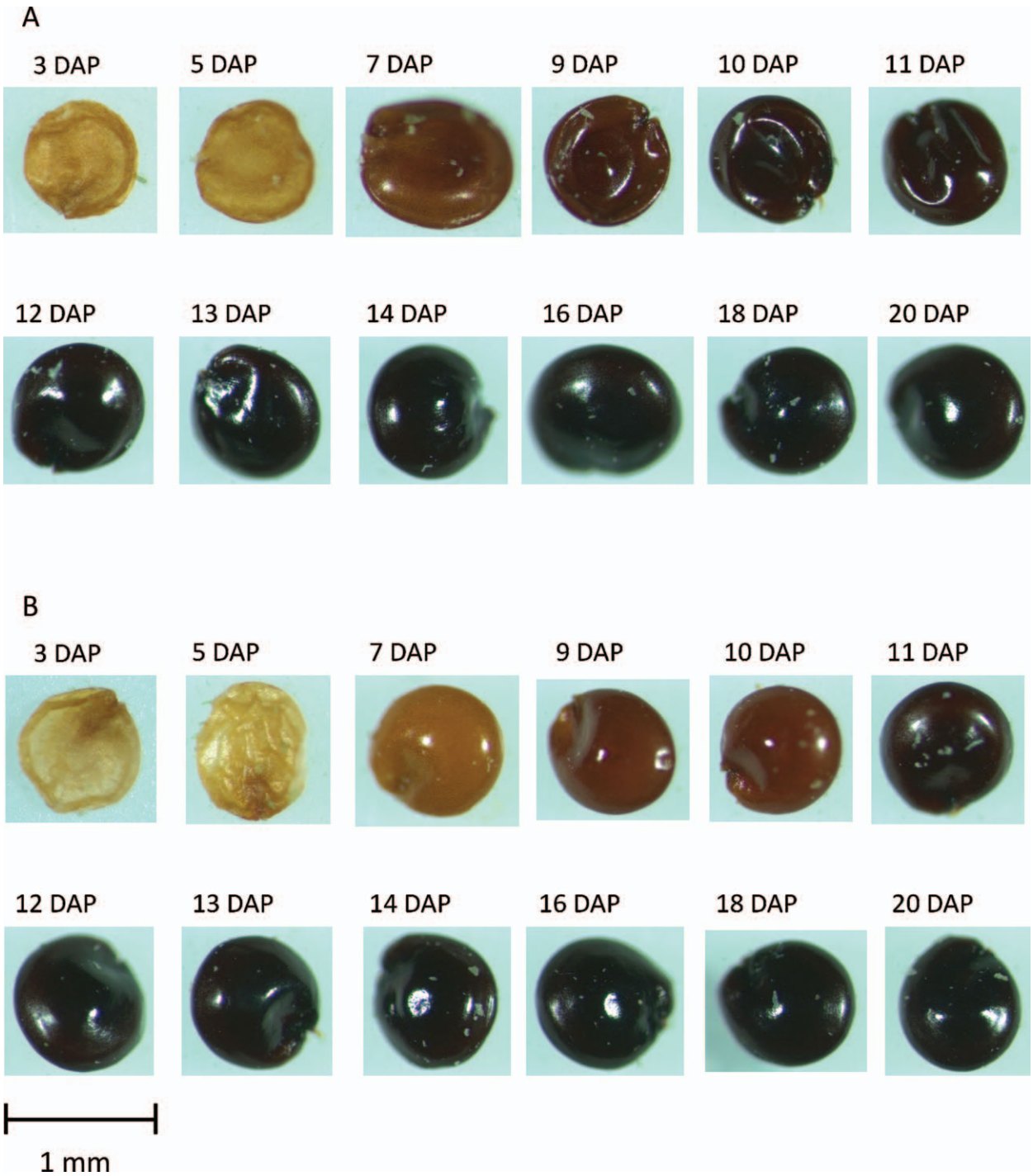

Figure 1. Pictures of seeds collected at the most prevalent maturity levels at selected day after pollination (DAP) receiving either a 48-h postharvest treatment of 30 C (A) or a 48-h postharvest treatment of −20 C (B).

did not begin to germinate until 9 DAP, at which time approximately 32% of those seeds at the MPML demonstrated viability (Figure 3B). Germination of these oven-treated seeds was maximized by allowing one more day of on-plant maturation, as germination reached about 79% by 10 and 11 DAP, after which point the percentage dropped slightly, hovering near 70% until 24 DAP when it again approached 80%.

For the cold-treated seeds, germination began by 10 DAP with about 5% of the seeds at the MPML germinating. By 11 DAP that percentage had increased to 13%, and at 12 DAP it reached about 40%, where it remained until 15 DAP. The percentage of cold-treated seeds germinating again increased at 16 DAP to over 50%, after which point it continued to gradually increase to eventually match that of the oven-treated seeds near 80% for the 24, 30, and 62 DAP harvest times.

The trends obvious from these data are that the seeds stored at 30 C postharvest for 48 h became viable at a time between 7 and 9 DAP, which was at least 1 d earlier than the cold-treated seeds became viable. It is also interesting to note that by 12 DAP the oven-treated seeds reached their maximum germination percentage near 80%, whereas the cold-treated

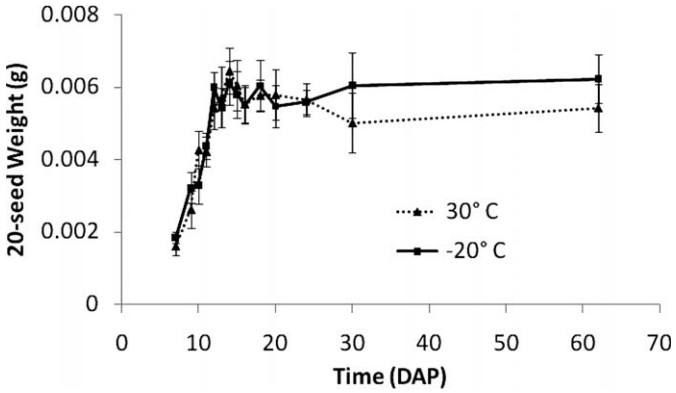

Figure 2. Mean 20-seed weight vs. days after pollination (DAP) for seeds receiving a 48-h postharvest treatment of storage at 30 C (▲) or −20 C (■). Vertical bars represent ± SEM (n = 5).

seeds approached this apparent limit to germination more gradually, only reaching this level by approximately 3 wk after pollination. This could suggest either that cold temperatures caused damage to the seeds that prevented them from germinating, or that the oven-treated seeds continued to mature for some time as the plant material dried after the harvest had been made. However, based on the fact that nearly 80% germination was attained by oven-treated seeds by just 12 DAP, whereas cold-treated seeds required approximately twice that time to attain the same germination percentage, it seems more likely that freezing caused some damage to the otherwise viable seeds until about 24 DAP, after which time they became immune to freezing damage.

TZ Tests. None of the ungerminated seeds analyzed from the 7 DAP harvests showed any signs of staining for either of the postharvest treatments (data not shown). This was expected because none of the seeds harvested 7 DAP germinated. By 10 DAP, only 17% of the cold-treated seeds were viable, while 83% of the oven-treated seeds were viable. This is in fairly close agreement with the viability estimates that may be inferred from the germination data in Figure 3. Analysis of ungerminated seeds harvested 13 DAP showed that cold-treated and oven-treated seeds were 83 and 100% viable, respectively. By 30 DAP, 100% of the seeds were viable, indicating the on-plant maturation was complete. As for the extra 10-seed samples collected by pooling seeds that had not been subjected to germination testing from each of the five females at 13 DAP, the oven-treated seeds showed 100% staining, while the cold-treated seeds showed only 60% staining, still in close agreement with the germination data in Figure 3.

Dormancy. Results of the dormancy test, in which oven-treated seeds were tested for germination without stratification, revealed an intriguing pattern of dormancy vs. time after pollination. The seeds are apparently dormant soon after fertilization occurs, as very few of the seeds harvested at 10 and 15 DAP germinated after 7 d of germination testing (Figure 4). Interestingly, by 30 DAP, approximately 35% of the tested seeds germinated, and the percentage of seeds germinating from the 62 DAP harvests was nearly identical to that of the 30 DAP harvests. It has been shown that these oven-treated seeds are indeed mature by 10 DAP (Figure 2). In fact, nearly 80% of oven-treated seeds harvested at 10 DAP

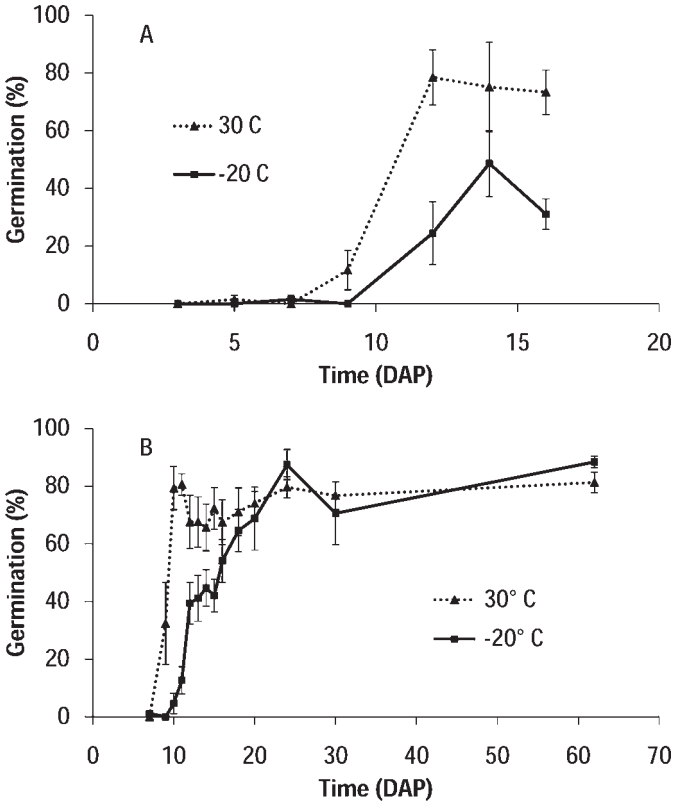

Figure 3. (A) Mean germination percentage of seed samples vs. days after pollination (DAP) from the first run of the experiment receiving a postharvest treatment of 30 C for 48 h (▲) or −20 C for 48 h (■). Seeds were first stratified for 10 d, then incubated at 35/30 C day/night for 7 d during the germination testing. (B) Mean germination percentage of seed samples from the second run of the experiment receiving a postharvest treatment of 30 C for 48 h (▲) or −20 C for 48 h (■) vs. DAP. Seeds were first stratified for 30 d, then incubated at 35/30 C day/night for 7 d during the germination testing. Vertical bars in both panels represent ± SEM ([A] n = 6, [B] n = 5).

germinated after stratification, and of those that did not germinate during germination testing, 83% were viable based on TZ testing. Thus, the fact that none of these seeds germinated under ideal conditions without being stratified indicates a high level of dormancy in the young seeds. Apparently the dormancy level began to decrease at some point between 15 and 30 DAP. Perhaps this could be beneficial to the species, because if young seeds had low levels of dormancy they would germinate in the fall and would probably be killed by frost before producing seeds. It should be noted, however, that this trend of dormancy vs. time may be biotype-specific. In fact, differing levels of dormancy have previously been reported for three other waterhemp populations by Leon et al. (2006), in which the authors suggest that seed dormancy is an adaptive trait that may be influenced by agricultural practices.

The data from this study suggest that waterhemp seeds may become mature as soon as 9 DAP. Thus, once pollen begins to spread in a field containing female waterhemp plants, a very narrow window of time exists in which a farmer can work to prevent seed production. Seed maturation biology has been studied in many other species, including some important weeds in agronomic cropping systems, and the results show that the time required for seed maturation after pollination varies among species. For instance, Egley (1976) reported that prickly sida (*Sida spinosa* L.) seeds are incapable of

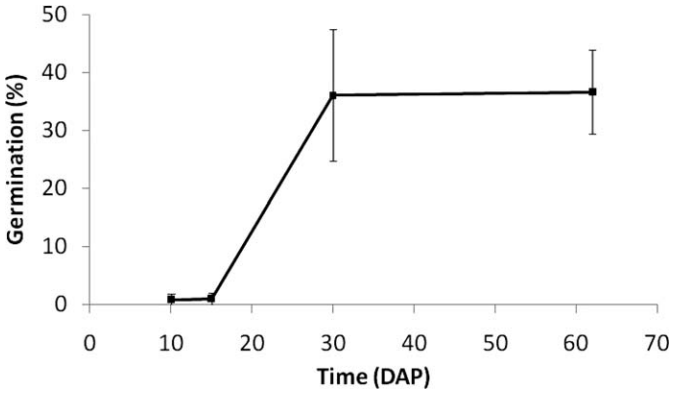

Figure 4. Mean germination percentage of nonstratified seeds receiving a postharvest treatment of 30 C for 48 h vs. days after pollination (DAP). Vertical bars represent ± SEM ($n = 5$).

germination until 12 d after anthesis, and that the seeds attain their maximum dry weight by 14 d after anthesis. Prickly sida seeds are capable of 80% germination at 12 to 16 d after anthesis, after which time germination drops to nearly 0%, presumably due to the onset of dormancy (Egley 1976). Chandler et al. (1977) reported that in purple moonflower (*Ipomoea turbinata* Lag.), seeds first acquire the ability to germinate at 20 d after anthesis. They also reported that maximum germination occurs at 26 d after anthesis, and that maximum dry weight occurs at 34 d after anthesis. Similarly, Jayasuriya et al. (2007) reported that in pitted morningglory (*Ipomoea lacunosa* L.), germination does not occur until at least 20 DAP. These seeds reach their maximum dry mass by 22 DAP and their maximum germination percentage at 24 DAP, but by 30 DAP the seeds no longer germinate unless manually scarified, indicating that dormancy is due to impermeable seed coats (Jayasuriya et al. 2007). Even in mouse-ear cress [*Arabidopsis thaliana* (L.) Heynh.], a species known for its short life cycle, Koorneef et al. (1989) found that seeds do not become viable until at least 13 DAP when dried immediately after harvest. Thus, our results indicate that waterhemp seeds become mature in as little time or less than that required for seeds of these other species.

One implication of the results of this study is directly applicable to scientists crossing waterhemp plants to perform progeny analysis or for bulk seed production. For rapid generation advancement in this species, one apparently need not wait more than 14 DAP to collect seeds from female plants. In fact, it is possible to collect many mature seeds by as early as 10 DAP if these seeds are dried immediately after harvest and then stratified for at least 10 d. Thus, potentially in as little as 20 d after flowering begins, one may begin growing the next generation of waterhemp plants. For long-term seed storage, however, as may be desirable in situations where the objective of the cross is simply to increase seed supply, it may be advisable to wait until at least 20 DAP before collecting seeds. Our results suggest that by this time the seeds may be safely stored at low temperatures without any obvious negative effects on viability.

From this study we conclude that waterhemp seeds become mature less than 2 wk after pollination has occurred. Seed color darkens and seed weight increases linearly until approximately 12 DAP. Finally, waterhemp seeds exhibit high levels of dormancy at an early age, and these dormancy levels begin to drop between 15 and 30 DAP. More research is needed to determine whether environmental or biotypic effects significantly impact seed maturation time in waterhemp.

Sources of Materials

[1] LC1 professional growing mix, Sun Gro Horticulture Canada Ltd., 52130 RR 65, P.O. Box 189, Seba Beach, AB 70E 2BO, Canada. Distributed by Sun Gro Horticulture Distribution Inc., 15831 N.E. 8th St., Suite 100, Bellevue, WA 98008.

[2] Scotts Osmocote Classic 13-13-13 slow-release fertilizer, The Scotts Company LLC, 14111 Scottslawn Rd., Marysville, OH 43041.

[3] SPOT Insight QE color model 4.2.1, Diagnostic Instruments, Inc., 6540 Burroughs St., Sterling Heights, MI 48314.

[4] Nikon SMZ800 stereoscopic zoom microscope, Nikon Inc., 1300 Walt Whitman Rd., Melville, NY 11747-3064.

[5] SPOT imaging software, version 3.5.0 2002, Diagnostic Instruments, Inc., 6540 Burroughs St., Sterling Heights, MI 48314.

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