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While They Were Asleep: Do Seeds After-Ripen in Cold Storage? Experiences With *Calendula*

Mark P. Widrlechner
USDA-ARS North Central Regional Plant Introduction Station, Ames, Iowa 50011-1170 U.S.A.
Email: isumw@iastate.edu

INTRODUCTION

Methods to break seed dormancy are of great interest to plant propagators, with many papers on this topic presented at past I.P.P.S. meetings. For example, in Vol. 54 of our Combined Proceedings of the International Plant Propagators' Society, there were reports on embryo culture to avoid dormancy (Douglas, 2004) and recommendations on dormancy-breaking techniques for *Helleborus* (Bush, 2004), *Salvia* (Navarez, 2004), and many wildflowers and grasses native to the North Central U.S.A. (Diboll, 2004). As propagators, we typically want quick methods that consistently result in high germination rates without large labor inputs. But if we can afford to be more patient, some seeds may eliminate their primary dormancy mechanisms during storage. This progressive loss of dormancy after maturity in "air-dry" seeds is known as after-ripening (Murdoch and Ellis, 2000). Typically, after-ripening is thought to occur under warm, dry conditions (Foley, 2000; Probert, 2000), but the literature of after-ripening is somewhat confusing. Simpson (1990) defined after-ripening in a more general way as "loss of the dormant state over some period of time through exposure of the seeds to a set of environmental conditions after maturation and separation from the parent plant." The term has even been used to describe combinations of warm storage and the effects of stratification (Baskin and Baskin, 1988).

Murdoch and Ellis (2000) and Probert (2000) noted that the rate of after-ripening increases with temperature in a predictable manner, and many studies on after-ripening focus on storage conditions at or above room temperature in order to get the most rapid loss of dormancy (Ellis et al., 1983; Liao et al., 2000). Probert (2000) reported that optimal rates of after-ripening commonly occur between 40 and 50 °C. However, there are also a few reports of slow after-ripening processes occurring at lower temperatures (below 10 °C), within a range typically used for medium-term seed storage (Widrlechner, 1991). For example, Schonbeck and Egley (1980) reported that, between 0 and 5 °C, after-ripening of redroot pigweed (*Amaranthus retroflexus* L.) seeds proceeded much more slowly than at higher temperatures, but that it was clearly observable after 6 months of storage. Cohn and Hughes (1981) were able to detect a loss of primary dormancy in a weedy rice (*Oryza sativa* L.) after 11 months of dry storage at 5 °C. And, by testing a range of different temperatures between 8 and 38 °C on dormancy-loss rates in malting barley (*Hordeum vulgare* L.), Favier and Woods (1993) found that the same loss of dormancy that took 10 days to achieve at 38 °C was observed in 100–120 days at 8ºC.

Murdoch and Ellis (2000) and Probert (2000) pointed out that seed moisture content is another important determinant of the rate of dormancy loss in after-ripening, reviewing evidence that after-ripening typically proceeds most quickly when cereals are held at 11%–15% moisture content, but slowing at lower moisture contents, and minimal below 8%, with some exceptions (Foley, 1994). So as seeds are dried and cooled to increase their longevity in storage, the after-ripening process could be

slowed two ways. Do these two phenomena (low temperature and low moisture content) interact to eliminate after-ripening, or do they just slow it down? Those of us who study ornamentals are often patient people, but how often do we have enough patience to design relevant experiments that might last 10, 20, or more years?

Fortunately, sometimes we don't need to design new experiments to gather sound data that shed light on slow-moving phenomena. In this paper, I present an example using historical germination data from the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) to help determine whether seeds in medium-term storage are just "asleep" (i.e., quiescent) or are also slowly losing their dormancy. I will focus solely on seeds of the ornamental genus *Calendula*, which includes both annuals and half-hardy perennials, native to the Mediterranean region and deserts of northern Africa and the Middle East. Although I have found no published reports of after-ripening in *Calendula*, it seems a likely candidate for investigation since after-ripening is widespread in species adapted to survive seasonal drought (Probert, 2000).

I have been working with *Calendula* seed production for more than 20 years and, during that time, have come to realize that when a sound seed lot is produced with a low initial-germination rate, if I am patient, I typically will be rewarded. Thus, when the topic of after-ripening being a warm-temperature phenomenon was discussed at the 2005 Eastern Region Annual Meeting in Atlantic City, I remembered *Calendula* and decided to evaluate our germination data to see whether it would be possible to document slow after-ripening under our medium-term storage conditions.

MATERIALS AND METHODS

Germination data from all seed lots of *Calendula* accessions regenerated and stored at the North Central Regional Plant Introduction Station were downloaded from the internal database of the Germplasm Resources Information Network (GRIN). Seeds were stored at 4 ºC and at 40% relative humidity (RH) until 1991 and at 25% RH thereafter. Germination data were retained for further analysis only when the same seed lot was tested on three or more occasions over at least 9 years of storage. This resulted in the retention of 81 tests of 24 stored seed lots, including seven accessions of *C. officinalis* L., six of *C. arvensis* L., five of *C. suffruticosa* Vahl, two of putative hybrid populations, and two unidentified to species.

Specific test conditions were documented for all but six of the 81 tests. Of those with documented test conditions, each was conducted on 200-seed samples, either as two replicates of 100 or four reps of 50. All were conducted in plastic boxes on top of moist blotter paper with alternating light and darkness, and germination counts were made at 7, 14, and 21 days after test initiation. The most common test condition (49 of 81 tests) was at 20 °C, with 8 to 12 h of light daily and the addition of 0.1% KNO_3. The other tests were conducted at 15 °C (6 tests) or alternating 20/30 °C (3 tests) instead of at 20 °C, or involved the addition of a 7-day, moist pre-chill before the test, either at 20 °C (12 tests) or at alternating 20/30 °C (5 tests).

Although the GRIN database includes germination data on normal and abnormal seedlings and dormant and dead seeds, only the normal seedling data were considered to be consistently reliable. The inventory lot code for the seed sample, its age at the time of the test (rounded to the nearest half-year), and the percentage of normal seedlings were copied to an Excel spreadsheet for manipulation. Data on the percentage of normal seedlings were then standardized, a process that al-

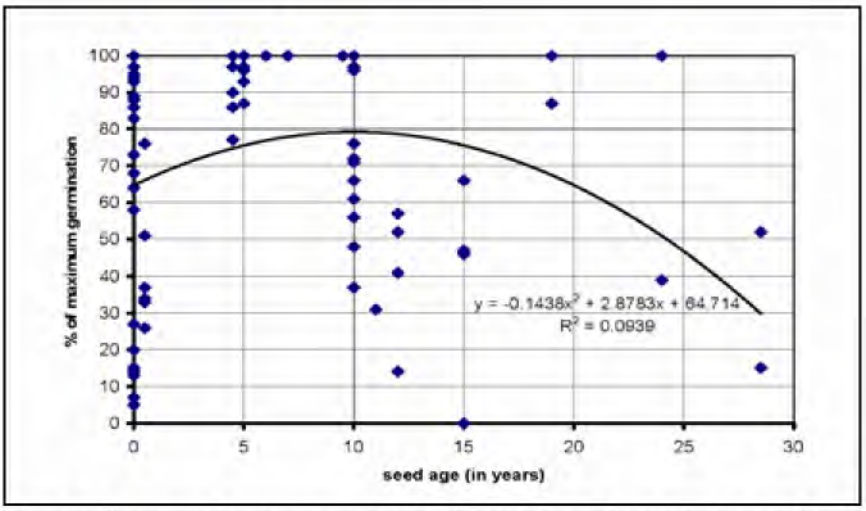

Figure 1. Percentage of maximum germination of *Calendula* seed lots in relation to seed age.

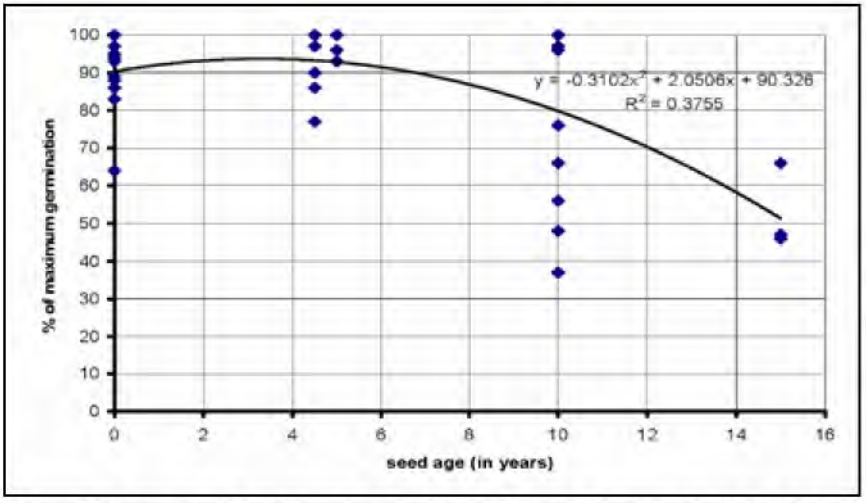

Figure 2. Percentage of maximum germination of *Calendula* seed lots with high initial germination tests (56% to 93%) in relation to seed age.

lows data from different tests to be compared. This was accomplished by coding the highest percentage recorded for each seed lot as "100%" and coding all tests with lower percentages as a percentage of the highest test result. Standardized data, denoted as percentage of maximum germination, and seed lot age were transferred to SAS (SAS Institute, 2003) for both linear and quadratic regression analyses. If the percentage of maximum germination is a product of the proportion of viable seeds (which decreases through seed aging) and the proportion of nondormant seeds (which increases through after-ripening), then one might expect a quadratic

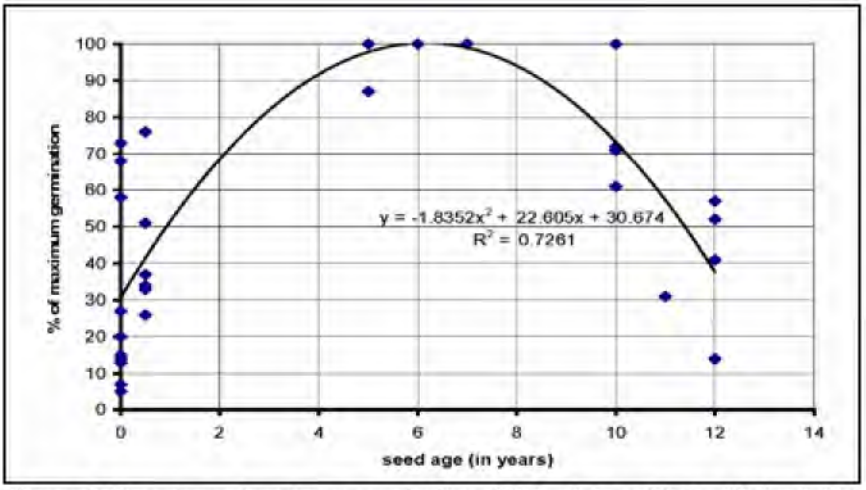

Figure 3. Percentage of maximum germination of *Calendula* seed lots with low initial germination tests (3% to 43%) in relation to seed age.

relationship, beginning with low values, rising to some peak as dormancy is eliminated, and then falling as seeds die in storage.

RESULTS AND DISCUSSION

When seed age was plotted against the percentage of maximum germination for all 81 tests (Fig. 1), no clear pattern was evident. The linear regression was not significant (data not shown), but the quadratic regression (Fig. 1) was significant at the $p < .05$ level, suggesting a weak relationship. Perhaps most importantly, this plot does indicate that *Calendula* seeds are relatively long-lived (25 to 30 years) under these storage conditions.

I then created two subsets of accessions from the entire data set: 11 lots with initial percentages of normal seedlings (raw data) above 50% (range 56% to 93%), and 10 lots with initial percentages below 50% (range 3% to 43%). Three lots were omitted from further analysis because they did not include test results conducted on seeds that were less than 1 year old. There were no statistically significant associations between individual species or for wild vs. domesticated populations and assignment to the two subsets.

Seed lots with high initial germination (Fig. 2) showed only very modest after-ripening effects, with an estimated peak of 94% of maximum germination attained about 3.5 years after seed production, but a significant quadratic regression ($p < .001$) and a weaker linear relationship ($R^2 = .25$). In contrast, seed lots with low initial germination (Fig. 3), likely the most dormant ones and perhaps also of lower overall quality, showed strong (and significant, $p < .0001$) increases in normal germination during initial storage, with an estimate of 100% of maximum germination reached about 6 years after seed production, but also a more rapid decline after reaching that peak.

This retrospective analysis strongly suggests that some *Calendula* seed lots after-ripened over a period of years while held at 4 ºC and 40% RH until 1991 and

25% RH thereafter. However, the diverse germination test conditions incorporated into this analysis add a degree of uncertainty to the findings. And the possibility of some alternative explanation, such as the death of seed-borne pathogens in storage allowing the percentage of normal seedlings to increase during storage, cannot be evaluated from these data. Therefore, it would be prudent to consider these findings more as a source of testable hypotheses about slow after-ripening, rather than as definitive conclusions. Ideally, the resulting hypotheses should be used to design long-term storage experiments on well-defined, healthy seed lots with all germination tests conducted under uniform conditions.

Two specific ideas for practical propagation also emerge from this study. First, typical dormancy-breaking techniques may not be required for seeds that have spent many years in cold storage. Second, if one receives new seeds with unknown dormancy requirements, it might be wise to retain a subset in cold, dry storage for at least a few years. And finally, as an important sidelight to this work, there is evidence from rice (Cohn and Hughes, 1981) that low-temperature after-ripening is more effective when seeds are first held at room temperature for a week before transfer to cold storage. As a standard practice with ornamental seed regenerations at the NCRPIS, we hold new lots at room temperature for at least 2 months before transfer to cold storage to allow for some degree of more rapid after-ripening.

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