Involvement of Endogenous Abscisic Acid in Onset and Release of *Helianthus annuus* Embryo Dormancy

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**ABSTRACT**

Mature seeds of *Helianthus annuus* L. exhibit dormancy that is eliminated during storage in dry conditions. **In vitro** culture of immature embryos isolated at different times after anthesis showed that the youngest embryos are able to germinate, but within the third week after pollination, dormancy progressively affected most of the embryos. A radioimmunoassay showed that the endogenous abscisic acid (ABA) level, which increased sharply in the first half of the development period, fell at precisely the moment when embryo dormancy became established. An application of fluridone, before the increase of ABA level, prevented both ABA synthesis and development of embryo dormancy. Applied later, after the rise of the ABA level, fluridone could not prevent embryo dormancy development. Dormancy thus appears to be dependent on ABA synthesis but not concomitant with its accumulation; it must therefore be induced by ABA during maturation. Furthermore, a preincubation in water allowed dormant embryos to germinate. This acquisition of germinability could not be directly related to a leaching of free ABA. Possible effects of this treatment are discussed.

The classic concept of the hormone balance theory supposed that induction, maintenance, and release of seed dormancy were regulated by the simultaneous action of promotive and inhibitory hormones. Recent work with hormone deficient mutants of *Arabidopsis thaliana* and *Lycopersicon esculentum* led Karssen and Groot (8) to a revision of this theory. In particular, it appears that ABA is responsible for the onset of dormancy in developing seeds but that it does not control the maintenance of dormancy in mature seeds. However, the authors noted that this conclusion might be valid for all species with so-called ‘coat-imposed dormancy’ but that direct evidence is missing to invoke the same hormonal control for embryo dormancy.

Our first objective, then, was to look for the involvement of ABA in the onset of the dormancy of an embryo. Our plant material, *Helianthus annuus* seeds, exhibited at harvest a dormancy which could be eliminated by storage in dry conditions, and **in vitro** culture showed that embryos themselves were dormant. Furthermore, in this species, the lack of dormancy shown by a nondormant white mutant strain was correlated by Wallace and Haberlan (16) with the absence of any detectable amounts of colored carotenoid pigments. In the light of recent knowledge about the synthesis of ABA through the carotenoid pathway (17), it may be suggested that the lack of dormancy must be correlated to the lack of ABA synthesis. This species appeared to be a good material to try to modify the physiological behavior of the embryo through an alteration of ABA synthesis. So from this perspective, we applied to the seeds during their development a solution of fluridone, a pyridinone inhibitor of carotenoid biosynthesis, in order to decrease their endogenous ABA levels. This approach allowed us to demonstrate the responsibility of ABA in the induction of the dormancy of the embryo.

On the other hand, these dormant embryos could be germinated after a preincubation in water. This treatment, which evokes the leaching of an inhibitor, was imposed to discuss the involvement of ABA in maintenance of dormancy.

**MATERIALS AND METHODS**

**Plant Material**

Plants of *Helianthus annuus* cv Mirasol were grown in the field during summer. In September flowers of d sterile plants were manually pollinated by pollen harvested on small f sterile flowers (seeds generously provided by Cargill). Flowers were tagged and embryos were isolated from seeds harvested at different times after pollination. For each age (10, 13, 17, 21, 26, 32, or 36 DAP) 20 to 30 embryos, *i.e.* a sample considered to be representative of the population, was used for **in vitro** culture or extraction. Furthermore, all the experiments were carried out twice or three times in different years (1986, 1987, 1988, 1989).

**In Vitro Culture**

Twenty-four embryos were aseptically isolated and cultured on Heller’s medium (macro- and microelements), supplemented with sucrose (20 g dm⁻³) and agar (6 g dm⁻³). The cultures were carried out at 23°C under white fluorescent light (45 W m⁻², 16 h d⁻¹). An embryo was considered to have germinated when the elongation of the radicle was clearly visible (2.0 mm). Growth of the seedling was recorded during a 1 month period.

In some cases, an incubation of 20 embryos in 100 mL water during 24 h was performed on a rotative shaker (3 rev. min⁻¹).

1 Abbreviations: DAP, day(s) after pollination; RIA, radioimmunoassay; fluridone, 1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4-(1H)-pyridinone.
The pellet for stirred chilled as control for Procedure Extraction (b).

DAP 3.

Figure 1. Changes with time after pollination in the fresh (○) and dry (■) weights and in water content (□) expressed as percent of fresh weight.

Figure 2. Changes with time after pollination in total embryo germination percentage observed after 14 d of culture on basal medium.

Figure 3. Changes with time after pollination in embryo ABA content for control seeds (a) and for seeds treated with fluridone on the 8th DAP (b). (Data are means of 8 individual estimations ± SE.)

Extraction Procedure

Thirty isolated embryos were ground in a mortar with chilled 80% methanol containing 2,6-di-t-butyl-4-methyl phenol as anti-oxidant (100 mg/L). The homogenate was stirred for 2 h at 4°C and centrifuged for 10 min at 2000g. The pellet was reextracted twice with the same volume of cold 80% methanol. The supernatants were collected and evaporated under reduced pressure at 40°C. The remaining aqueous extract was adjusted to pH 3.0 and extracted four times with diethyl ether (v/v).

Endogenous ABA Determination by Radioimmunoassays

ABA was quantified by RIA performed exactly as described earlier (12). Anti-ABA antibodies were obtained by immunization of rabbits with (+) ABA-human serum albumin conjugate obtained via a mixed anhydride reaction. The tracer was the iodinated (125I) derivative of the conjugate obtained by coupling (+) ABA to the peptide glycy1-l-tryosin. Radioimmunological incubations were performed by equilibrium dialysis.

A study of the cross-reactivities of structurally related compounds showed the specific binding of (+) ABA, when the acid function was free, esterified or linked by amid linkage. The conversion of the carboxyl group of ABA into an amide inducing an increase in sensitivity of the free (+) ABA estimation, allowed ABA values in an extract to be calculated by differential measurement before and after amidation (12).

Each value was the average of results obtained from four different dilutions of the extract (two replicates for each).

Fluridone Application

Fluridone, generously provided by Lilly and Co., was applied in 1% (v/v) acetone-water with a paintbrush. One-half of the seeds of a flower were treated with 100 μg/mL fluridone. Then the seeds were allowed to develop in situ in the field.

RESULTS

In vitro Physiological Behavior of Embryos Isolated at Different Times after Pollination

Nearly invisible to the unaided eye at the 4th d after pollination, the embryo developed very rapidly and its fresh weight reached its maximum (about 55 mg), on the 22nd d. The dry weight became stable (around 40 mg) from the 26th d, and water content decreased progressively till the 44th d (Fig. 1)

The change of the germination percentages observed for excised embryos after 14 d of culture is shown in Figure 2. The youngest cultured embryos were isolated 7 d after polli-
1166 Plant or dormant agitated Figure 4. B, 1989

Figure 4. Changes during culture of total germination percentage of dormant embryos isolated 26 DAP and cultured on basal medium directly (a), on a medium supplemented with GA$_3$ at $2.75 \times 10^{-6}$ M (b1) or $5 \times 10^{-6}$ M (b2), or on basal medium after a preincubation in agitated water during 24 h (c1) or during 3 d (c2). A, 1987 experiment; B, 1989 experiment.

Figure 5. Changes with time after pollination in ABA leached in water during a 24 h incubation of isolated embryos (data are means of 8 individual estimations ± SE).

nation (7–8 mg fresh weight). Most of them were able to ‘germinate’ but their behavior was heterogeneous: growth of root was slow and late; the hypocotyl grew irregularly; no epicotyl was observed. All the embryos isolated 10 and 13 d

Figure 6. Changes, during culture, of total germination percentage of 36-d-old embryos isolated from control seeds (a) or fluridone-treated seeds (b, c, d) and cultured on basal medium (a, b) or on a medium supplemented with GA$_3$ (2.75 × 10$^{-6}$ M) (c) or ABA (3.75 × 10$^{-7}$ M) (d). After pollination (about 30–35 mg) germinated; many of them gave a normal plantlet with typical elongation of primary root, hypocotyl, and epicotyl. Some of the oldest already showed some difficulty of growth, in particular an inverse gravitropism of the roots and a twisted hypocotyl. Furthermore, the maximum germination percentage, obtained during the first week of culture for 10-d-old embryos, required 2 weeks for 13-d-old embryos. The germination rate continued to slow down progressively with older embryos. The 17-d-old embryos showed very peculiar behavior: deep greening and considerable enlargement of cotyledons occurred in all of them during the first week of culture; the growth of a tiny root was observed later for 50% of them; no hypocotyl elongation was observed. Twenty-one-d-old embryos germinated at a low rate. Other embryos showed the typical asymmetrical behavior of cotyledons: the cotyledon in contact with culture medium became large and green, whereas the upper one remained small and white. On the 26th d after pollination, dormancy was well established, and most of the embryos remained white and did not germinate.

ABA Level Changes with Time from Pollination

Changes in ABA levels showed a pattern similar to that which was often described during seed development (Fig. 3, curve a). The steep rise observed from the 7th until the 13th d was followed first by a sharp drop then by a slight shoulder as the seeds matured.

Consequences of an Incubation of the Embryos in Agitated Water

If, before being cultured in agar medium, isolated embryos were incubated in agitated water, their germination was improved, whatever their age (Table I). However, at the moment when embryo dormancy developed, difficulty of growth appeared; atypical germinations with tiny roots preceded by greening and enlargement of cotyledons were then observed in about 50% of the samples. When embryo dormancy was
well established, this incubation in agitated water allowed the normal germination to occur very rapidly; at this moment, gibberellic acid also induced a high germination percentage. The results obtained in two different years can be compared in Figure 4. In 1987 (Fig. 4A), a 24 h incubation or a relatively low concentration of gibberellic acid (2.75 \times 10^{-6} \text{ M}) was sufficient to allow a high germination percentage to be observed. In 1989 (Fig. 4B), the dormancy was deeper than in 1987; a longer incubation (72 h) and a higher concentration of gibberellic acid (5 \times 10^{-5} \text{ M}) were necessary.

When the water used for incubation of embryos was analyzed by RIA, free ABA could be detected at a level which increased till a maximum for 13-d-old embryos and then decreased and fluctuated at a lower level, reached for 21-d-old embryos (Fig. 5).

**Physiological Consequences of an Application of Fluridone on Maturing Seeds**

The physiological consequences of an application of fluridone to maturing seeds depended on the time of application. Applied early, on the 8th (1988) or 10th (1987) d after pollination, fluridone prevented the development of embryo dormancy. Embryos allowed to continue their development on the plant and isolated when they were 36 d old (*i.e.* near the maturity of the seeds) were able to germinate in much higher percentage (Fig. 6, curve b) than control embryos originating from non-treated seeds (Fig. 6, curve a). The germination percentage could still be enhanced a little by addition of GA_{3} in the culture medium (Fig. 6, curve c) or by a 24 h preincubation in water (Fig. 7). Similar improvement could also be observed for embryos isolated earlier during the development (18–25 DAP). On the contrary, germination was nearly (Fig. 6, curve d) or totally inhibited by ABA introduced in the culture medium at, respectively, 3.75 \times 10^{-7} \text{ M} and 3.75 \times 10^{-8} \text{ M}.

If the application of fluridone was repeated two, three, or four times at weekly intervals, high percentages of germination were again observed. Moreover, water contents of the embryos regularly determined from the day of fluridone application to the 44th d were not significantly modified by these treatments. A single application of fluridone on the 19th or 25th DAP was ineffective (Table II). Germination percentages of mature embryos were not improved compared to the control.

**Changes of ABA Levels after Fluridone Application**

ABA levels were determined at different times after a single treatment applied on the 8th DAP (Fig. 3, curve b). Whatever the age of the embryo, the ABA content was low, and in particular, the maximum ABA content described in control 13-d-old embryos was not observed.

**DISCUSSION**

Cultured *in vitro*, 7-d-old embryos of *Helianthus annuus* showed some difficulties of growth, linked to an incomplete morphogenesis. This type of development is known as ‘precocious germination’ (15), and some complex medium with growth substances and vitamins would be needed to obtain normal viable plants (1).

The very rapid embryogenesis allowed the embryos to exhibit normal development as soon as they were 10 or 13 d old. Germination was rapidly obtained for nearly all the 10-d-old embryos on the third day of culture, but it was delayed for the 13-d-old embryos, when ABA content was at its maximum—a lag phase occurred, before germination could take place in all the individuals. A similar lag phase proportional to the ABA content has been previously described in non-dormant embryos of *Phaseolus vulgaris* (14).

Later on, between the 13th and the 26th DAP, the physiological status of the embryos completely changed; the appearance of abnormal germinations and the lowering of the germination percentages obtained even after 1 month of culture were the symptoms of an embryo dormancy that developed early in the course of seed maturation and increased progressively within a 10-d-long period (Fig. 2). Nevertheless, this embryo dormancy became established at the moment when the endogenous free ABA level fell (Fig. 3).

Since many arguments have recently appeared in favor of ABA biosynthesis through a carotenoid pathway (13), fluridone, a pyridinone inhibitor of carotenoid biosynthesis (2), was applied on the seeds. If the application of fluridone took place early, before the increase of ABA level, it prevented both ABA synthesis (Fig. 3, curve b) and development of...
embryo dormancy (Fig. 6, curve b). However, the germination of these nondormant embryos could still be inhibited by exogenous ABA at 3.75 × 10⁻⁷ or × 10⁻⁶ m. Applied later, on the 19th or 25th DAP, after the rise of the ABA level, fluridone did not prevent development of embryo dormancy (Table II). Dormancy thus appears to be dependent on ABA synthesis but not concomitant with its accumulation; dormancy must therefore be induced by ABA during maturation. This perspective was introduced by Karsen et al. (7) and Groot (6). Studies with the ABA mutant of Arabidopsis presented the first clear evidence that endogenous ABA is essential for the onset of dormancy during seed development.

Fong et al. (5) were able to induce vivipary in developing maize seeds by fluridone on condition that it was applied early. Their results indicate that there are important developmental changes occurring at the 11th DAP which reversibly commit the immature embryo to vivipary or dormancy. Our results proved that changes did not appear suddenly in sunflower seeds but were established progressively as ABA increased. Even in the case of a first application on the 8th DAP and of repeated applications, vivipary could not be observed in H. annuus seeds. Water content itself changed in a way similar to that which was observed in control. It may be that the behavior of the two species is different, or the time of application was already too late. The second hypothesis is supported by the persistence of a slight embryo dormancy since germination could be slightly improved by addition of exogenous GA₃ or by preincubation in water.

Indeed, for dormant H. annuus embryos, a 24 h long incubation in water can induce germination (Table I). The improvement of germination percentage by culture on a liquid medium previously observed for Taxis baccata (9) or Pyrus malus (3) embryos could be related to a leaching of ABA. It be so, germination could be obtained by simply the leaching of the inhibitor, that is to say that ABA still controlled the maintenance of dormancy. This point of view is not shared by Karsen and Groot (8) from their work with mutants. But it should be noticed that for T. baccata or P. malus embryos, not only free ABA but also its conjugates must be taken into account (4, 10). In the case of H. annuus embryos, RIA allowed ABA to be detected in the incubation water at a level proportional to the endogenous level of free ABA in the embryo (Fig. 5), but antibodies did not cross-react with oxidative products (phaseic acid, dihydrophaseic acid), and the whole ABA metabolism should be taken into account (11). Further studies, including metabolism of 2-¹⁴C-ABA, are currently in progress to look into the involvement of ABA in the control of maintenance of dormancy, taking into account biosynthesis, release from conjugates, and catabolism.

The germination of dormant H. annuus embryos could also be obtained by addition of exogenous GA₃ in the culture medium. Karsen and Groot (8) proposed that on the one hand, ABA does not control the maintenance of dormancy, and on the other, GAs are absolutely required for germination.

In this hypothesis, the role of preincubation in water should also be explained by the induction of an increased sensitivity to endogenous GAs. The observation that the elimination of a deep dormancy (Fig. 4B) needed either a longer preincubation in water or a higher concentration of GA₃, rather than a weak one (Fig. 4A) argues in favor of this hypothesis. Endogenous GAs will be analyzed to provide an experimental basis for discussion of this point.

**LITERATURE CITED**