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Restoration of Seed Germination at Supraoptimal Temperatures by Fluridone, an Inhibitor of Abscisic Acid Biosynthesis

Toshihito Yoshioka[1], Takashi Endo and Shigeru Satoh

Laboratory of Bio-adaptation, Graduate School of Agriculture, Tohoku University, Aoba-ku, Sendai, 981-8555 Japan

Fluridone, an inhibitor of ABA biosynthesis, restored the seed germination of lettuce (*Lactuca sativa* L. cv. Grand Rapids) and many other plant species at supraoptimal temperatures. ABA content in lettuce seeds after imbibition quickly decreased at 23°C, but not at 33°C (a supraoptimal temperature). Fluridone caused a decrease in ABA content at 33°C, which suggests that the maintenance of high ABA content could be responsible for high-temperature inhibition of germination of lettuce seeds. This probably results from an increase in the rate of ABA biosynthesis at the higher temperature. The present study indicates that ABA plays a decisive role in the regulation of seed germination at supraoptimal temperatures.

Key words: Abscisic acid (ABA) — Fluridone — Lettuce (*Lactuca sativa*) — Seed germination — Thermodormancy — Thermoinhibition.

The range of temperature suitable for seed germination varies with the plant species. Germination of nondormant seed is inhibited at high temperatures that do not prevent normal growth of shoots and roots. This inability of seeds to germinate at supraoptimal temperatures is called high-temperature inhibition or thermoinhibition (Reynolds and Thompson 1971, Mayer and Poljakoff-Mayber 1982, Abeles 1986, Valdes and Bradford 1987, Gallardo et al. 1991). In some species, after a long exposure to high temperature, secondary dormancy (thermodormancy) is induced, and the seeds fail to germinate even after transfer to lower temperatures (Khan and Samimy 1982).

Lettuce achenes (seeds) germinate at 10 to 22°C but not at temperatures above 25–30°C; the threshold temperature varies with the cultivar (Reynolds and Thompson 1971, Gray 1975, Thompson et al. 1979, Abeles 1986). This threshold is raised or lowered by application of growth regulators such as gibberellins, cytokinins, ethylene, and ABA. In lettuce seeds, gibberellins (Hegarty and Ross 1979), cytokinins (Reynolds and Thompson 1971, 1973), and ethylene (Abeles and Lonski 1969, Abeles 1986) expand the range of temperature at which they can germinate in darkness; ABA decreases the temperature range (Reynolds and Thompson 1971, 1973).

Exogenous ABA inhibits germination of lettuce seeds induced by GA_3 or red-light (Khan 1968, Sankhla and Sankhla 1968); the level of endogenous ABA decreases more rapidly under conditions that allow germination than under those that inhibit it, although a decrease in the endogenous level of ABA is not always correlated with germination (Braun and Khan 1975). Khan (1975) pointed out a similarity between the effects of ABA and high temperatures. However, it is still uncertain which of the endogenous hormones play decisive roles in thermoinhibition of seed germination.

Fluridone, 1-methyl-3-phenyl-5-[3-trifluoromethyl-(phenyl)]-4-(1*H*)-pyridinone, is an inhibitor of phytoene desaturase, which converts phytoene to phytofluene in the pathway of carotenoids biosynthesis (Bartels and Watson 1978, Fong and Schiff 1979). Carotenoids are the main precursors of ABA in plants (for review, Quatrano et al. 1997). Thus, the inhibition of carotenogenesis should also prevent the biosynthesis of ABA. Recently, fluridone has been found to be a useful tool in such studies (Stewart and Voetberg 1987, Xu and Bewley 1995, Popova and Riddle 1996).

In the present study, we examined the effects of fluridone on germination of lettuce seeds at a supraoptimal temperature and analyzed how the ABA content in lettuce seeds was affected by temperature and application of fluridone. The effects of fluridone on the seeds of various other kinds of plants at supraoptimal temperatures were also examined.

Materials and Methods

Plant seeds—Lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds were purchased from Takii & Company, Ltd. (Kyoto, Japan). Seeds of the other horticultural crops, *Freesia hybrida* Hort., *Chrysanthemum parthenium* Bernh., and *Cryptomeria japonica* Hassk., were obtained from Sakata Seed Co. (Yokohama, Japan). All seeds were stored at 4°C in a desiccator.

Weed seeds were collected in the suburbs of Sendai, Japan, at seasons of maturation in 1995 and 1996. After harvest, they were left to dry for several months at 20–25°C, and then stored as above. The species used were *Agrostis alba* L., *Bromus catharticus* Vahl., *Cardamine flexuosa* With., *Cerastium glomeratum* Thuill., *Conyza canadensis* (L.) Cronquist, *Dactylis glomerata* L., *Festuca rubra* L., *Gnaphalium japonica* Thunb., *Lolium perenne* L., *Medicago sativa* L., *Plantago lanceolata* L., *Poa annua* L., *Poa pratensis* L., *Senecio vulgaris* L., *Stellaria neglecta* Weihe,

[1] Corresponding author: fax 81-22-717-8834; e-mail yoshi@bios.tohoku.ac.jp

Taraxacum officinale Weber, *Trifolium repens* L., *Vicia angustifolia* L., and *Youngia japonica* (L.) DC.

Germination test—In most experiments, 25 or 50 seeds were selected for uniformity in size and sown in a square petri dish (5×5 cm^2) with two layers of filter paper and 4 ml of water or fluridone solution at designated concentrations. The petri dish was wrapped in a sheet of aluminum foil and incubated in darkness at temperatures of 18, 23, 28, or 33°C. At designated times after the start of incubation, germinated seeds were scored. Germination tests were made in triplicate, and data are shown as the mean ± SE.

In the experiment in which the effect of fluridone on the sensitivity of lettuce seeds to ABA was examined, seeds were incubated with 0.4 M polyethyleneglycol in the presence or absence of 30 μM fluridone at 33°C for 120 h in darkness. The polyethyleneglycol was used to prevent seed germination during the incubation with fluridone. Then, after being rinsed with water and blotted on filter paper, the seeds were subjected to a germination test at 23°C for 24 h in the presence or absence of 10, 30, or 100 μM ABA.

Since most seeds sensitive to high temperature are also sensitive to light (Mayer and Poljakoff-Mayber 1982), we conducted germination tests in the dark to avoid complications caused by the effect of light.

Assay of ABA contents—Three 1 g (initial dry weight) replicates of lettuce seeds were incubated at 23 or 33°C with or without 30 μM fluridone in darkness. The seeds were collected at designated times after the start of incubation and stored at −30°C until use.

The ABA contents were determined as follows. Each frozen sample was pulverized with a mortar and pestle in liquid N_2, and ABA was extracted with 9 ml of 80% methanol. To the extract, 2,640 ng of *trans,trans*-ABA was added as an internal standard. The extract was evaporated under vacuum to the aqueous solution (< 1 ml), which was passed through a Supelclean LC-18 SPE tube (1 ml, Supelco) and eluted with 1 ml of 60% methanol. Then the sample was passed through a Supelclean LC-NH_2 SPE tube (1 ml, Supelco) and eluted with 1% of acetic acid in methanol. The eluate was concentrated to 50 μl under vacuum and the concentrate was taken up in 100 μl of 20% methanol.

The solution was analyzed by HPLC (Shimadzu LC-10, column: Wakosil-II 5C18 HG, 4.6 mm i.d. × 25 cm; column temperature 40°C; flow rate 1.5 ml min^{-1}; step-wise linear gradient elution with methanol, 20 to 35% at 0 to 0.5 min, 35 to 40% at 0.5 to 15 min, 40 to 100% at 15 to 15.1 min, and 100 to 35% at 15.1 to 25 min; detection: UV 254 nm). *trans,trans*-ABA was eluted at 9.7 min and *S*-(+)-ABA at 12.0 min. The amount of ABA determined by HPLC assay was nearly the same as that determined by an enzyme-linked immunosorbent assay with the Phytodetek-ABA kit (Idetek, San Burno, CA, U.S.A.). The ABA fraction [*S*-(+)-ABA], separated by HPLC, was collected, methylated with diazomethane, and identified by GC-MS with a Shimadzu QP-2000 instrument equipped with a column DB-1 (J & W Scientific, 0.25 mm i.d. × 15 m, 0.25 mm film thickness) operating at 70 eV. The putative methylated ABA fraction showed an m/z spectrum identical to that obtained for the authentic *S*-(+)-ABA methyl ester: m/z (relative intensity); 278 (M^+, 1.2), 260 (5.9), 246 (4.5), 205 (8.6), 190 (100), 162 (45.8), 147 (13.4), 134 (53.1), 125 (40.9).

Chemicals—Fluridone was a gift from Mr. Kazuma Yamauchi of Dow Chemical Japan Co., and *S*-(+)-ABA from Professor Yasuo Kamuro of Gifu University. A specimen of *trans,trans*-ABA was purchased from Sigma Chemical Co.

Results and Discussion

Stimulation by fluridone of seed germination at supraoptimal temperatures—Fig. 1A shows the germination percentages of lettuce seeds in the presence or absence of 30 μM fluridone. In the absence of fluridone, 100, 92.3, 48.5, and 3.2% of the seeds germinated at 18, 23, 28, and 33°C, respectively. These results agree with those reported previously (Reynolds and Thompson 1971, Gray 1975, Thompson et al. 1979, Abeles 1986).

On the other hand, in the presence of fluridone at 30 μM, 99.3, 100, 98.2, and 97.6% of the seeds germinated at 18, 23, 28, and 33°C, respectively (Fig. 1A). In another experiment at 33°C, 0, 22.7, 56.3, and 97.6% of the lettuce seeds germinated in the presence of 0, 0.3, 3, and 30 μM fluridone, respectively.

We also used various species of winter crops and winter weeds to examine the effect of fluridone on seed germination at supraoptimal temperatures. *Chrysanthemum parthenim* and *Freesia hybrida* are hardy ornamental crops, and *Cerastium glomeratum* is a winter vegetable. The seeds of these species must be sown in cool seasons to avoid high-temperature inhibition of germination. The other plants used were winter weeds, which are known to germinate in spring and/or autumn, times of low maximum temperatures.

Results obtained with eight typical weeds are shown in Fig. 1B–I. In the absence of fluridone, 98.1% of *Stellaria neglecta* seeds germinated at 18°C, but only 12.7% at 23°C, and none at 28 and 33°C. In the presence of 30 μM fluridone, 100% of the seeds germinated at 23°C, but 0% at 28 and 33°C (Fig. 1B). In all other plant species presented in Fig. 1, the promoting effect of fluridone at supraoptimal temperatures is evident, although the range of temperature at which the promotion was differed from species to species.

Similar results were obtained in 12 other species (data not shown); *Agrostis alba, Cerastium glomeratum, Chrysanthemum parthenim, Conyza canadensis, Cryptotaenia japonica, Dactylis glomerata, Festuca rubra, Freesia hybrida, Medicago sativa, Plantago lanceolata, Trifolium repens*, and *Vicia angustifolia*. In contrast, no effect of fluridone was observed in 2 gramineous weeds, *Bromus catharticus* and *Lolium perenne* (data not shown). The seeds of these species were in deep primary dormancy after their shattering from the mother plants.

Effects of temperature and fluridone on the time course of lettuce seed germination—Fig. 2 shows the time course of lettuce seed germination at 23°C and at 33°C in the presence or absence of 30 μM fluridone. Although fluridone stimulated germination at 33°C, the timing was markedly delayed as compared with that at 23°C. Nearly 100% germination occurred by the 24th h after the start of imbibition at 23°C in the absence of fluridone, but similar

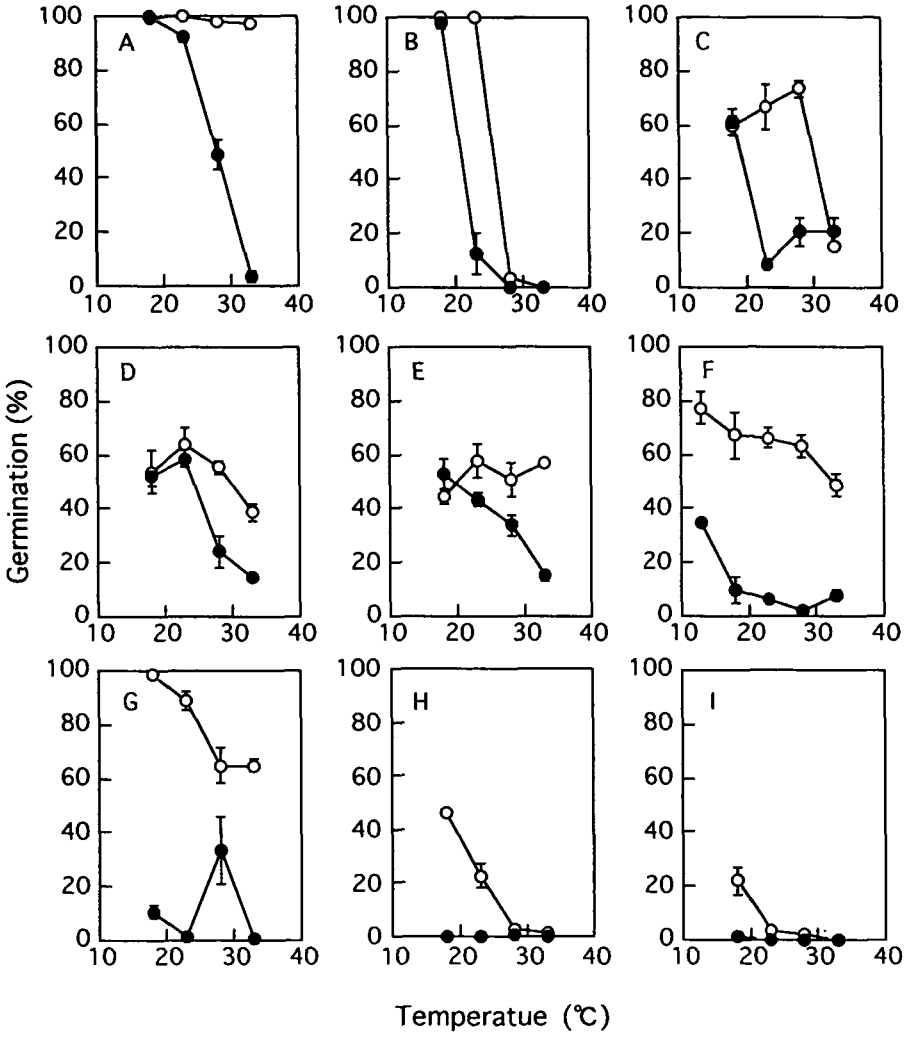

Fig. 1 Restoration by fluridone of seed germination at supraoptimal temperatures. In all experiments except for those with *Poa pratensis, Traxacum officinale*, and *Cardamine flexuosa*, 50 seeds of each respective species were incubated with (○) or without (●) 30 μM fluridone at given temperatures for two weeks in darkness. For *P. pratensis, T. officinale*, and *C. flexuosa*, 25 seeds were used, and the incubation period was extended to four weeks for *C. flexuosa*. Data are the mean ± SE of three replicated dishes. A, *Lactuca sativa*; B, *Stellaria neglecta*; C, *Gnaphalium japonicum*; D, *Poa annua*; E, *Youngia japonica*; F, *Traxacum oficinale*; G, *Senecio vulgaris*; H, *Cardamine flexuosa*; I, *Poa pratensis*.

germination was attained only at the 360th h at 33°C in the presence of 30 μM fluridone. In another experiment at 23°C, fluridone at 30 μM did not affect the rate and percentage of germination of lettuce seeds (data not shown). This result suggests that the delay of germination at 33°C in the presence of fluridone was not caused by phytotoxic effects of this chemical other than the inhibition of ABA biosynthesis.

Changes in ABA content of lettuce seeds incubated at different temperatures with or without fluridone—Since fluridone is an inhibitor of carotenoid and ABA biosynthesis (Bartels and Watson 1978, Fong and Schiff 1979, Stewart and Voetberg 1987, Le Page-Degivry et al. 1990, Xu and Bewley 1995, Popova and Riddle 1996) and exogenously-applied ABA inhibits seed germination (Khan 1968, 1975, Reynolds and Thompson 1971, 1973), we examined the effects of high temperature and fluridone on the ABA content in lettuce seed. Fig. 3 shows the changes of ABA content per one gram of initial dry weight of seeds during incubation.

Dry lettuce seeds contained 133.1 ng g^{-1} of ABA. The ABA content in seeds incubated at 23°C decreased soon after the start of imbibition. The values were 105.7 and 37.1 ng g^{-1} at the sixth and twelfth h of incubation, respectively. When seeds were imbibed and incubated at 33°C, ABA content remained constant at the initial level for 120

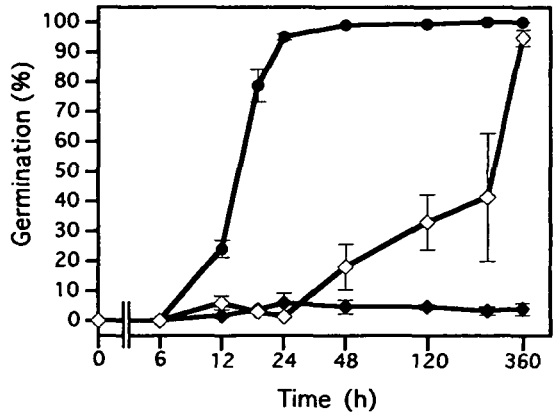

Fig. 2 Time course of germination of lettuce seeds at 23°C and 33°C in the presence or absence of 30 μM fluridone. Note that the abscissa is shown on a log scale. Each point is the mean±SE of triplicate petri dishes, each with 100 seeds. ●, 23°C without fluridone; ◆, 33°C without fluridone; ◇, 33°C with 30 μM fluridone.

h of incubation. Then it decreased to 109.1 ng g^{-1} at the 240th h and 82.0 ng g^{-1} at the 360th h of incubation. In the seeds imbibed and incubated at 33°C with 30 μM fluridone, the ABA content decreased more slowly than that at 23°C, but, after 48 h of incubation, the ABA content was 40.2 ng g^{-1}, which was comparable to the value attained in the seeds incubated at 23°C for 12 h. There might be two causes of the delay in the decrease of ABA content; the high temperature could affect the rate of metabolism (and degradation) of ABA and/or fluridone could not completely inhibit the ABA synthesis. We did not further investigate these possibilities. However, the delay in the decrease of ABA content at 33°C with fluridone clearly would correspond to the delay in seed germination under these conditions (Fig. 2).

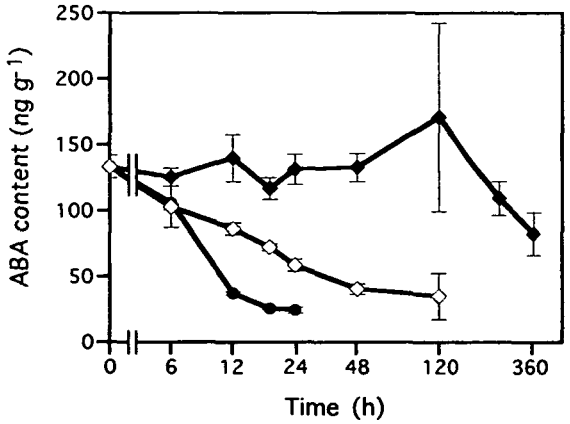

Fig. 3 Changes in ABA contents in lettuce seeds at 23°C and 33°C in the presence or absence of 30 μM fluridone. Seed samples of 1 g initial dry weight were incubated for designated periods, and their ABA contents were determined at time points as indicated. Note that the abscissa is shown on a log scale. Each point is the mean±SE of triplicate samples, each with 1 g of initial dry weight. ●, 23°C without fluridone; ◆, 33°C without fluridone; ◇, 33°C with 30 μM fluridone.

We also determined the amount of ABA in the water substrata in which 50 lettuce seeds had been incubated for 18 h at 23 and 33°C. The amount of ABA in each incubation medium was less than 10.2 ng (data not shown). This suggests that the decrease in the amount of ABA in the seeds during incubation was not caused by leakage from the seeds.

Fig. 4 shows the relationship between the germination percentage and the ABA content in the seeds used in the experiment shown in Fig. 2 and 3. ABA content decreased with the increase in germination percentage. Non-germinated seeds contained more than 50 ng g^{-1} of ABA. Note that all of the data obtained with the seeds incubated at 33°C in the presence of 30 μM fluridone and at 23°C without fluridone were nearly on the same curve. This suggests that the germination of lettuce seed is mainly controlled by its ABA content.

Effect of fluridone on the sensitivity to ABA of lettuce seeds—It has been reproted that the manipulations of maternal ABA levels by spraying with ABA in *Brassica napus* L. (Juricic et al. 1995) and with fluridone in *Sorghum bicolor* (L.) Moench. (Bench-Arnold et al. 1995) modified the sensitivities of seeds to ABA. Therefore, we examined the effect of fluridone on the sensitivity of lettuce

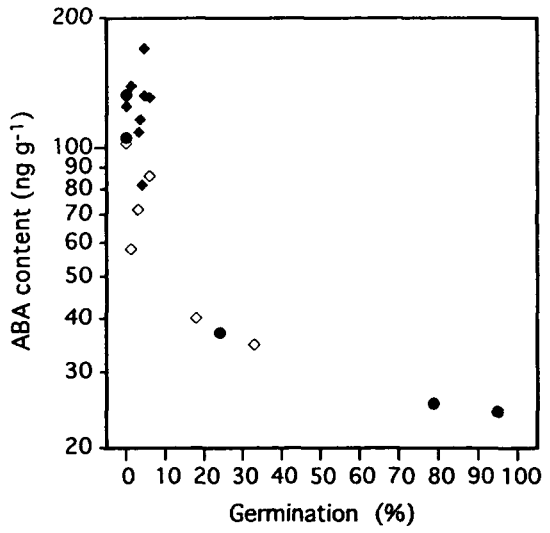

Fig. 4 Relationship between germination percentages and ABA contents in lettuce seeds. Data from Fig. 2 and Fig. 3 were replotted. The data used were those at 0, 6, 12, 18, and 24 h for the seeds incubated at 23°C without fluridone (●); at 6, 12, 18, 24, 48, 120, 240, and 360 h for the seeds incubated at 33°C without fluridone (◆); and at 6, 12, 18, 24, 48, and 120 h for the seeds incubated at 33°C with 30 μM fluridone (◇).

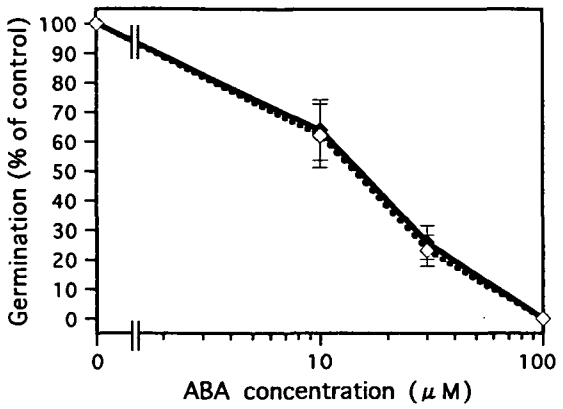

Fig. 5 No effects of fluridone on sensitivity to ABA of lettuce seeds. Lettuce seeds were pre-incubated at 33°C for 120 h with 0.4 M polyethyleneglycol with (◇) or without (◆) 30 μM fluridone and then were allowed to germinate at 23°C for 24 h in the presence of ABA at designated concentrations. Each point is the mean ± SE of triplicate petri dishes, each with 50 seeds.

seeds to ABA (Fig. 5). Lettuce seeds pre-incubated in the presence or absence of 30 μM fluridone responded equally to exogenously applied ABA, indicating that there was no difference in their sensitivity to ABA.

At 33°C, the ABA content in the lettuce seeds remained unchanged during the incubation but decreased in the presence of fluridone, an inhibitor of ABA biosynthesis. This means that the metabolism (and degradation) and synthesis of ABA are balanced in the lettuce seeds incubated at 33°C and that continuous synthesis is necessary for the continued inhibition of germination at the supraoptimal temperature.

It is of interest to compare the present findings with the report on *Helianthus annus* L. that the steady production of ABA is responsible for the maintenance of embryo dormancy (Le Page-Degivry et al. 1990). Toyomasu et al. (1994) reported that exogenously applied gibberellin and light, which promote germination, caused a decrease in the endogenous level of ABA in photoblastic lettuce seeds. These data support our assumption that ABA plays a decisive role in regulation of seed germination at supraoptimal temperature.

In seed development, ABA is considered to be synthesized within the seed-surrounding tissues or translocated from the mother plant (for review, Kermoda 1995). Recently, it was suggested that the early steps of ABA biosynthesis are located in chloroplast membranes (Marin et al. 1996). On the other hand, in lettuce seed germination, the putative ABA biosynthesis occurred in the seeds by the sixth h of imbibition. Nevertheless, the plastids of lettuce seeds probably do not develop into mature stages during the early phases of germination, based upon the results of Whatley (1974) for *Phaseolus vulgaris* L., in which plastids remained as proplastids until the 24th h of imbibition, although an increase in size and an association with the rough endoplasmic reticulum were obtained. Therefore, further research will be needed to localize the site of synthesis of this hormone during seed germination.

Since ABA and gibberellins share the early part of the mevalonate pathway (or isopentenyl pyrophosphate pathway) for their biosynthesis in plants, the inhibition of ABA biosynthesis by fluridone might increase gibberellin biosynthesis by causing a transition of the flow of intermediates from ABA biosynthesis to gibberellin biosynthesis. However, recent studies with gibberellin-deficient tomato and *Arabidopsis thaliana* L. mutants suggested that the synthesis of gibberellins may not be involved in breaking dormancy; their action is limited to late germination processes such as radicle growth (Hilhorst and Karssen 1992). The contribution of gibberellins, if any, in the germination stimulated by fluridone at supraoptimal temperatures is open to future studies.

The restoration by fluridone of the seed germination at supraoptimal temperature was observed in 17 out of 19 weed species. The seeds used were those of the winter annuals or winter perennials, which do not germinate during summer in their habitats. Thus, the regulation of ABA content in response to high temperatures is considered a contributory factor in determining their germination phenology. The high-temperature inhibition of seed germination was not restored by the treatment with fluridone in *Bromus catharticus* and *Lolium perenne*, which produce deep dormant seeds. The non-effect of fluridone might be explained by the fact that the germination timing of winter annuals is regulated not only by the high-temperature inhibition of germination but also by the onset of dormancy.

The seeds induced to germinate by fluridone developed albino seedlings and soon died. Therefore, fluridone or other herbicides with a similar action mechanism, such as norflurazon and diflufenican, could be used as a new method for weed control by decreasing the number of soil-buried seeds.

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