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Respiration and Protein Synthesis in Dormant and After-ripened Seeds of *Avena fatua*[1]

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ABSTRACT

Dormant seeds of *Avena fatua*, which do not germinate when allowed to imbibe water, have a respiration rate only about 20% less than that of imbibed nondormant (after-ripened) seeds in the period before actual germination and are capable of synthesizing protein at a rate comparable to that of the nondormant seeds. An increase of protein synthesis is observed in nondormant seeds at the beginning of root protrusion. Autoradiography of seeds administered ^{3}H-leucine shows that protein synthesis occurs in the axis part of the embryo, the scutellum, the coleorhiza, and the aleurone layer. Dormancy in seeds is not a state of general inactivity; rather, it must be due to some specific metabolic block.

In an attempt to gain insight into the biochemical aspects of seed dormancy, we have recently made a comparative study of carbohydrate metabolism in dormant and after-ripened seeds of *Avena fatua* L. (wild oat) (5). Now we have investigated two other major metabolic processes associated with cell growth and seed germination, namely, respiration and protein synthesis.

Unlike similar stages in the life cycle of some microorganisms, namely bacterial spores, which are often described as metabolically arrested (13), the dormant wild oat seeds respire at a readily measurable rate and are capable of synthesizing protein at a rate comparable to that of nondormant seeds in the period before actual germination. They are thus not in a state of general inactivity or gene repression (*cf.* Ref. 1), which should be manifest in an inability to synthesize proteins; their dormancy must therefore be due to some specific metabolic block.

MATERIALS AND METHODS

The Seeds. Most experiments were done using two batches of seeds of *A. fatua* L. (wild oat) type Montana. One, harvested in 1963, was after-ripened (at room temperature in dry condition) and gave 100% germination within 1 day. The other, harvested in 1966 and stored at −15 C prior to use, was deeply dormant; it did not germinate even after 1 week. As will be described in connection with the results, a 1968 crop was used for the later experiments.

Measurement of Oxygen Uptake. The uptake of O_2 was measured by means of a Gilson differential respirometer. Sterile precautions were taken as described previously (4). Each flask contained 10 seeds. Two flasks were included in each treatment.

Incorporation of ^{14}C-Leucine into Protein. Lots of 10 seeds were husked, pricked at the dorsal side of the endosperm (a measure which facilitates entry of the applied compounds without affecting germination), sterilized in 1% NaOCl for 2 min, washed, placed in a 25-ml Erlenmeyer flask with 2 ml of water, shaken at 21 ± 1 C for a period of time, then transferred to 1 ml of 1 mM leucine with 2.5 μc of uniformly labeled ^{14}C-leucine (specific radioactivity 290 mc/mmole, New England Nuclear Corp.), and further shaken for 2 hr. The seeds were then washed in ice-cold, nonradioactive leucine solution, homogenized, extracted in 4 ml of 0.2 M NaCl plus 0.1 mM leucine, and centrifuged at 12,000*g* for 15 min. The pellet was suspended in 4 ml of the salt solution and recentrifuged. One twentieth of the pooled 12,000*g* supernatant fraction was precipitated with an equal volume of 15% trichloroacetic acid, and the precipitate (soluble protein) was collected on a Millipore filter. The filter was dried and the radioactivity was determined in a Nuclear Chicago gas flow detector with a counting efficiency of 40% for carbon-14. The pellet was suspended in water and precipitated in the same way as above. Approximately 80% of the radioactivity associated with the acid-insoluble material could be released by pronase, a potent proteolytic enzyme of bacterial source. Thus, this material is considered as insoluble protein.

Microautoradiography of Seeds Exposed to ^{3}H-Leucine. To locate the sites of protein synthesis, the seeds were exposed to L-leucine-4,5-^{3}H (specific radioactivity 6.0 c/mmole, Schwarz BioResearch), then washed in nonradioactive leucine, fixed, dehydrated and infiltrated with paraffin according to the technique of Feder and O'Brien (7). Sections 5 to 8 μ thick were placed on a slide, dewaxed, coated with Kodak nuclear track emulsion type NTB 3, exposed, stained with toluidine blue-acid fuchsin, mounted, and examined under the microscope (10).

RESULTS

Respiratory Intensity. The rate of respiration (O_2 uptake) in dormant seeds was low as compared to that of nondormant seeds; the difference could be detected from the early hours of soaking (Fig. 1). In both kinds of seeds, the rate increased with time. However, in the dormant ones it reached a steady state of 9 μl hr^{-1} per 10 seeds in 12 hr while in the nondormant ones it continued to increase linearly up to 36 hr, and only then sloped off, at a steady rate of 36 μl hr^{-1} per 10 seeds. Since root protrusion in nondormant seeds took place at approximately 16 hr

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following soaking, the difference in the respiratory intensity during the latter hours must be largely the effect of germination.

Rate of Protein Synthesis. Lots of 10 seeds were preincubated for 6, 10, or 16 hr and then exposed to ^{14}C-leucine for another 2 hr at 21 ± 1 C. The incorporation of the label into trichloroacetic acid-insoluble material was determined. Table I shows the result of a typical experiment. Within the limits of error, the rates of amino acid incorporation in the two types of seeds are comparable up to 12 hr of soaking. Obviously the low rate of respiration in dormant seeds did not limit the rate of protein synthesis. A dramatic increase in incorporation was observed in the nondormant (after-ripened) seeds after 16 hr of imbibition, that is, at the beginning of root protrusion.

The newly synthesized protein in *A. fatua* seeds seems to be stable. In a pulse-chase labeling experiment (Table II), we exposed the seeds to ^{14}C-leucine from the 6th to the 8th hr following soaking, then homogenized one lot and measured the radioactivity incorporated into protein. The other lot was washed, "chased" in ^{12}C-leucine for 4 hr, and then processed in the same manner. The radioactivity in the protein, instead of decreasing, somewhat increased after the chase. This suggests that at least during the 8- to 12-hr period, the protein already synthesized was not degraded to any significant extent, and that part of the free ^{14}C-leucine taken up into the protein precurser pool earlier was further incorporated into new protein while, as shown by the drop in unincorporated radioactivity inside the seeds, another, much larger part of free ^{14}C-leucine must have leaked out of the seeds.

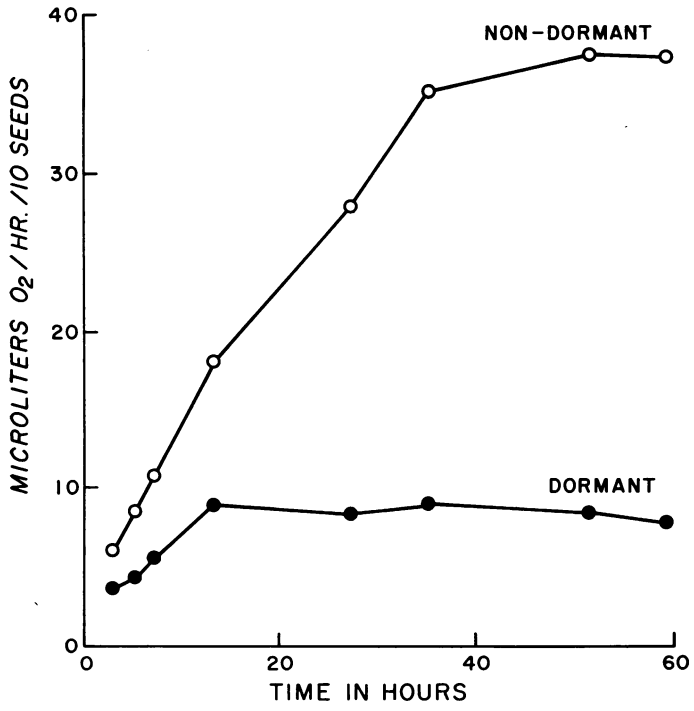

FIG. 1. Time course of respiration of dormant and nondormant seeds of *A. fatua* at 21.5 C.

Table I. *Rate of Protein Synthesis in Nondormant (After-ripened) and Dormant Avena fatua Seeds*

Exposure to Radioactivity	Incorporation into Protein	
	Nondormant	Dormant
	cpm/10 seeds	
From 6th to 8th hr	1660	2110
From 10th to 12th hr	1950	1520
From 16th to 18th hr	6180	1610

Table II. *Incorporation of ^{14}C-Leucine into Proteins in Avena fatua Seeds in a Pulse-Chasing Labeling Experiment*

Seeds were preincubated at room temperature for 6 hr, then shaken in 2 ml of 0.5 mM leucine with 10 μc of ^{14}C-leucine for 2 hr, washed and (*a*) homogenized, or (*b*) further shaken in 0.5 mM carrier leucine for 4 hr before homogenization.

Seeds and Treatment	Incorporation into Proteins		Unincorporated Radioactivity Inside the Seeds
	Soluble	Insoluble	
	cpm/10 seeds		
Nondormant			
(*a*) 6 $\underline{^{14}C}$ 8	4870	2720	8090
(*b*) 6 $\underline{^{14}C}$ 8 $\underline{^{12}C}$ 12	7030	2960	3040
Difference (*b*) − (*a*)	+2160	+240	−5050
Dormant			
(*a*) 6 $\underline{^{14}C}$ 8	3480	2600	6920
(*b*) 6 $\underline{^{14}C}$ 8 $\underline{^{12}C}$ 12	6710	2960	1330
Difference (*b*) − (*a*)	+3230	+360	−5590

Sites of Protein Synthesis in the Seed. In an experiment designed to determine the rate of protein synthesis in each organ or tissue shortly before root protrusion, after-ripened seeds were soaked for 16 hr, then exposed to L-leucine-4,5-^{3}H (10 μc in 1 ml of 1 mM leucine) for 2 hr, and a microautoradiograph was prepared. The result shows that protein synthesis occurs in all parts of the embryo, including apical meristem, coleoptile, primary leaf, radicle, root cap, and scutellum as well as aleurone layer (Fig. 2). Interestingly, the most active synthesis takes place in the coleorhiza, a tissue surrounding the root cap and radicle. This tissue appears to disintegrate as the embryonic root emerges and hence is a senescent tissue. In fact, autolysis is already evident in some cells (the empty cells) in coleorhiza shown in this micrograph.

Similarily prepared microautoradiographs of dormant seeds administered labeled leucine show that protein synthesis also occurs in the entire embryo, the coleorhiza, and the aleurone layer. However, there is more incorporation of the labeled precursor in the scutellum and the aleurone layer, and somewhat less incorporation in the coleorhiza in dormant seeds, as compared with nondormant seeds (data not shown).

Experiments with a 1968 Crop. The foregoing experiments were carried out with seeds harvested in different years: a 1963 crop representing after-ripened (nondormant) seeds, and a 1966 crop representing dormant seeds. The question may be raised as to whether the observed differences in behavior are merely a reflection of seeds matured in different set of conditions and have no bearing on the problem of dormancy. Therefore, a similar study was made with both dormant and nondormant seed from the same year. After harvest in the fall of 1968, one lot of the seeds (*A. fatua* L. type Montana) was stored at 28 C, 50% relative humidity, for 8 months; these seeds after-ripened and germinated 70 to 85% in 3 days. One lot was kept in a freezer at −15 C for the same period of time; this lot remained dormant but viable. The average rate of O_2 uptake in dormant and after-ripened seeds, during the 2.5th to the 13.5th hr following imbibition, were 7.6 and 9.4 μl hr^{-1} per 10 seeds, respectively; viz., there was a 24% increase in $Q0_2$ as the seeds had undergone after-ripening, confirming the early observation made by Atwood (2).

The after-ripened seeds incorporated an increasing amount of ^{14}C-leucine as incubation proceeded, especially after 3 days,

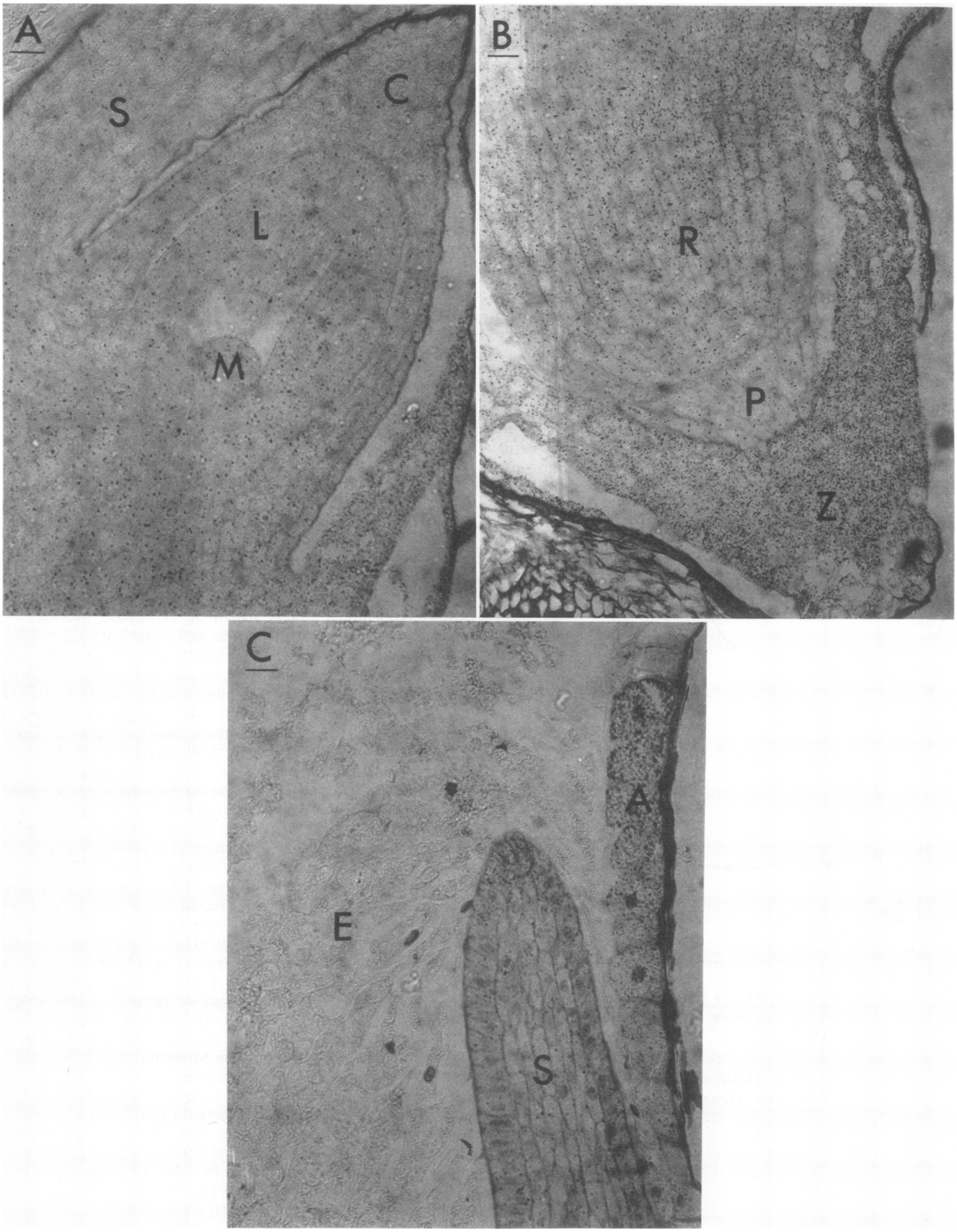

FIG. 2. Microautoradiographs showing the upper (A) and lower (B) portion of an embryo and the endosperm (C) of a nondormant *A. fatua* seed, which had been exposed to ^{3}H-leucine (10 μc in 1 ml of 1 mM leucine) from 16 to 18 hr following imbibition. Unincorporated ^{3}H-leucine was washed off. The label incorporated into proteins appears as black dots. S: Scutellum; C: coleoptile; P: root cap; Z: coleorhiza; E: starchy endosperm; A: aleurone layer. × 100.

when most seeds had germinated. When the seeds were treated with 1 mM GA_3, they germinated faster (80% in 1 day), and amino acid incorporation increased already in the 1st day. Incorporation in dormant seeds, instead of increasing, showed a slight reduction as incubation went on. Chloramphenicol (about 10 μg/ml), cycloheximide (about 10 μg/ml), and actinomycin D (100 μg/ml) inhibited the incorporation ^{14}C-leucine into protein by 30, 91, and 34%, respectively (Table III).

It is noteworthy that in this experiment, during the 10- to 12-hr period, after-ripened seeds incorporated 32% less radioactivity than did dormant seeds, and that gibberellic acid caused a further reduction of incorporation in the after-ripened seeds continuously treated with GA_3. The amount of radioactivity incorporated is a combined function of the rate of protein synthesis and the specific radioactivity of ^{14}C-leucine in the seeds. In order to compare the rate of protein synthesis, the specific radioactivity of ^{14}C-leucine in each treatment has to be the same. To achieve this, an excessive quantity of carrier (nonradioactive) leucine (5 mM) was included in the isotopic medium to render the contribution from the endogenous amino acids to alteration of the specific radioactivity of the applied ^{14}C-leucine insignificant. Then the trend was altered: there was approximately the same amount of incorporation of labeled precursor into protein in dormant and after-ripened seeds; in the GA_3-treated seeds, there was 50% more incorporation as compared with the nontreated control (Table IV). It is thus apparant that dormant and after-ripened seeds have similar rates of protein synthesis (in the early hours), but their endogenous levels of amino acids are different. Prolonged treatment with GA_3 resulted in an increased synthesis of protein. Reduced incorporation observed when a low level of carrier amino acids was used could be the result of expansion of amino acid pool caused by GA_3, presumably through enhancement of proteolytic activity.

Table III. *Rate of Protein Synthesis of Avena fatua Seeds, 1968 Crop*

The seeds were exposed to ^{14}C-leucine (2 μc in 2 ml of 1 mM leucine) for 2 hr at 22 ± 0.5 C. Treatment with 1 mM gibberellic acid (GA_3), and with 100 μg/ml actinomycin D was continuous. To test the effect of cycloheximide and of chloramphenicol, the seeds were shaken in a 100 μg/ml solution of the respective inhibitor for a period of 30 min preceding exposure to the labeled leucine, and then a concentration of 10 μg/ml of the inhibitor was included in the isotopic medium. Figures in parentheses denote the percentage of seeds germinated at that time.

Seeds and Treatment	Incorporation into Protein during			
	10th–12th hr	22th–24th hr	46th–48th hr	70th–72th hr
	cpm/10 seeds			
After-ripened	1320	2190	2200	5820
	(0)	(0)	(40)	(70)
After-ripened, in GA_3	910	6020		
	(0)	(80)		
Dormant	1930	1810	850	1300
	(0)	(0)	(0)	(0)
Dormant, in chloramphenicol	1360			
	(0)			
Dormant, in cycloheximide	174			
	(0)			
Dormant, in actinomycin D	1270			
	(0)			

Table IV. *^{14}C-Leucine Incorporation in 1968-harvested Avena fatua Seeds in Presence of High Concentration of Carrier Leucine*

The seeds were shaken in 0.5 ml of 5 mM leucine with 2 μc of ^{14}C-leucine from the 10th to the 12th hr following soaking at 22 ± 1 C.

Seeds and Treatment	Incorporation into Protein		
	Soluble	Insoluble	Total
	cpm/10 seeds		
After-ripened	1274	1162	2436
After-ripened, in 1 mM GA_3	2107	1556	3663
Dormant	1401	1291	2692

DISCUSSION

Dormant seeds of *A. fatua* can incorporate isotopically labeled amino acids into trichloroacetic acid-insoluble material. This reaction is almost completely inhibited by cycloheximide, and pronase releases 80% of the radioactivity associated with the acid-insoluble material. Therefore, the incorporation observed is a true indication of protein synthesis. Since chloramphenicol at a concentration inhibitory to bacterial growth only slightly suppresses the incorporation of the labeled precursor, it is unlikely that the synthesis of protein is due to bacterial contribution.

The data with the 1963 versus the 1966 crop and dormant versus after-ripened grains of the 1968 crop qualitatively agree with each other. In both sets, dormant seeds are metabolically active and are able to respire and to synthesize protein.

When the seeds were exposed to actinomycin D during the first 12 hr of imbibition, the incorporation of ^{14}C-leucine was somewhat reduced. This suggests that some DNA-dependent RNA synthesis is required for the maximal rate of protein synthesis in the seeds (*cf.* Ref. 3).

Gibberellic acid probably sets in motion a sequence of reactions leading to seed germination. Enhanced protein biosynthesis is observed well before root protrusion has taken place; therefore, it could be an early preparative step toward germination. In isolated aleurone layers of barley and wild oat, gibberellins stimulate synthesis of amylases (12, 15, 14, 11) and protease (9). It has been stated that gibberellic acid promotes seed germination by enhancement of hydrolase synthesis (1, 8). However, when the time course of amylase development was followed in the whole grains, it was found that the increase of amylase activity occurred 1 or 2 days after root protrusion, an accepted criterion for seed germination. Thus, germination precedes amylase synthesis. The development of amylase is evidently brought about in the endosperm in response to a stimulus from the growing embryo, most likely gibberellins (16), and the buildup of amylases is a postgermination phenomenon, essential for seedling growth but not a limiting factor in initiation of seed germination (6). The question of how gibberellins induce germination in dormant seeds remains moot.

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