Symposium: Deterioration Mechanisms in Seeds

Protein Degradation During Seed Deterioration

John P. Cherry

Southern Regional Research Center, ARS, U.S. Department of Agriculture, P.O. Box 19687, New Orleans, LA 70179. Present address: Eastern Regional Research Center, ARS, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118.
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The aging of seeds involves degradative changes that lead to reduced seed germination, decreased seedling vigor, or death; these changes can, in many cases, be accelerated by microorganisms (1,4,13,14,24,28). Seed deterioration rates increase when moisture and temperature conditions are favorable for microorganisms (4,14,21).

At this time, the relative importances of seed aging, infection by microorganisms, or a combination of both, are poorly understood. Seed aging involves a definite sequence of physiological and ultrastructural changes that occur independent of microorganisms (17,20,22,24,26,28), but pathogens produce metabolites (eg, pectic enzymes, proteases, toxins, etc.) that greatly increase the rate of deterioration of the host's cellular structures (20,22,26,28). Autolysis also becomes a factor after host enzymes (such as proteases) are released through disrupted cell membranes (27,28).

The complex mechanism(s) operating during host-pathogen interactions are far from understood. Microbial proteases facilitate invasion of plant tissues (23,27–29) and, along with polysaccharide-degrading enzymes such as endopolygalacturonases, the hydrolysis of proteins both within and between the host cell walls. These hydrolysis mechanisms enable the microorganism to enter plant cells. However, plant cells often produce inhibitors of endopolygalacturonases (15,27). Inhibitors of trypsin and chymotrypsin control plant proteases (27). Many of the proteases of microorganisms have trypsin- or chymotrypsinlike specificities and when challenged with inhibitors from the plant, usually are inhibited (27).

Basha and Cherry (3) showed that the proteolytic activity in the total extract (in 1 M NaCl-20 mM sodium phosphate buffer, pH 7.0) of 10-day-old peanut cotyledons was totally and partially inhibited when mixed with an equal portion of extracts from dormant seeds or from 2- and 4-day germination, respectively.

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Extracts from seeds germinated for 6 days or more were only weakly inhibitory. The mechanism producing increased proteolytic or decreased inhibitory activities in germinated seeds is unclear at this time. Changes could involve one or more of the following mechanisms: Breakdown of stored inhibitors of proteolytic enzymes following germination, synthesis of more enzymes, and activation of enzyme precursors. It is speculated that similar processes occur during host-pathogen interactions.

Most of the seed proteins are stored within protein bodies; during imbibition and germination they are hydrolyzed to polypeptides and free amino acids by proteases and peptidases and translocated to the developing embryonic axis (3,16,18,19,25). During this process, the protein bodies swell, their external membranes remain intact, and small vacuoles form within them, fuse, and become central vacuoles. Protein hydrolysis can occur either within or peripheral to the protein bodies, and the vacuole fusion process may begin before degradation of the proteins is complete.

Ghetie (16) proposed that within the protein bodies, peptide bonds were hydrolyzed after initial "double activation" of both the proteins and specific proteolytic enzymes. This activity depolymerized proteins from their native conjugates and dissociated them into polypeptides that were further hydrolyzed to free amino acids. Hara and Matsubara (18,19) characterized two proteolytic activities, I and II, in globulin degradation in pumpkin seeds. Activity I, detected in dry seeds, hydrolyzed α and β subunits of the globulins to form Fα,β, and activity II, not observed in dry seeds, hydrolyzed Fα,β to low-molecular-weight polypeptides and amino acids. During germination, a proteolytic enzyme with *N*-α-benzoly D,L-arginine *p*-nitroanilide (BAPA) activity appeared, which suggested that it might be involved in the digestion of small polypeptides (19). Purified trypsin inhibitor did not affect the proteolytic activities of I, II, or BAPA. As germination proceeded, trypsin inhibitor activity gradually decreased.

Cherry and co-workers (5–12) examined selected biochemical changes that occur in fungus-contaminated peanut seeds. They showed that peanut seeds infected for various lengths of time with *Aspergillus parasiticus, A. oryzae, A. flavus, Rhizopus oligosporus,* or *Neurospora sitophila* underwent sequential protein, enzyme, lipid, amino acid, and fatty acid changes that differed from standard profiles of otherwise similarly treated uninoculated seeds. Peanut seeds artificially inoculated with fungal conidia and incubated at 29 C for time intervals ranging from 2 to 18 days supported luxuriant growth of the fungi (7,10,11). The biochemical changes included decomposition of proteins and lipids to varying quantities of lower-molecular-weight components, free amino acids, and fatty acids; quantitative depletion of low-molecular-weight polypeptide components; and depletion of some

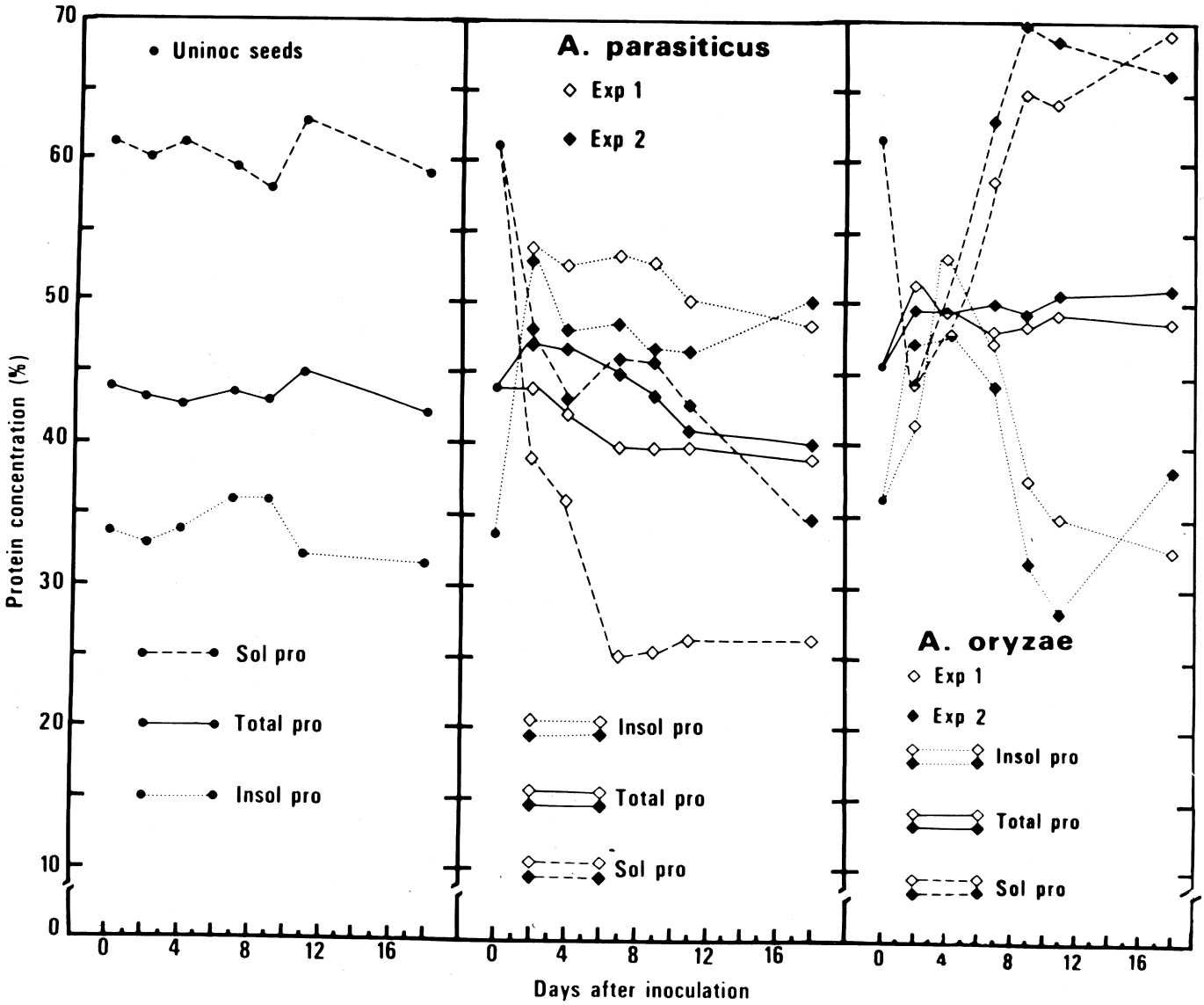

Fig. 1. Quantitative changes in proteins (macro-Kjeldahl) from whole (Total), and pH 7.9 (I = 0.01) sodium phosphate buffer-soluble (Sol), and -insoluble (Insol) fractions from peanut seeds uninoculated (Uninoc) and inoculated (Inoc) for varying time intervals to 18 days with *Aspergillus parasiticus* and *A. oryzae*. Data are from experiments 1 and 2 of Cherry et al (10,12) presented to show the degree of variability between tests.

enzymes, intensification of others, and/or production of new enzyme systems. These changes suggested that the biochemical mechanisms operative in the saprophyte-seed interrelationships functioned very efficiently and systematically for the growth of the fungus at the expense of the seed. Transformations detected by gel electrophoresis (eg, catabolism of peanut proteins) coincided with enzyme changes in extracts of fungal tissue from the seed surface. Moreover, many of the enzyme systems of peanut seeds remained active during the infection period. A closer look at these data is presented in the following discussions.

Quantitative Protein Assays

Quantitative protein assays showed that peanut seeds inoculated with *A. parasiticus* or *A. oryzae* and incubated for time intervals ranging to 18 days showed decreases in percentages of proteins extractable in pH 7.9 (I = 0.01) sodium phosphate buffer during the early stages (from 0 to 4–6 days) of the infection period (Fig. 1) (9–11). Simultaneously, there was an increase in protein content of buffer-insoluble fractions. During longer infection periods, certain fungus-contaminated seeds showed a progressive decrease in soluble protein that leveled off at the later stages of infection (eg, by *A. parasiticus*; Fig. 1; 11,12), while others increased in soluble proteins (*A. oryzae*; Fig. 1; 10). These changes varied between experiments, particularly in those with *A. parasiticus* (Fig. 1) (12).

Free Amino Acid Assays

During rapid development of *A. parasiticus* and *A. oryzae* in peanut seeds, most of the free amino acids increased to levels greater than those in uninoculated seeds (10,12). During later stages of infection, little further change in protein content of seed components was noted. However, many of the free amino acids declined. In spite of the differences in changes in soluble protein induced by the two fungi (Fig. 1), the accompanying increases in free amino acid content were similar throughout the infection period.

Protein Gel Electrophoretic Assays

The polyacrylamide gel electrophoretic patterns of buffer-soluble proteins from uninoculated seeds did not change significantly during the 0 to 18 days of incubation (9–11). Evidently, incubation at high humidity and 29 C did not cause sufficient environmental change within the seeds to allow enough proteolysis to be detectable by gel electrophoresis. Protein changes were noted in extracts of seeds infected by *A. parasiticus* or *A. oryzae* during the test period (Fig. 2). With *A. parasiticus*, these changes were more rapid in experiment one than experiment two (cf Figs. 1 and 2). With *A. oryzae*, the major difference between the two experiments was that the lower-molecular-weight components in region 2.0–6.0 cm disappeared more rapidly in experiment one than two.

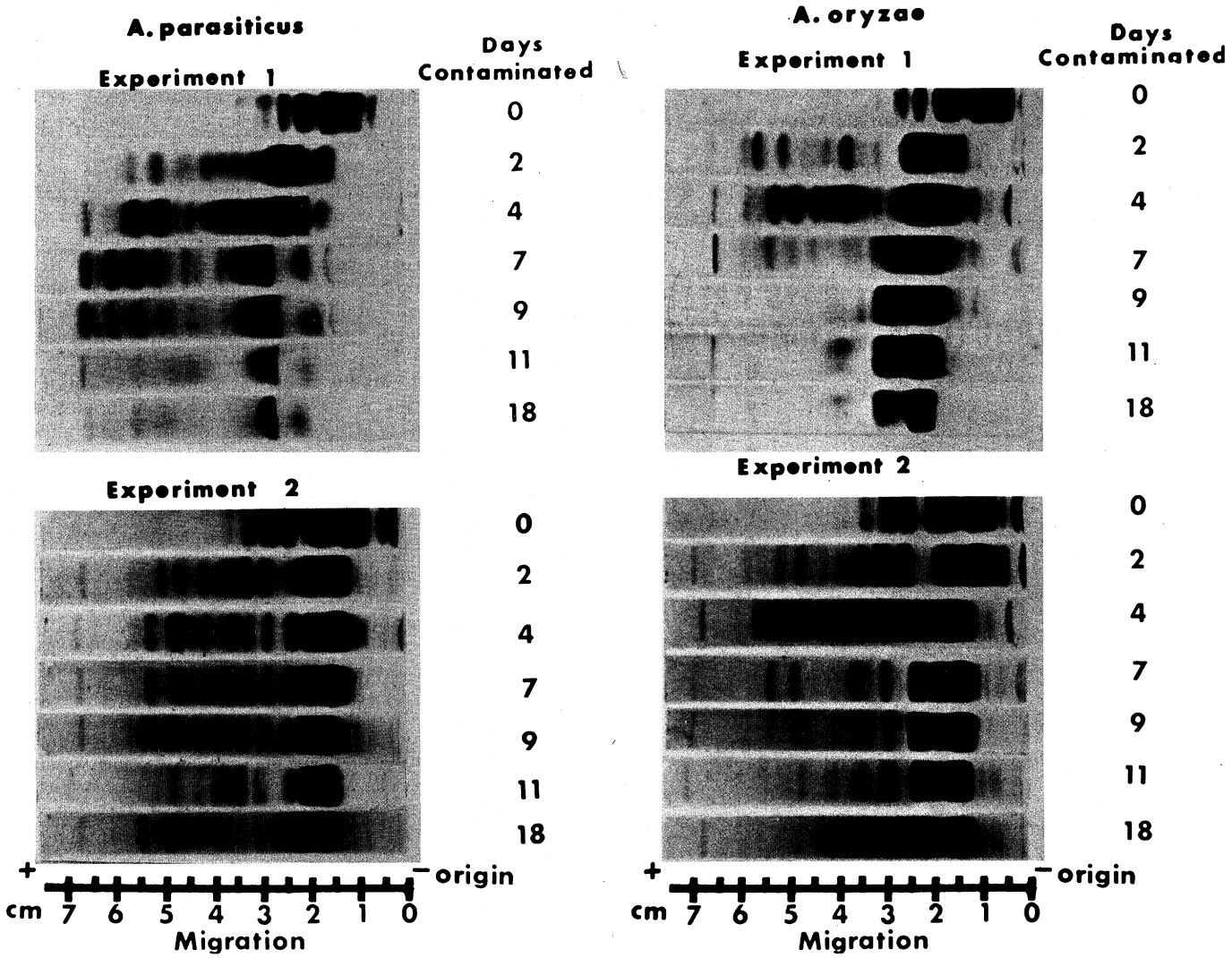

Fig. 2. Polyacrylamide disc-gel electrophoretic patterns of pH 7.9 (I = 0.01) sodium phosphate buffer-soluble proteins of peanut seeds inoculated for varying time intervals to 18 days with *Aspergillus parasiticus* and *A. oryzae*. Data are from experiments 1 and 2 of Cherry et al (10,11).

In experiment one with *A. parasiticus*, the major storage globulin, arachin (region 0.5–1.5 cm) (2), was not clearly discernable in the gel patterns after day 2 of infection (Fig. 2). Simultaneously, a pair of dark-staining components and a group of low-molecular-weight proteins appeared in regions 1.5–2.5 and 3.0–7.0 cm, respectively. During days 7–18, the components in these two regions of the gel slowly declined or disappeared. A band in region 2.5 cm was the only major component in the gel patterns of peanut seeds after 18 days of infection.

In other experiments with *A. parasiticus* and *A. oryzae*, proteins were degraded more slowly, but showed similar new polypeptide components in region 0.0–1.0 cm and increased mobility and poor resolution of the bands in region 1.0–2.0 cm as the infection progressed. Proteins in region 0.0–2.5 showing increased mobility and poor resolution are probably arachin components that were only partially hydrolyzed. As portions of these components were removed, their size, conformation, and electrical charge gradually changed and caused an increase in electrophoretic mobility. At the same time, bands normally located in region 2.0–3.5 cm disappeared and a new group of polypeptides appeared in region 3.5–7.0 cm. In the later stages of infection, many of the proteins in the lower half of the gel patterns were difficult to distinguish.

Differences in activities of proteases produced by *Aspergillus* spp. and other seed pathogens have been reported (23,29). Although certain proteolytic enzymes are common to both *A. parasiticus* and *A. oryzae*, each species also produces an array of proteases that differ from those of other members of the group. Thus, differences in electrophoretic patterns of soluble proteins from peanut seeds inoculated with these two fungi are probably a result of varying types and amounts of proteases produced by the fungi during the 18-day test period. However, variations in the release and activities of peanut seed proteases cannot be discounted and may contribute to the observed variability.

Enzyme Gel Electrophoretic Assays

Changes that occur in enzymes of buffer extracts from peanut seeds infected by *A. parasiticus* were studied (11). The esterase activities, for example, detected on electrophoretic gels depicted changes such as depletion, intensification, and production of new multiple molecular forms. After 2 days, esterase activity increased both quantitatively and qualitatively in gel patterns and continued to intensify during days 3–5. This intensification was the same for whole seed, cotyledonary, and axial tissue extracts prepared with or without mycelial growth from the seed surface.

Certain esterases of the peanut seeds remained active during the infection period and had mobilities similar to those of esterases extracted from fungal tissue collected from the exterior surface of peanut seeds. However, certain esterase bands in gel patterns could distinguish fungal tissue grown on Czapek's solution from that collected from the surface of peanut seeds. These variations in banding patterns of tissue collected from two distinct sources may reflect differences in the nutritional needs of the fungus grown under two conditions, or may result from a differing adaptive response to different environments and nutrient availability.

Other enzymes included in studies of the effects of fungi on peanut seeds were peroxidases, leucine aminopeptidases, oxidases, and catalases (5,6,9,11). Similar to the esterases, these enzymes showed many changes in multiple molecular forms depicting degradation of protein peptides (leucine aminopeptidases), hydrolysis of ester linkages (esterases), hormonal interaction, and/or oxidation of organic substrates with hydrogen peroxides (peroxidases and oxidases), and decomposition of toxic substances such as hydrogen peroxide (catalases).

As with the fungus-infected seeds, protein extractability declines in the cotyledons during the germination period (3). Major changes in protein composition of seed extracts were shown at and after day 4 by gel electrophoresis (Fig. 3) (3). The arachin components and the bands in region 2.0–3.5 cm decreased quantitatively in the gel patterns, and simultaneously, numerous proteins appeared in region 3.5–7.0 cm. Also, as with the fungus-infected seeds, the appearance of small protein components in the gel patterns of extracts from germinated seeds indicated hydrolysis of the major storage proteins by proteases and peptidases to polypeptides of various sizes and to free amino acids (3,5–7,9–12).

Similarities were noted in the gel patterns of proteins in extracts from germinating seeds and those infected with fungi (cf Figs. 2 and 3). This was especially noted in the gels of seeds infected with *A. parasiticus* and those of the germinated seeds. The major bands in regions 1.0 and 2.0 cm, especially the one in region 2.0 cm, declined during the test period. Although, a number of small components appeared in region 2.5–7.0 cm, two dark-staining bands appeared in region 5.0–6.0 cm during the early phases of protein hydrolysis. One major difference between the two hydrolysis processes was that the increased mobility of the arachin components (region 0.5–2.0 cm to 2.0–3.0 cm) in gels of extracts from fungus-infected seeds (especially those of *A. parasiticus*) did not occur in those of germinated seeds. This suggested that the proteases of fungi alone or in combination with seed enzymes hydrolyze the proteins in a different manner than those of the peanut seeds. The fungal proteases, or their combination with seed enzymes, cause major changes in size, shape, and/or conformation of proteins that result in their having increased mobilities and diffused bands on electrophoretic gels. These types of dramatic changes in the major globulins are not noted in germinating seeds.

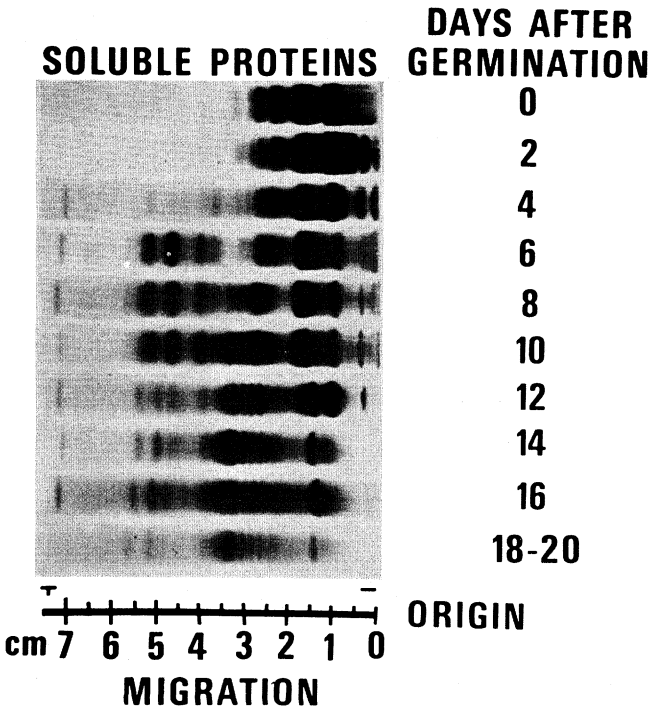

Fig. 3. Polyacrylamide disc-gel electrophoretic patterns of 1 M NaCl-20 mM sodium phosphate buffer (pH 7.0)-soluble proteins of peanut seeds germinated for varying time intervals to 18 days. Data are from Basha and Cherry (3).

Conclusions

No doubt, microorganisms face a complex array of inhibitory mechanisms during invasion of plant cells. In some way, either through the production of toxins or by one of the mechanisms suggested above to occur in germinating seeds to overcome inhibitor activity(ties), the microorganisms surmount these interfering conditions and grow luxuriantly on seeds. This is followed by decomposition of proteins to low-molecular-weight components, depletion of the latter, and the formation of free amino acids. In addition, there is a depletion of some enzymes, the intensification of others, and/or production of new multiple molecular forms of enzymes. Many of these enzymes in seeds remain active during the infection period. Thus, the biochemical

mechanisms operating in the saprophyte-seed interaction very efficiently and systematically enhance fungal growth at the expense of the seed reserves.

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