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Mechanisms of Starvation Tolerance in Pearl Millet

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ABSTRACT

The response of pearl millet (*Pennisetum glaucum* [L.]) seedlings to prolonged starvation was investigated at the biochemical and ultrastructural level. After 2 days of darkness the bulk of the seedling carbohydrate reserves were depleted. After 8 days in the dark the respiratory rate had declined to less than 50% of its initial value and the plants had lost half of their total protein content. Unlike the situation with carbohydrate depletion, protein loss was restricted to specific organs. The secondary leaf and stem (including the apical meristem) showed little or no protein loss during this period. In the primary leaf, seed, and roots, protein loss was substantial. In spite of the high rate of protein degradation in the primary leaf and roots, these organs showed no ultrastructural changes suggestive of tissue, cellular, or subcellular degradation. In addition, ribulose bisphosphate carboxylase was not preferentially degraded during starvation and only a small decline in chlorophyll content was observed after 8 days in the dark. During the period from 8 to 14 days, cell death started at the tip of the primary leaf and gradually spread downward. Both shoot and root meristems remained alive up to 14 days. Consequently, the eventual death of the plant was due to the loss of the carbohydrate-producing regions rather than the meristems. We suggest that these results provide an explanation for the high degree of starvation tolerance exhibited by pearl millet.

Carbohydrate starvation is a fact of life for most higher plants. Since plants manufacture their own carbohydrates through photosynthesis, any condition which lowers the photosynthetic rate can lead to starvation. These conditions can include loss of photosynthetic tissue through microbial, insect, or herbivore attack or reduction in light intensity via shading by neighboring plants. In these situations the process of starvation begins with a decline in storage carbohydrate content and ends with the death of the organism.

Starvation has been extensively studied in animals (reviewed in 4, 16). In plants, the largest body of literature pertaining to starvation has come indirectly from work on dark-induced leaf senescence (9, 12, 19–21). In addition to these long-term leaf studies, carbohydrate starvation has also been investigated in short-term leaf studies (14), in various organs of intact plants (7), in attached roots (2), in excised roots (17, 18), and in cell suspension cultures (6, 10, 11, 15). These studies have shown that the growth or respiration rates of the tissue or cells are immediately reduced by the lack of carbohydrate. Following carbohydrate depletion, net protein and lipid breakdown commences. This self-consumption is evident at the ultrastructural level and, at least in wheat and barley, consists initially of the selective degradation of chloroplasts and RuBPcase[2] (9, 20, 21).

We have observed that seedlings of pearl millet are better able to survive starvation than many other species, including wheat and barley (C. Baysdorfer, unpublished data). In the present study we have investigated the mechanisms of starvation tolerance in this species, focusing primarily on the question of preferential degradation of specific organs, tissues, cell types, cellular organelles, and proteins. Our results show that, unlike the situation in wheat and barley, chloroplasts and RuBP carboxylase are not preferentially degraded in pearl millet. In addition, we show that specific organs are protected from the loss of protein which occurs during starvation, and that even in those permanent organs showing the greatest protein loss, structural and functional integrity are maintained.

MATERIALS AND METHODS

Plant Materials. Pearl millet (*Pennisetum glaucum* (L.) R. Br., Tift $23B_1E_1$) seedlings were grown in culture tubes as previously described (2). The plants were transferred to darkness (<3 μE m^{-2} s^{-1}) at 7 DAP and then harvested at various times thereafter. Following harvest, tissue samples were either quick-frozen in liquid N_2 and stored at −80°C or fixed for electron microscopy. For respiration measurements, about 80 plants were removed from the culture tubes and put in 96-hole pipette-tip racks suspended over 0.5 L aerated one-half strength Hoagland solution. Growth conditions for these plants were the same as for plants left in culture tubes and by 8 DAP the leaf area index was 1.5. The plants were transferred to darkness at this point and respiration rates measured at 0, 2, 5, and 8 d after transfer. Respiration rates were calculated by placing the racks in a dark, sealed chamber (volume 5 L) and, after equilibration, measuring the difference in CO_2 between incoming and outgoing air using a differential IR gas analyzer (model 865, Beckman Inst.).[3] The rate of air flow through the chamber was 3 L min^{-1} and the temperature was 25°C.

Dark survival time was determined by removing plants (n = 5) from the dark after varying lengths of time and returning them to the light for 1 to 2 weeks. Surviving plants resumed growth from shoot and root meristems. Root elongation was determined by measuring the length of the primary root (n = 6) following harvest after varying lengths of time in the dark.

Metabolite Measurements. Chl, sucrose, starch, glucose, and fructose were extracted and assayed as previously described (1). Metabolite values represent the mean of four plants per treatment. Residual dry weights were determined as previously described (2).

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[2] Abbreviations: RuBPcase, ribulose 1,5-bisphosphate carboxylase; DAP, days after planting; PEPcase, phosphoenolpyruvate carboxylase.

[3] Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

Transmission Electron Microscopy. For transmission electron microscopy fresh tissue was obtained from the primary root (about 1 and 5 cm from the root tip) and the midportion and apical end of the first leaf. The tissue sgments (about 1–2 mm thick) were fixed in either 2 or 3% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.0) for 5 h at room temperature. After a 1 h wash in buffer the tissue was postfixed overnight in buffered 2% OsO_4 at 4°C. Dehydration was in a cold ethanol series and propylene oxide with embeddment in Spurr's epoxy resin (13). Serial sections were cut with a diamond knife on an American Optical Ultracut ultramicrotome, stained with uranyl acetate and lead citrate, and viewed and photographed with an Hitachi HU-11E microscope.

Protein Extraction and Analysis. For total protein analysis, samples were ground in a glass/glass homogenizer in 50 mM Tris (pH 7.0) and were centrifuged, and the supernatant was assayed by the Bradford method (3). Samples for two-dimensional electrophoresis were phenol-extracted (5). Depending on the organ analyzed, from 4 to 16 plants were used for each of the assays described above. Two-dimensional gels were run as previously described (2). After electrophoresis the gels were silver stained with Bio-Rad reagents according to the procedure of Merril *et al.* (8).

Experimental Design. With the exception of the respiration measurements, all experiments were conducted at the same time using the same groups of plants. The entire set of experiments was replicated twice. For certain experiments (survival, protein, TEM) additional replicates were also performed.

RESULTS

Effects of Starvation on Basic Physiological Processes. The effect of starvation on plant survival, root growth, and respiration is shown in Figure 1A. All the plants survived 10 d in darkness while most survived for 14 d. Occasionally, a plant recovered after 20 d in the dark. The first visual symptoms of stress were a slight yellowing of the leaves after 5 d; at 8 to 10 d necrosis started at the tip of the primary leaf and gradually extended downward so that by 14 d more than half of the primary leaf was necrotic. The shoot and primary root meristems remained viable for at least 14 d as determined by regrowth from these meristems following transfer to light. In addition, all nonnecrotic regions of the leaf regained a dark green color during recovery. Elongation of the primary root occurred at a rate of 3 to 4 cm/d in both controls (plants kept in a normal photoperiod during the experiment, data not shown) and in the stressed plants for the first day in the dark. Thereafter, root elongation rates dropped to zero (Fig. 1A). When the starved plants were returned to the light for 1 to 2 weeks, elongation of the primary root again reached a rate of 3 to 4 cm/d. These observations show that the functional integrity of all permanent organs is maintained for at least the first 8 d of starvation.

Whole plant respiration rates declined gradually throughout the experiment. By 8 d, the plants were respiring at 40% of their initial rate. Note that since the plants used for respiration measurements were 1 d older and were grown under more crowded conditions than those used for the other studies, it is not possible to directly equate the loss of carbon through respiration with the decline in carbohydrate content. Thus, only the trends in respiration are emphasized here.

The effects of starvation on leaf Chl content, whole plant protein content, and whole plant storage carbohydrate content are shown in Figure 1B. As noted visually, there was a very slight decline in leaf Chl starting at 5 d. Whole plant protein levels showed a gradual decline throughout the experiment. In contrast, whole plant carbohydrate levels dropped to less than 10% of their initial value by 2 d. The remaining carbohydrate is then depleted at a much slower rate.

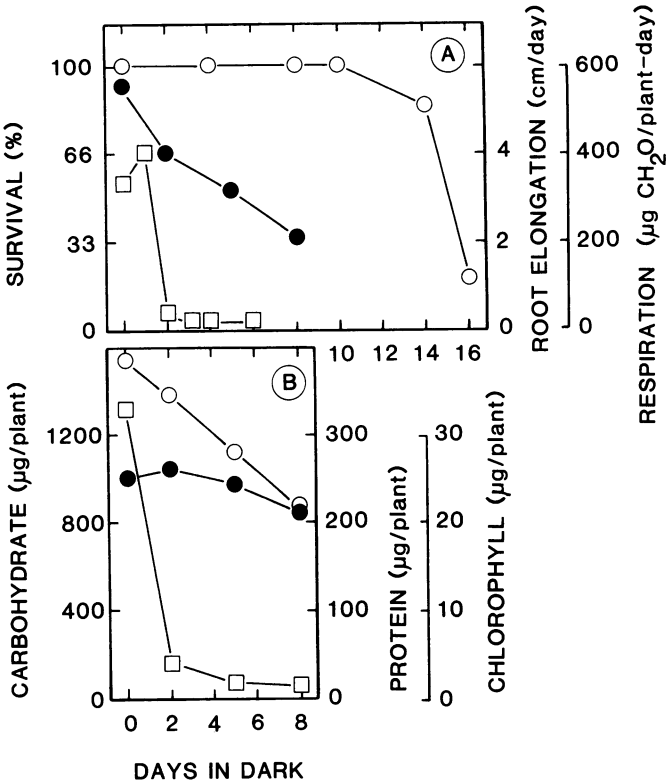


FIG. 1. Physiological parameters of starvation. A, Effect of starvation on survival (○), respiration rates (●), and root elongation rate (□); B, starvation effects on whole plant protein content (○), leaf Chl (●), and whole plant carbohydrate content (□).

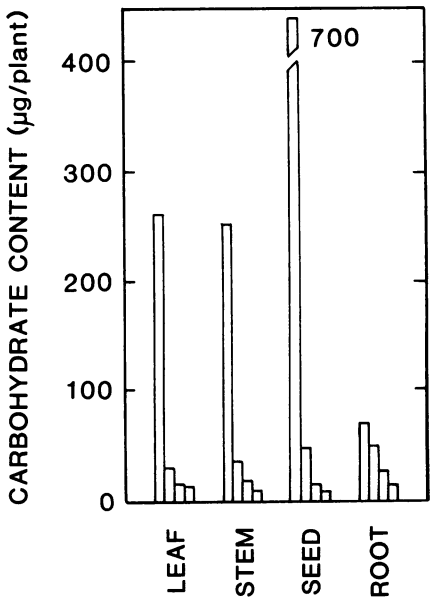
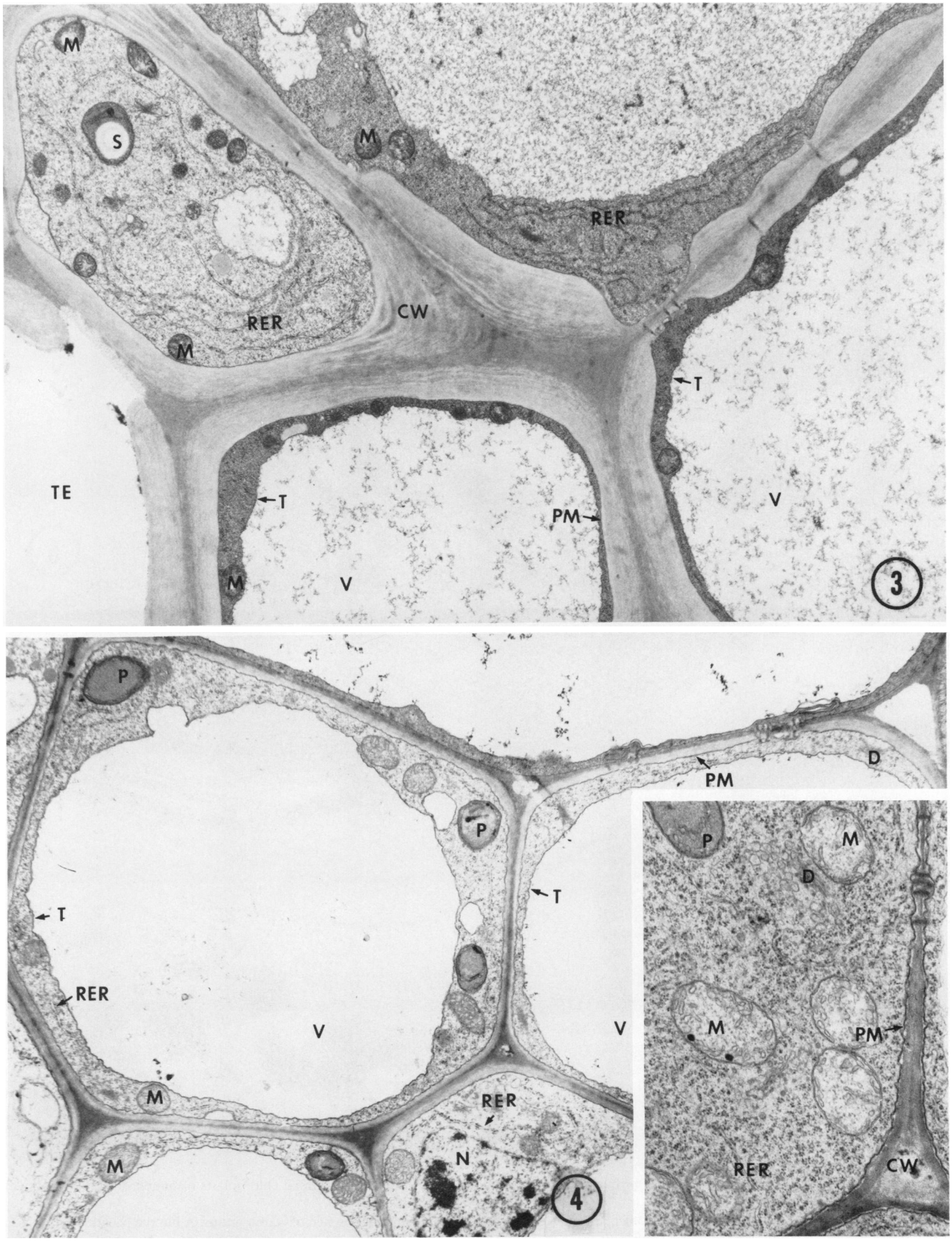


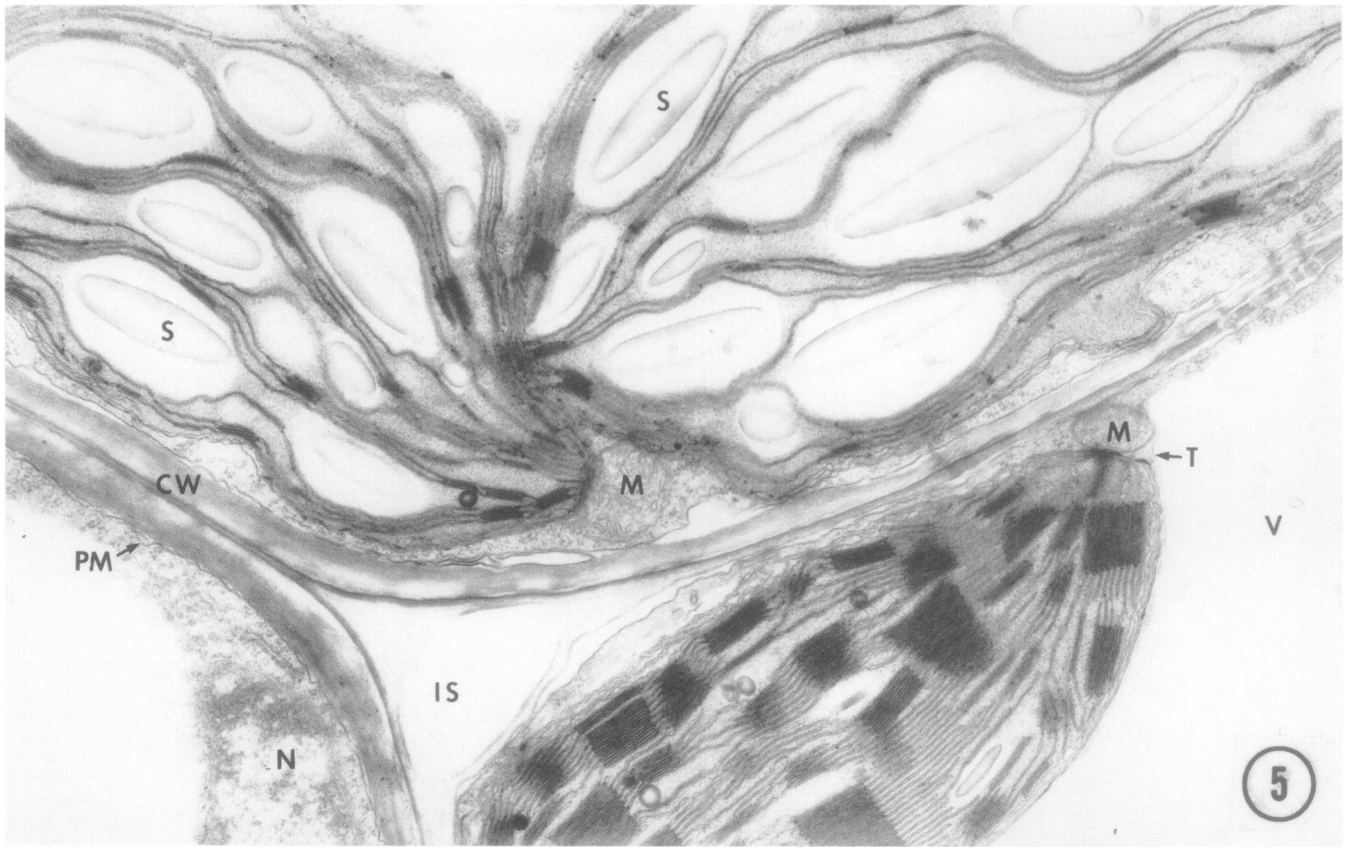
FIG. 2. Effect of starvation on carbohydrate content of various organs. For each organ, carbohydrates were analyzed at 0, 2, 5, and 8 d in the dark.

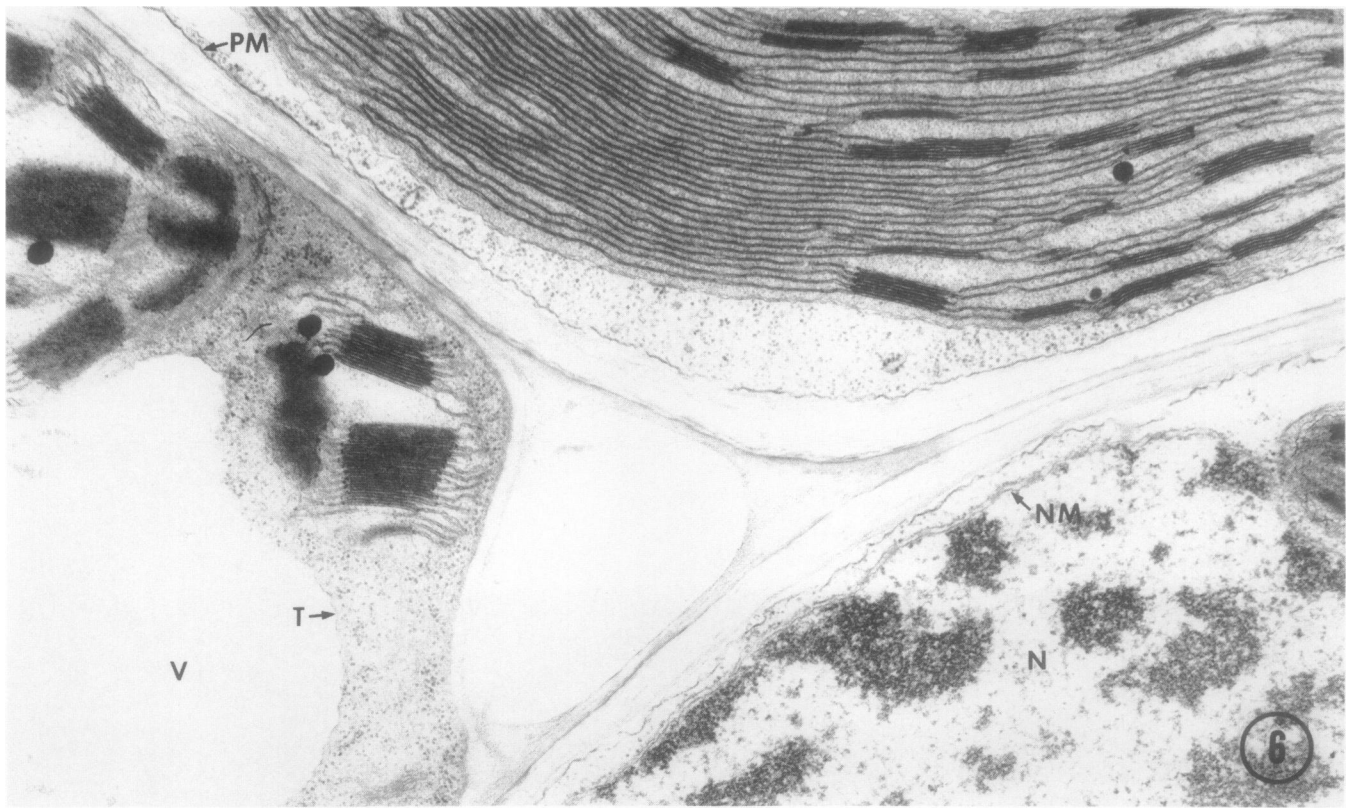
To determine if residual or structural dry weight decreases during starvation, this parameter was measured in different organs at 0, 2, 5, and 8 d. The values for 0 and 8 d, respectively, are listed below as a percentage of the total plant residual dry weight: primary leaf, 30 and 35%; secondary leaf, 5 and 6%; stem, 15 and 16%; seed, 24 and 15%; root, 26 and 28%. Values for plants harvested at 2 and 5 d were similar to those for the 8



FIGS. 3 and 4. Fig. 3. Transection of portion of root (about 5 cm from the tip) of plant after 0 d in the dark showing tracheary element (TE) and xylem parenchyma cells. ×12,000.

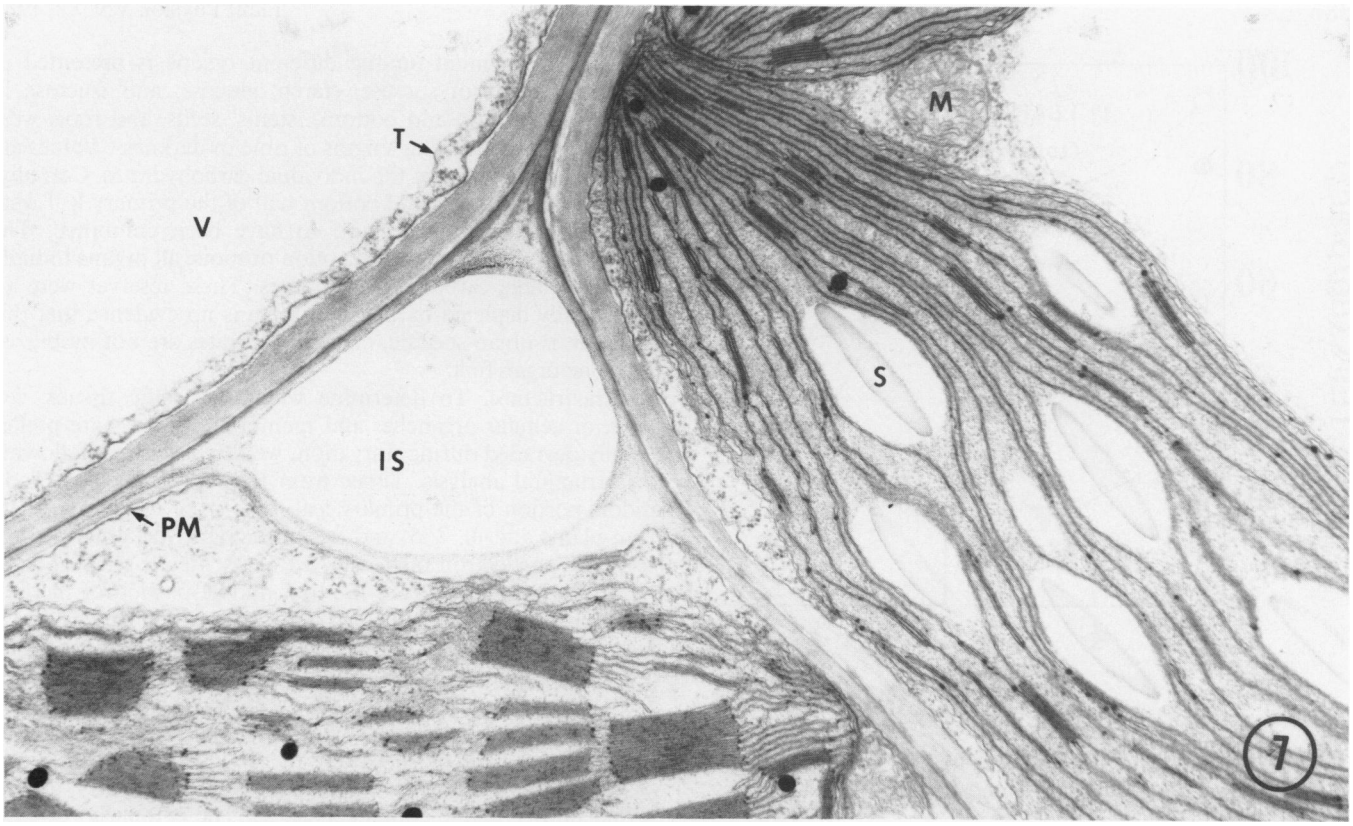
Fig. 4 and inset. Transection of portion of root (about 5 cm from tip) of plant after 8 d in the dark showing xylem parenchyma cells. CW, cell wall; PM, plasma membrane; RER, rough endoplasmic reticulum; S, starch grain; T, tonoplast; V, vacuole. ×8,000; inset, ×19500.

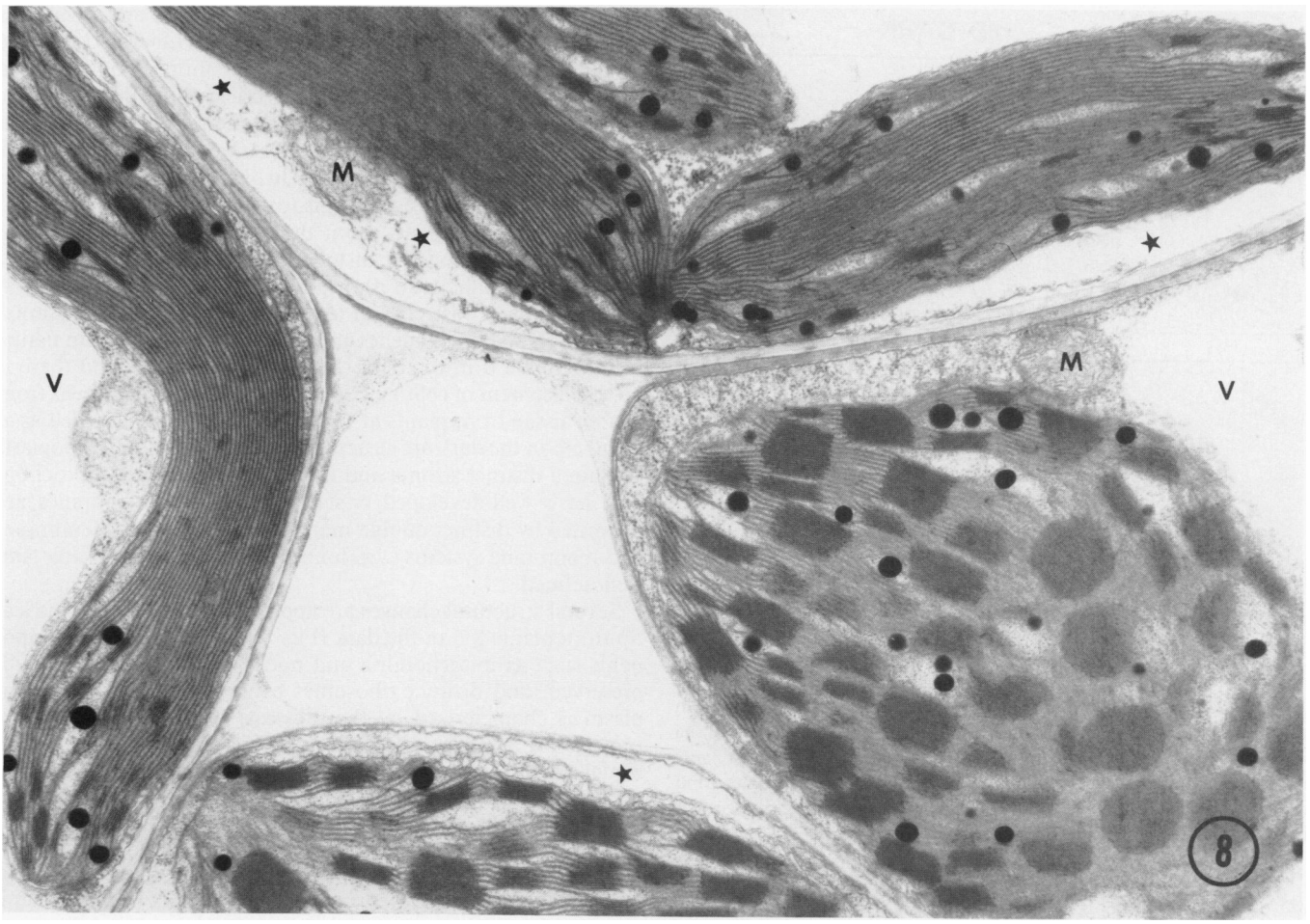




FIGS. 5 and 6. Fig. 5. Portion of midregion of primary leaf from plant after 0 d in the dark. Agranal chloroplasts of bundle-sheath cell (above) contain prominent starch grains (S). ×12,000.

Fig. 6. Portion of midregion of primary leaf from plant after 8 d in the dark. Other than absence of starch grains in bundle sheath chloroplasts (above), the cytoplasmic components appear similar to those in Figure 5. ×11,000. CW, cell well; IS, intercellular space; M, mitochondrion; N, nucleus; NM, Nuclear membrane; PM, plasma membrane; T, tonoplast; V, vacuole.





FIGS. 7 and 8. Fig. 7. Portion of tip of primary leaf from plant after 0 d in the dark. ×11,000.

Fig. 8. Portion of tip of primary leaf from plant after 8 d in the dark. Both bundle-sheath (above and left) and mesophyll (below) chloroplasts are characterized by numerous electron-dense osmiophyllic globules. Although the plastids, mitochondria nad ribosomes appear intact and discrete, prominent electron-translucent areas (stars) are conspicuous in the cytoplasm of both bundle sheath and mesophyll cells. ×9,000. IS, intercellular space; M, mitochondrion; PM, plasma membrane; S, starch grain; T, tonoplast; V, vacuole.

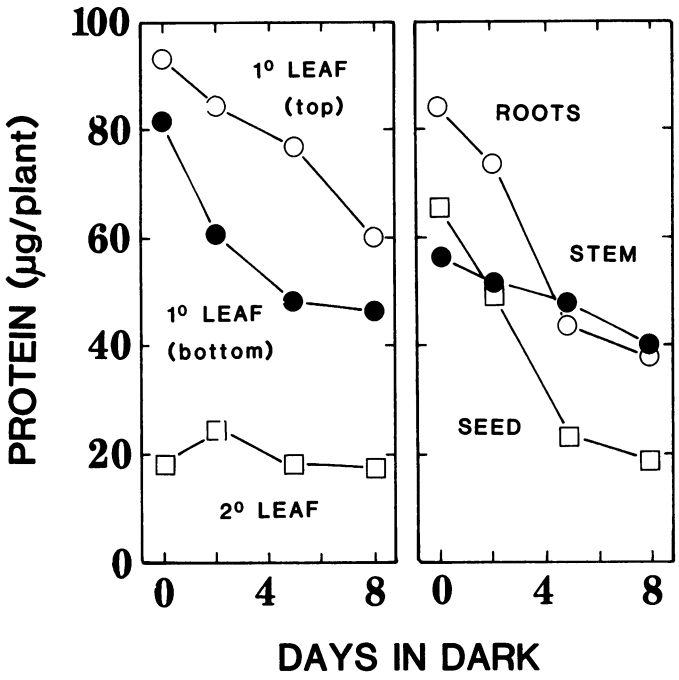


FIG. 9. Soluble protein content of various organs during starvation.

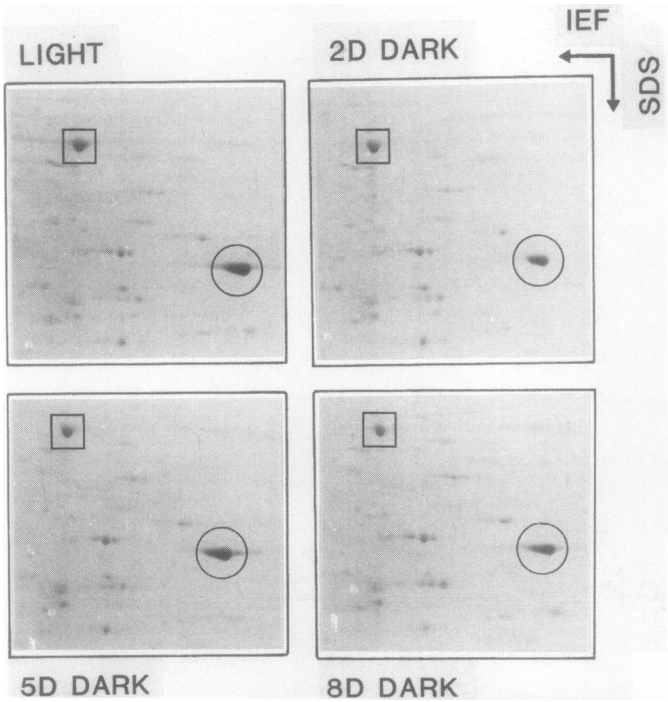


FIG. 10. Effect of starvation on leaf protein profile. Silver-stained gels of primary leaf protein after varying times in the dark. Sixty μg of protein were loaded onto each gel. Only that portion of the gel containing RuBPcase large subunit (○) and PEPcase (□) is shown. RuBPcase large subunit was identified on the basis of its abundance, mol wt, and isoelectric point. PEPcase was identified using antibody to maize leaf PEPcase.

d plants. Total residual dry weights at 0, 2, 5, and 8 d were 4.54, 5.05, 5.09, and 5.06 mg/plant, respectively. These results show that, unlike the situation with storage carbohydrates, structural carbohydrates are not broken down during starvation.

Carbohydrates Are Depleted Uniformly in Different Organs. A more detailed analysis of the effect of starvation on storage carbohydrate content among different organs is presented in Figure 2. Levels of sucrose, starch, glucose, and fructose in primary leaves (top and bottom), stems, seeds, and roots were determined after varying lengths of time in darkness. Values are expressed as the sum of the individual carbohydrates. Carbohydrate levels in the top and bottom half of the primary leaf were not significantly different and so have been combined. The results show that, with the exception of roots, all organs initially contained large carbohydrate reserves. These reserves were almost totally depleted by 2 d, and there is no evidence that this depletion is organ-specific, *i.e.* carbohydrates are not mobilized from one organ first.

Ultrastructure. To determine whether specific tissues, cell types, or cellular organelles and membrane systems are preferentially degraded during starvation, we performed the following ultrastructural analysis. Tissue from the root tip, mature root, middle portion of the primary leaf, and tip of the primary leaf from plants after 0, 2, 5, and 8 d in the dark was processed for transmission electron microscopy. Representative sections from the root (about 5 cm from the tip), middle portion of the first leaf and leaf tip at 0 and 8 d are shown in Figures 3 to 8. Parenchyma cells (including cortical, endodermal, pericycle, and vascular parenchyma) from mature root tissue at 0, 2, 5, and 8 d in the dark are characterized by a well-preserved, mostly parietal layer of cytoplasm with a large central vacuole and tonoplast (Figs. 3 and 4). During the period from 0 to 8 d, the vacuoles may contain flocculent material (Fig. 3) or appear devoid of any visible contents (Fig. 4). The cytoplasm of each cell is delimited by a distinct plasma membrane and includes a nucleus, plastids, numerous mitochondria with well-developed cristae, profiles of rough endoplasmic reticulum (ER; Figs. 3 and 4) and dictyosomes and associated vesicles (Fig. 4, inset). The only consistent difference observed between root tissue of plants 0 d in the dark and that from plants at 2, 5, and 8 d in the dark was the presence of starch grains in the control tissue and its absence in the dark treated plants (compare Fig. 3 with Fig. 4).

Examination of tissues from the midportion of leaves from plants at 0, 2, 5, and 8 d in the dark indicates that, except for the presence (plants at 0 d) or absence (plants at 2, 5, and 8 d) of starch, the ultrastructural features of the tissue were similar (*cf.* Figs. 5 and 6). Organelles and membrane systems from tissues from plants 8 d in the dark (Fig. 6) appear intact, and there is no displacement of cellular components. Likewise, the cells from leaf tip tissue from plants at 0 d in the dark (Fig. 7) as well as at 2 and 5 d in the dark are structurally well preserved. Chloroplasts contain a distinct stroma and thylakoid membranes, mitochondria have well-developed cristae, the nuclear membranes are delimited by distinct double membranes, and other membranes and membrane systems (*e.g.* tonoplast, rough ER) are clear and well-defined.

Several structural changes are apparent in tissues from the leaf tip from plants 8 d in the dark (Fig. 8). Although many components such as mitochondria and nuclei appear intact and well-preserved, and distinct ribosomes can be identified, the cytoplasm is characterized by the presence of numerous electron-transparent areas (starred regions in Fig. 8) which may be the initial stages of cellular degradation. Mesophyll chloroplasts appear much more rounded than in control tissue (or in tissue from plants 2 and 5 d in the dark), and both bundle-sheath and mesophyll chloroplasts appear electron dense and contain numerous, large osmiophillic bodies. Although the grana and thylakoid membranes are well preserved, there has been a general deterioration of the stroma such that the thylakoids have become closely appressed to one another (Fig. 8). Finally, although not illustrated, it was observed in some samples that the bundle-sheath chloroplasts which in control tissue occupy a centrifugal

position in the cell and lie adjacent to the mesophyll cells, have migrated to a centripetal position and lie adjacent to the vein.

Consequently, with the exception of the leaf tip tissues, there is no ultrastructural evidence for the preferential degradation of specific tissues, cell types, organelles, or membrane systems in either the roots or the main portion of the primary leaf in pearl millet for the period from 0 to 8 d. By comparison, Wittenbach *et al.* (21) observed that plastid degradation occurred by 3 to 5 d in the dark in young wheat seedlings.

Protein Degradation is Organ Specific. To determine if the gradual decline in total protein observed in Figure 1B was restricted to specific organs, total protein was measured from the top and bottom halves of the primary leaf, the secondary leaf, the stem, seed, and roots (Fig. 9). The results show that protein loss varied substantially between organs. Little or no change occurred in secondary leaves or the stem (which includes the shoot meristem). In contrast, protein levels declined by 40 to 70% in the primary leaf, seed, and root.

Pearl Millet Ribulose Bisphosphate Carboxylase is Not Preferentially Degraded during Starvation. There is considerable evidence that RuBPcase is selectively degraded during dark-induced senescence (starvation) in wheat and barley seedlings. In these species the soluble protein content of the leaves declines by 35 to 40% after 2 to 3 d in the dark and RuBPcase degradation accounts for 70 to 90% of this loss (9, 20, 21). We therefore conducted an analysis of the fate of RuBPcase in pearl millet. Figure 10 shows the effect of starvation on the total leaf protein profile. For RuBPcase large subunit, no major decline in staining intensity was observed during the period from 0 to 8 d. Since equal amounts of protein were loaded onto each gel, these results show that selective degradation of RuBPcase does not occur in pearl millet. Degradation of this enzyme, instead, appears to be at a rate equivalent to that of total protein.

DISCUSSION

The process of starvation in pearl millet can be considered to pass through three distinct stages. In the first stage (0–2 d) the bulk of the storage carbohydrate reserves are depleted. Coincident with this, respiratory rates start to decline and growth stops. These changes are not accompanied by any visual or ultrastructural alterations other than the disappearance of starch. Net protein degradation starts at this stage and a slight re-working of the protein profile is evident from other studies (2).

In the second stage of starvation (2–8 d), the small pools of carbohydrate that remain are depleted while respiratory rates decline gradually to about one-half their initial values. Given the lack of carbohydrate, it is clear that other compounds are being consumed during this stage. Protein is one of these, showing a net loss of 70% from seeds, 55% from roots, 40% from primary leaves, and little or no change in secondary leaves and stems (including meristems). Surprisingly, even the permanent organs showing the greatest effect of starvation in terms of protein loss (primary leaf, roots) do not show changes at the ultrastructural level suggestive of tissue or cellular degradation. Finally, selective degradation of RuBPcase does not occur in pearl millet, unlike the situation in wheat or barley where selective degradation of this enzyme during starvation does occur (9, 20, 21). As a consequence of the factors discussed above, the pearl millet seedling survives the loss of one-half of its total protein content (0–8 d) without predjudicing its future survival should the stress be removed.

In the third stage (8–14 d), necrosis starts at the tip of the primary leaf and spreads downward. The first ultrastructural changes that precede necrosis are an accumulation of osmiophilic bodies in the chloroplasts, stromal deterioration and the appearance of electron-transparent areas in the cytoplasm. Root and shoot meristems remain functionally intact throughout this period since all plants remaining alive after 14 d show regrowth from the original primary root tip and shoot meristem. Thus, in the last stage of starvation the death of the individual occurs not by the loss of meristematic regions but rather by the loss of the carbohydrate producing organs.

The results presented above reveal the broad outlines of a superbly crafted strategy of starvation tolerance. The basis of this strategy (carbohydrates used first, protein used last) is likely to be common to all plants. It is in the details of the execution that species differences are likely to be found. In the present study we have shown: first, that expanding leaves and stems (including the shoot meristem) are buffered from the loss of protein that occurs in other organs; second, that even in those permanent organs where protein loss is the greatest, ultrastructural and functional integrity remain intact. To our knowledge, similar studies have not been conducted with other species so we do not know whether these traits are unique to pearl millet. One trait which is unique to pearl millet, at least in comparison with wheat and barley (9, 20, 21), is the ability of this species to prevent the preferential degradation of RuBPcase and the loss of Chl during starvation. As a consequence, the functional integrity of the chloroplast is maintained. The survival value of this trait to a plant recovering from starvation is clear.

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