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Maintenance of Normal or Supranormal Protein Accumulation in Developing Ovules of *Glycine max* L. Merr. during PEG-Induced Water Stress[1]

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ABSTRACT

Protein accumulation in developing ovules of hydroponically grown soybean (*Glycine max* L. Merr.) plants was unaffected or enhanced by polyethylene glycol-induced water stress. The cultivar Wayne and the experimental variety '9656' were severely stressed by inclusion of PEG-6000 in the nutrient solution to water potentials as low as −20 bar. Leaves rapidly yellowed and abscised under these conditions. Fresh and dry weight of 'Wayne' ovules was reduced by severe stress, but protein content was unaffected. Ovules of 9656 were more resistant to severe stress: fresh weight and dry weight were unaffected by stress and protein content increased. Moderately stressed Wayne ovules behaved like severely stressed 9656 ovules: seed fresh weight and dry weight were unaffected and protein content increased. However, protein content did not increase if the plants were defoliated. No changes in seed protein quality were observed with stress, based on polypeptide banding patterns after one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Water stress has been shown to alter protein metabolism in vegetative tissues of many plant species (2, 15). Among the documented effects of water stress are: decreased tissue protein content (5, 10), decreased incorporation of amino acids into protein (3, 9), decreased nitrate reductase activity (19), increased ribonuclease activity (8, 12), dissociation of polyribosomes (13, 14), and altered mRNA transcription and translation (16). Changes in the pattern of protein synthesis during water stress have been observed in double-labeling experiments (6, 7) indicating that the effect is selective. This raises the possibility that water stress may alter the nutritional quality of high protein legume crops such as soybean, the mature seeds of which are 40 to 45% protein on a dry weight basis. However, little is known about the effect of water stress on storage protein synthesis and accumulation in developing legume seeds.

In this paper we report that protein accumulation in developing ovules of two soybean varieties is not prevented and can actually be increased by moderate or severe (leaves senesced) water stress. We also present evidence for stress-induced foliar nitrogen mobilization which appears to account for the maintenance of normal or supranormal seed protein accumulation.

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MATERIALS AND METHODS

Plant Material. Two varieties of *Glycine max* L. Merr. were used in this study. Seed of cv Wayne was purchased from the Agricultural Alumni Seed Improvement Association Inc., West Lafayette, IN. Seed of 9656, an experimental low linolenic acid variety, was provided by Dr. W. R. Fehr of the Iowa State University, Agronomy Department. Seeds were disinfected in 10% (v/v) commercial bleach solution for 10 min, rinsed thoroughly with deionized H_2O, sown in flats of perlite, and germinated in the greenhouse. Seedlings were transferred into the hydroponic system 2 weeks after sowing. The nutrient solution used was that described by Epstein (11) except that Fe-EDTA was replaced with Sequesterene 330 Fe (Ciba-Geigy Corp., Greensboro, NC) at a concentration of 12.5 mg/l. KNO_3 was the major N source in this solution. Young plants were grown in half-strength nutrient solution for the first 2 weeks after transfer into the hydroponic system and full strength solution thereafter. The pH of the solution was maintained between 6.2 and 6.5 and the solution was replaced twice a week. Aeration was provided by bubbling compressed air through the nutrient solution. Newly opened flowers were tagged daily with color-coded thread for approximately 1 month to provide a precise record of seed age at harvest.

Administration of Water Stress. Stress was applied stepwise by addition of PEG-6000 (Carbowax 6000, Union Carbide Corp.) to the nutrient solution in 2.5% (w/v) increments every 2 d to a final level of 12.5% PEG (about −9.2 bars). The PEG concentration was held at 12.5% for 10 to 13 d. For the severe stress experiment in the summer of 1981, the plants were grown in 2.5-L pots, and transpired solution was replaced with deionized H_2O every evening. During the day the solution level was allowed to drop due to transpiration thereby decreasing the water potential to −15 to −20 bars. For the moderate stress experiment in the summer and fall of 1982, the plants were grown in 5-L pots such that transpiration had little effect on the solution level and the water potential remained between −9 and −12 bars. At the end of the stress period, pods were harvested, sorted by age, shelled, and the seeds frozen in liquid N and stored at −30°C.

Determination of Fresh Weight, Dry Weight, and Protein Content of Seeds. Fresh weight was determined by weighing the frozen beans. Frozen seeds were then lyophilized to constant weight and dry weights recorded. Dry seeds of the same age from plants within a treatment were then pooled and ground to a fine powder in a Wiley Mill. Samples were prepared for protein determination by homogenizing 10 mg flour in a 1.5-ml ground glass homogenizer with 1 ml of the globulin extraction buffer described by Wolf and Briggs (24). The homogenate was poured into a 1.5-ml disposable centrifuge tube and centrifuged 5 min in an Eppendorf tabletop centrifuge. The layer of lipid on top of

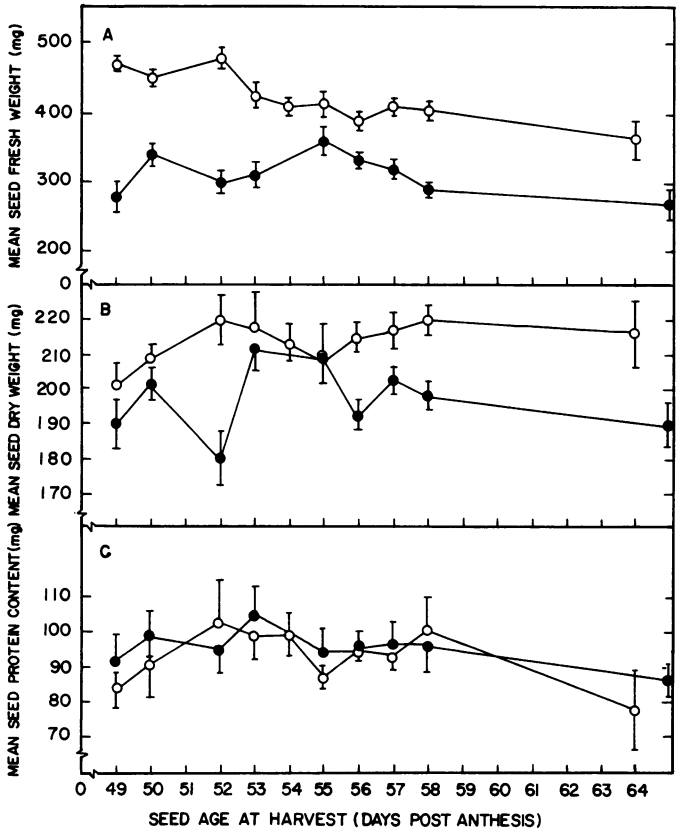

FIG. 1. Effect of severe water stress on fresh weight (A), dry weight (B), and extractable protein content of developing soybean (cv Wayne) ovules. Plants were hydroponically grown and stressed by inclusion of PEG-6000 in the nutrient solution as described in "Materials and Methods." Seeds from control (O) and severely stressed (●) plants were harvested 21 d after first addition of PEG. Data shown represents the mean ±SE among the five plants per treatment.

the supernatant was aspirated and the supernatant transferred to a fresh tube and centrifuged an additional 5 min. Remaining lipid was aspirated. Protein estimation was by the Coomassie blue dye-binding method of Bradford (4) (Bio-Rad Laboratories) with BSA as the standard.

SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE in 1-mm thick 12.5%T–2.6%C slab gels was essentially as described by Laemmli (17). Protein samples in globulin extraction buffer were mixed with an equal volume of electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated 3 min in a boiling water bath. Samples containing equal protein (commonly 20–50 μg) were then loaded on all lanes of the gel. Electrophoresis was at 15 mamp during stacking and 25 mamp until completion. Gels were stained in 0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid, and destained in 50% (v/v) methanol, 10% (v/v) acetic acid, followed by 7% (v/v) acetic acid, 5% (v/v) methanol.

RESULTS AND DISCUSSION

Previous work from this laboratory (1) has shown that cv Wayne ovules accumulate approximately 80% of their maximum fresh weight, dry weight, and protein between 25 and 50 d post anthesis. Sionit and Kramer (22) reported that it is this 'podfill' period that is most sensitive to drought-induced yield reductions in the field. Therefore, the initial experiment of this study was to examine the effect of severe water stress on ovule development during podfill.

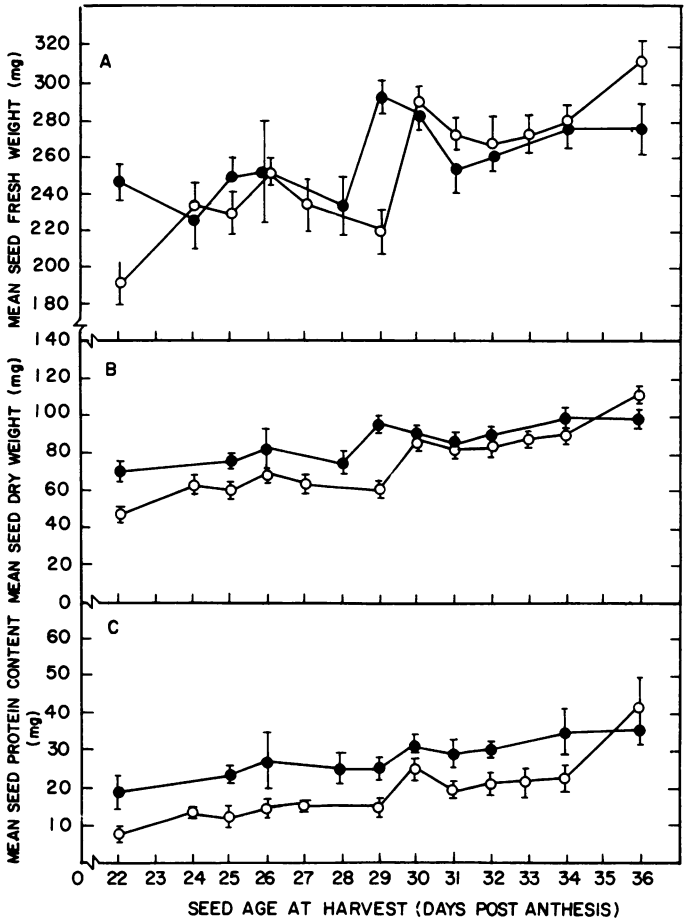

FIG. 2. Effect of severe water stress on fresh weight (A), dry weight (B), and extractable protein content of developing soybean (experimental variety 9656) ovules. Plants were stressed as described in Figure 1. Seeds from control (O) and stressed (●) plants were harvested 18 d after first addition of PEG. Data shown represents the mean ±SE among the five plants per treatment.

With severe stress (−16 to −20 bars) leaves rapidly yellowed and within 7 d of 12.5% PEG treatment most leaves had abscised and the distal end of the pods had begun to senesce and shrivel. No differences were observed between the two soybean varieties in this regard.

At the beginning of stress, Wayne seeds were in the podfill stage, their ages ranging from 28 to 44 d post anthesis. At harvest, the seeds had achieved their maximum size and were beginning to desiccate. Fresh weight was dramatically reduced by severe water stress in Wayne seed of all ages (Fig. 1A). Dry weight was also reduced, though not as dramatically as fresh weight (Fig. 1B). However, total protein per seed was unaffected by severe water stress even though the seeds weighed less than control ovules of the same age. Thus protein accumulation in developing ovules was resistant to water stress and was not inhibited as reported for protein synthesis in vegetative tissues (2, 15).

9656 ovules were severely stressed earlier in their development than were Wayne, the ovules ranging from 4 to 18 d post anthesis at the beginning of stress. The seeds had entered the podfill stage at harvest 18 d later. We believed this early stage of seed development might be very sensitive to severe stress, but this was not the case. Though 9656 plants were defoliated like Wayne plants by severe stress, the developing ovules were resistant to water stress. Fresh weight was unaffected by severe water stress in 9656 ovules of all ages (Fig. 2A). Dry weight accumulation was also resistant to severe stress (Fig. 2B), but seed protein content was

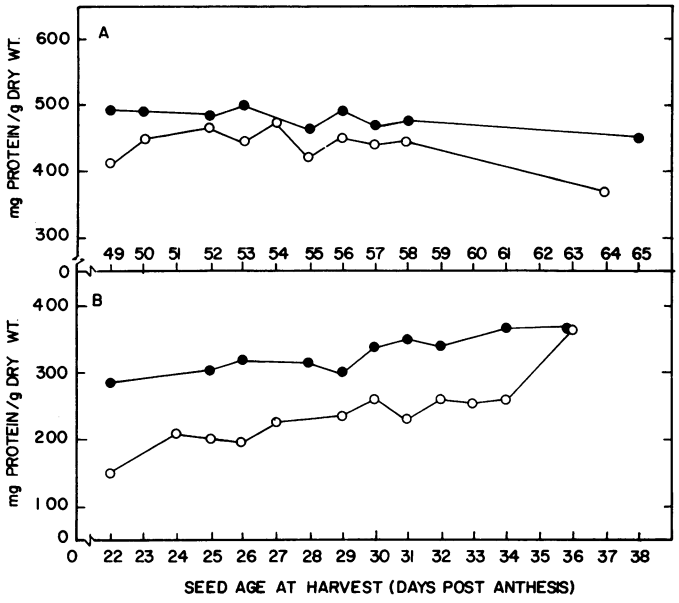

FIG. 3. Effect of severe water stress on relative protein content of cv Wayne (A) and 9656 (B) ovules. Wayne and 9656 plants were stressed and harvested as described in Figures 1 and 2, respectively. Control (O), severely stressed (●).

significantly higher in ovules of severely stressed *versus* control plants (Fig. 2C). Relative protein content (mg protein per gram dry weight) of developing ovules was greater for stressed than for control seeds of both Wayne (Fig. 3A) and 9656 (Fig. 3B). This demonstrated that protein accumulation was conserved more than some other metabolic path(s) that also contributed to dry weight. As a result protein comprised a greater percentage of seed dry weight after stress. Preliminary evidence suggested that in Wayne, the lipid content per seed was reduced with severe stress (data not shown), which may partially account for the observed increase in relative protein content.

Maintenance of normal or supranormal protein accumulation in developing ovules while the leaves of the plant senesced was surprising. The ovules did not appear to be 'escaping' stress because the distal end of the pods senesced and shriveled during the experiment. In Wayne, seed fresh and dry weight were reduced with lethal stress, indicating that stress affected the ovules. The fact that 9656 ovules were stressed earlier in their development than Wayne ovules may account for the observed difference in varietal response.

Since the plants were grown without benefit of *Rhizobium* infection, it is assumed that nitrate reductase (and its associated enzymes) activity accounted for all nitrogen used by the plant. Morilla *et al.* (19) have shown that nitrate reductase activity in maize is severely inhibited by mild water stress; however, no data are available for nonnodulated hydroponically grown soybeans. The possibility that ovules might be able to reduce nitrate is precluded by the work of Thompson *et al.* (23). They showed that soybean cotyledons cultured *in vitro* were incapable of growing with nitrate as the sole nitrogen source. However, if cotyledons were supplied with reduced nitrogen, preferably glutamine, they accumulated protein faster than did cotyledons developing on the plant, suggesting that availability of reduced nitrogen limits the rate of protein accumulation in developing ovules *in vivo*. Such considerations lead us to hypothesize that during severe water stress, protein accumulation in developing ovules continues to be dependent upon reduced nitrogen mobilized from vegetative tissues of the plant. Elimination of secondary sinks, such as expanding leaves, coupled with efficient mobilization of nitrogen from vegetative tissues, could result in

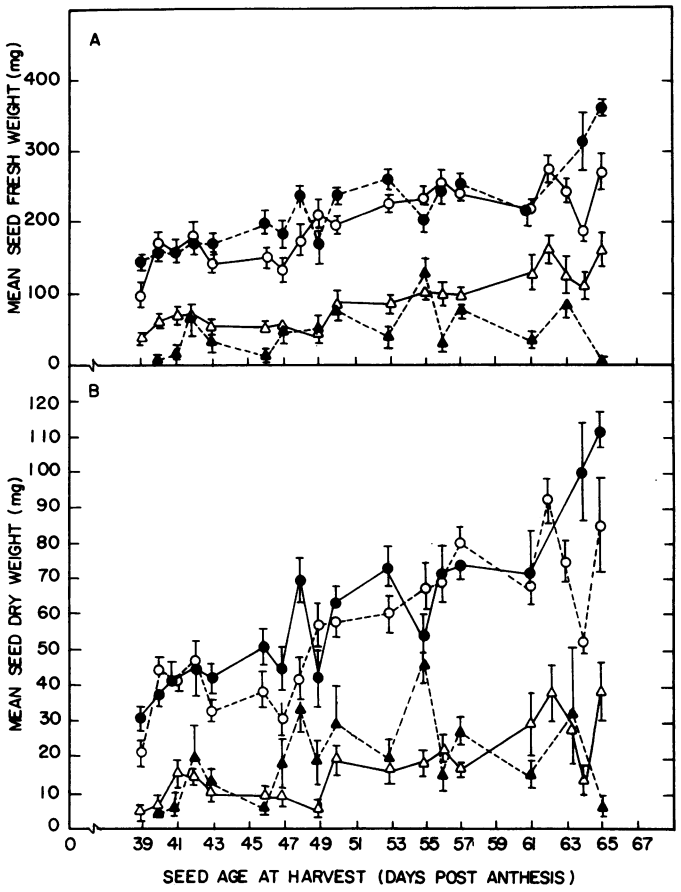

FIG. 4. Effect of moderate water stress and defoliation on fresh weight (A) and dry weight (B) of developing soybean (cv Wayne) ovules harvested 21 d after first addition of PEG-6000 to the nutrient solution. Treatments were; control (O); stressed, (●); defoliated (Δ); and stressed, defoliated (▲). Data shown represents the mean ±SE among the five plants within a treatment.

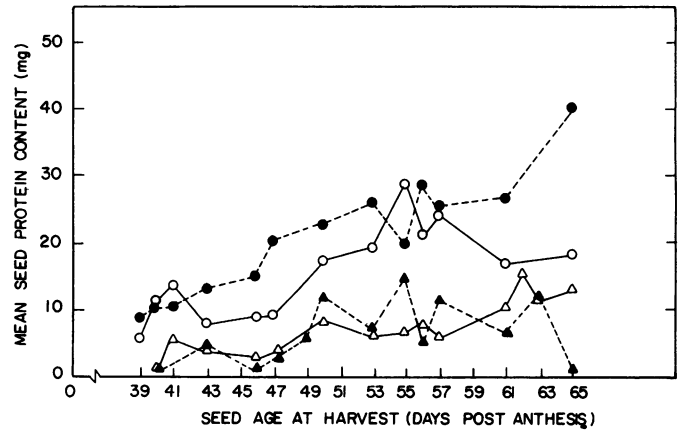

FIG. 5. Effect of severe water stress and defoliation on extractable protein content of developing soybean (cv Wayne) ovules. Plants were stressed and harvested as described in Figure 4. Treatments were: control (O); stressed (●); defoliated (Δ); and stressed, defoliated (▲). Data was not collected individually for each plant within a treatment; instead seeds of the same age within a treatment were pooled and then analyzed for protein content. As a consequence, the standard error of the mean was not computed as in Figures 1 and 2.

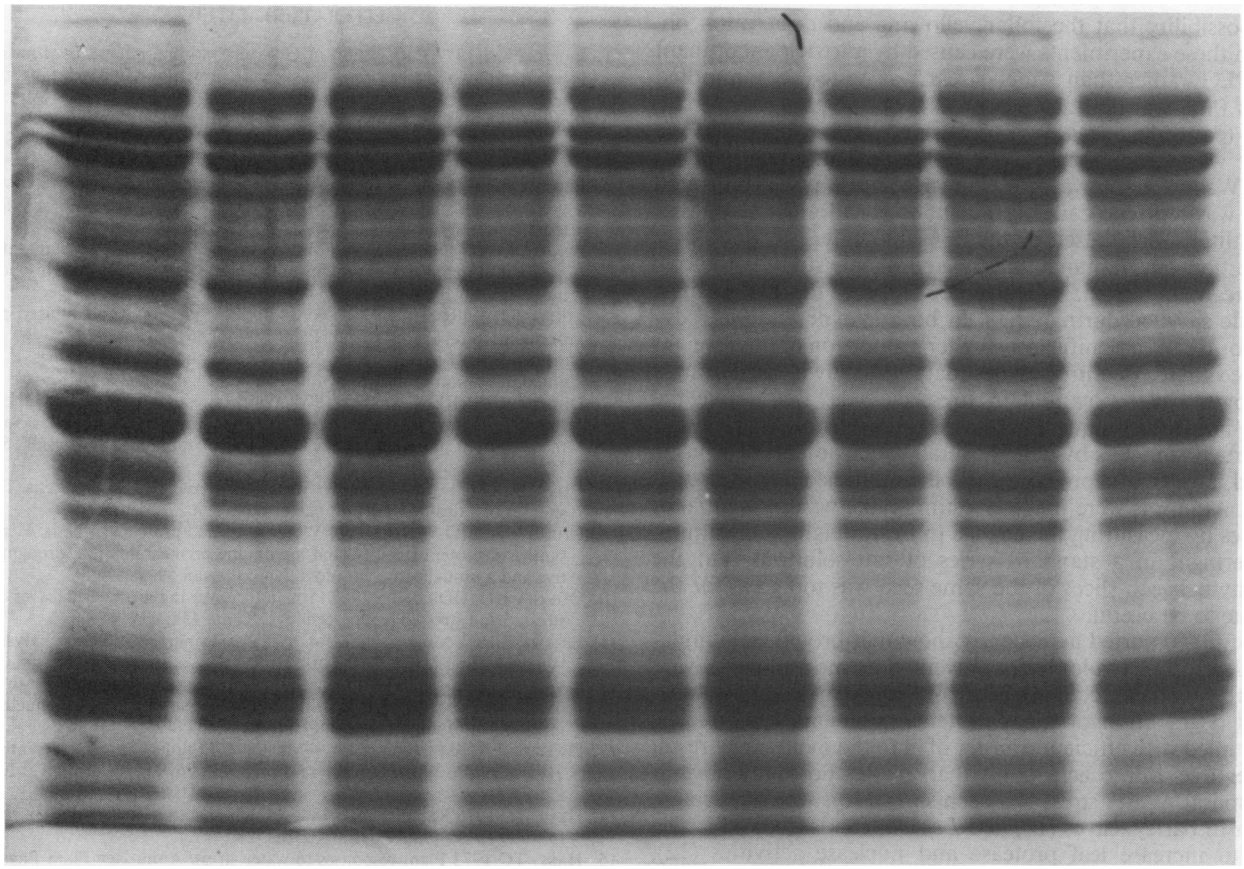

FIG. 6. One-dimensional SDS-PAGE analysis of seed proteins from control and severely stressed soybean (cv Wayne) plants. Plants were stressed and seeds harvested as described in Figure 1. Lane 1: 49-d-old, control; 2: 49-d-old, stressed; 3: 54-d-old, control; 4: 54-d-old, stressed; 5: 59-d-old, control; 6: 59-d-old, stressed; 7: 64-d-old, control; 8: 64-d-old, stressed; 9: fully mature and dried, control.

higher free amino acid pools than ovules normally have at their disposal for protein synthesis, which might account for increased protein accumulation in 9656 ovules with severe stress.

Observation of foliage during severe stress indicated that salvaging of metabolites was occurring. The first visual symptom of stress was chlorosis along the margins of fully-expanded leaves. Chlorosis rapidly spread to interveinal areas of the leaves and was followed by interveinal necrosis. During these changes, the major leaf veins remained green and turgid, giving the leaves a skeletal appearance. Leaf veins were the last laminar tissues to senesce, and leaf abscission followed shortly. From first observation of chlorosis to abscission took approximately 7 d in 12.5% (w/v) PEG (in 2.5-L pots). Progressive yellowing of leaves also occurs during monocarpic senescence, when N is known to be mobilized from leaves to seeds and protein accumulation in seeds proceeds rapidly (20). Stress-induced mobilization of N may be similar, if not identical, to the salvaging that occurs during monocarpic senescence.

To test the hypothesis that it is primarily leaves, and not stems or roots, which supply developing ovules with reduced N during stress, a defoliation experiment was performed in the summer and fall of 1982. Plants (cv Wayne only) were grown in 5-L pots so that transpiration had little effect on the solution level. Stress was moderate causing interveinal chlorosis within 7 d of 12.5% PEG treatment. At first addition of PEG (2.5% w/v), half the plant were defoliated, giving four treatments: control; stressed; defoliated; and stressed, defoliated. Expanding leaf buds were removed daily from defoliated plants for the duration of the experiment. Twenty-one days after first addition of PEG, seeds were harvested and compared for fresh weight, dry weight, and protein content as before.

Moderate water stress had far less impact on fresh weight accumulation than did defoliation (Fig. 4A). Comparing defoliated and intact plants separately, moderate water stress decreased the fresh weight of ovules on defoliated plants, but had no effect on fresh weight of ovules developing on intact plants. Seed pods of moderately stressed, defoliated plants rapidly withered and senesced, whereas pods remained green and turgid with all other treatments. Thus the presence of leaves was necessary for pods to withstand stress. Without leaves, ovules rapidly desiccated, resulting in decreased seed fresh weight.

Dry weight accumulation in developing Wayne ovules was reduced by defoliation but was not further reduced by stress (Fig. 4B). There was no difference in seed protein content of stressed *versus* unstressed defoliated plants (Fig. 5). However, in intact plants seed protein content was higher with stress. Thus, leaves appeared to be the source of N mobilized to ovules during stress.

It is possible that higher seed protein content with stress might be due to pod shedding resulting in fewer seeds with more nitrogen available to them than is usually the case. However, no significant difference in seed number with stress was found in any of our experiments (data not shown).

When proteins of stressed and control seeds were analyzed by one-dimensional SDS-PAGE, no differences in protein banding patterns were detected. A gel comparing control and severely stressed Wayne seed proteins from ovules of several different ages is shown in Figure 6. There are no major differences in the polypeptide banding patterns in any of the lanes. This was also true for seed proteins of severely stressed 9656 and defoliated Wayne (gels not shown).

The possibility that the foliar yellowing and desiccation observed in these experiments were caused by a toxic contaminant in the PEG rather than water stress was tested. Six hundred grams of PEG was dialyzed against 2 L of full-strength nutrient solution (equivalent to a 30% PEG solution) for 48 h, and the dialysate was used to replace the nutrient solution in which a mature Wayne plant was growing in a 2.5-L pot. Transpired solution was replaced daily with distilled H_2O for 7 d. No foliar abnormalities were caused by the PEG dialysate.

A question that remains unanswered in this study is the role of osmoregulation in the maintenance of source to sink transport and ovule survival during stress. In both Wayne and 9656 dry weight comprises a significantly higher proportion of fresh weight in stressed than in control ovules (data not shown) suggesting a much lower water potential in stressed seeds. But unlike the pressure-bomb technique for leaves, there is no accurate and nondestructive means by which overall water potential, osmotic potential, and turgor pressure can be simultaneously measured in seeds. Attempts were made to estimate the water potential of seed slices by measuring the change in fresh weight after immersion overnight in a standard series of salt solutions, but the variability between slices of the same seed was too great for the technique to be useful.

The results reported here clearly show that protein accumulation in developing ovules is not prevented by moderate or severe water stress, and can actually be increased by stress.

Rapid degradation of foliar proteins, pigments, and nucleic acids coupled with efficient transfer of N to the ovules may result in higher free amino acid pools than ovules normally have at their disposal. Water stress has been shown to stimulate protein turnover in barley leaves (10) and *Lemna* fronds (5), and is also known to increase leaf protease and nuclease activities (12), therefore such a N salvaging scheme is plausible. Pate and Flinn (21) have shown, using long-term pulse-chase experiments with $^{15}NO_3$ and $^{14}CO_2$, that during normal seed development in *Pisum*, N is readily mobilized from leaves to ovules, but carbon is not. This may explain the (tentative) reduction in oil content observed in ovules of severely stressed 'Wayne' plants, since oil is the major storage form of carbon in soybeans.

Accelerated protein synthesis or decelerated protein turnover could explain stress-induced increases in seed protein content. However, Madison *et al.* (18) were unable to detect any turnover of soybean seed proteins during normal seed development. Therefore it would appear that any observed increase in seed protein content is the direct result of more rapid protein synthesis.

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