Effects of Water Stress on the Activities of Three Enzymes in Maize Seedlings

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ABSTRACT

Changes in the activities of three enzymes (nitrate reductase, L-phenylalanine ammonia-lyase, and a dehydrogenase adenine dinucleotide-oxidase complex) were measured during development of water stress in young maize (Zea mays) plants.

L-Phenylalanine ammonia-lyase and nitrate reductase activities decreased markedly with water deficits of 10 to 20%. The activities did not reach zero at water deficits as high as 50%, but appeared to approach a new steady state. Partial to complete recovery of enzyme activity occurred 24 hours after rehydration of the stressed plants. The oxidase activity did not respond to water stress in the same manner as that of the other two enzymes.

It is suggested that the level of enzyme activity is a consequence of an equilibrium between the rates of synthesis and degradation, and that progressive tissue dehydration reduces both the enzyme synthesis and the enzyme-inactivating systems.

MATERIALS AND METHODS

The dominant tall form of the d, dwarf maize (Zea mays) was grown in vermiculite in the greenhouse. Nutrient requirements were provided with a modified Hoagland's solution 1 (9). Moisture stress was initiated by withholding water for varying periods beginning approximately 10 days after germination. In early experiments, the plants remained in the greenhouse until sampled for assay, but in later experiments they were transferred to a growth chamber when water stress was initiated. The chamber environment was 27 C, 30 to 50% relative humidity, and a light period of 12 hr/day of 1000 ft-c. The plants were sampled at the middle of the light period.

In the rehydration experiments, the vermiculite base was saturated with water 24 hr prior to sampling.

Water deficit was estimated by the method of Weatherly (21). Duplicate or triplicate 5-cm sections were taken from the midportion of the third leaf of two or three plants. The original weight was determined immediately after harvest, and the tissue was floated in deionized water for 4 hr. At that time, the saturated weight was taken and the tissue was dried at 95 C for 24 hr for dry weight determination. The water deficit was expressed as the ratio of water taken up divided by the final water content, multiplied by 100.

The midportion of the leaf blades from four to six seedlings was used as the source of tissue for nitrate reductase and NADH oxidase. PAL was prepared from the leaf sheaths of the same or comparable seedlings. Nitrate reductase activity was determined by the method of Hageman and Plesher (6), except that the tissue was ground in a chilled mortar with pestle and sand. PAL was prepared and assayed as described by Reid and Marsh (15). NADH oxidase activity was determined in the same extract used for nitrate reductase. The reaction mixture for the oxidase assay contained 200 μmoles of tris buffer, pH 8.2; 20 μmoles of (NH₄)₂SO₄; 0.66 μmole of NADH; plus enzyme in a final volume of 3 ml. The reaction was run at 30 C and was initiated by the addition of NADH. Changes in absorbancy at 340 nm were followed spectrophotometrically for 5 min.

One unit of activity for all three enzymes was defined as the amount of enzyme which catalyzed the formation of 1 μmole of product in 1 min under standard conditions. Protein was determined by standard biuret or Lowry methods (12) with bovine serum albumin as a standard.

RESULTS

The results of water deficit measurements (Fig. 1) indicated a diurnal stress, presumably caused by high transpiration during the day, followed by turgor recovery associated with low transpiration at night. The varying water content of the tissue introduced a problem in selecting the most suitable basis for expressing the results of the enzyme activity determinations. Because of the large amount of water lost from the desiccated

1 Part of this material was taken from a thesis by John M. Bardzik submitted to the University of Massachusetts in partial fulfillment of the requirements for the M.S. degree.

9 Abbreviation: PAL, L-phenylalanine ammonia-lyase.
tissue (up to 40–50% in some instances, cf. Figs. 1 and 4D), presentation of the data on a fresh weight basis did not appear to be valid. Expressing the activities on an organ or seedling basis proved impractical, and therefore the results of the enzyme assays are presented on a protein basis.

The seedlings under water stress, with but one exception (day 7), contained less nitrate reductase activity than the control (Fig. 2). Not only was nitrate reductase activity low in wilted samples taken near midday (points indicated by "p" in Fig. 2), but the activity remained low the following morning (indicated by "a" in Fig. 2), even though the leaves had temporarily regained turgidity overnight. On the 6th day and again on the 7th day after the initiation of this experiment, conditions prevailed which caused the specific activity of nitrate reductase in the control tissue to increase almost 2-fold during the time from midmorning to early afternoon (Fig. 2). In the stressed tissue, however, this increase in nitrate reductase activity did not occur. The high degree of fluctuation in both water deficit and nitrate reductase activity, especially during days 5 to 7, possibly was caused by varying weather conditions, which were only slightly modified by the greenhouse. In order to eliminate these environmental variables, all further experiments were conducted in a growth chamber.

An experiment was designed to determine the effect of increasing severity of water stress upon the activities of nitrate reductase, PAL and the NADH oxidase complex. Water was withheld from the plants for varying periods up to 6 days, at which time all samples were harvested and the water deficit and enzymatic activities were determined. Typical data from this type of experiment are presented in Figure 3. PAL activity was decreased markedly at approximately 10% water deficit while nitrate reductase activity was similarly decreased at 20% water deficit. In this experiment the activity of PAL appeared to reach a new steady state level at about one-half that of the control plants when the water deficit was in the range of 20 to 40%. The activity of nitrate reductase also decreased to about 50% of that of the control values and fell only slightly between water deficits of 20 and 40%. On the other hand, the activity of NADH oxidase in this experiment remained near the control level.

A time course study was conducted to establish the rate of loss of the enzymatic activities in plants subjected to stress conditions. Ideally, the rate of loss of enzyme activity in tissue maintained at a constant level of water deficit should have been investigated. However, establishing a constant level of water deficit, other than near saturation, is not practical because of the daily fluctuations, presumably associated with transpiration (Fig. 1). It is possible, however, to follow changes in enzyme activity with time as the tissue becomes progressively more and more desiccated. The results of such an experiment are presented in Figure 4. Water was withheld from the plants at day zero, and PAL, nitrate reductase, and NADH oxidase activities as well as water deficits were determined in both control and stressed plants over the next 4 to 5 days. Control values on day 1 for nitrate reductase, PAL, and NADH oxidase were 0.140, 17.3, and 36.5 milliunits/mg protein, respectively, and remained relatively constant throughout the duration of the experiment. Experiments were also included to determine the effect of relief from water stress conditions on these enzymatic activities. On each day, starting the 1st day after the initiation of the experiment, a part of the population of stressed plants was watered, and 24 hr later tissue from these rehydrated plants was also assayed for enzyme activity and water deficit. Control plants here, as in the other experiments, were watered daily.

The activities of both nitrate reductase and PAL decreased with time and desiccation, but in this case the initial rate of loss of nitrate reductase activity was faster than that of PAL. Four days after the experiment was initiated the water deficit was 50%. The experiment was not carried beyond this point because the tissue appeared almost dead. Upon watering, however, these severely stressed plants recovered turgor and eventually resumed growth. Even at the most advanced stages of desiccation studied, both nitrate reductase and PAL re-
mained partially active. In this experiment, the activity of nitrate reductase appeared to reach a minimal level (on days 3 and 4) below which it did not fall, even though during this time the water deficit increased from 25 to 50%.

Watering the plants resulted in essentially complete recovery of turgor within 24 hr, as shown by the data presented in Figure 4D. Concomitant with this regain of turgidity was a relatively rapid increase in the activities of nitrate reductase and PAL. The recovery of nitrate reductase activity was especially dramatic considering the fact that the tissue had been subjected to a 50% water deficit on day 4.

In this experiment, the activity of the NADH-oxidase complex was also reduced by the water stress. The reduction in activity, however, was smaller and less consistent than that of the other enzymes studied.

**DISCUSSION**

Our observations suggest that water stress induced a new and lower level of activity of two of the enzymes studied, nitrate reductase and PAL. A third activity, NADH oxidase, although reduced in some instances, did not exhibit a consistent decrease upon desiccation. The protein responsible for the latter activity was not characterized, but it is likely that several enzymes functioned in this oxidation reaction. It is also conceivable that the water stress inactivated some NADH oxidases and concomitantly activated others. However, the relatively high NADH oxidase activity at all stages of water stress serves to illustrate that desiccation does not automatically cause a reduction of all enzymatic activities.

Both nitrate reductase and PAL are reported to be enzymes formed and inactivated within a few hours and subject to control mechanisms which as yet are only poorly understood (3, 11, 17, 23, 24). A number of factors lead to enhanced activity of both enzymes. The available evidence suggests that the increase in activity is due to synthesis of new protein and not to activation of a pre-existing protein (1, 24). Recently, evidence has accumulated that the loss of activity is also an active process (14, 20, 23, 24). The addition of cycloheximide to maize leaf sheaths prevented the loss of PAL activity normally observed in excised tissue (14, 16). This observation suggested that the activity of a PAL-inactivating system was dependent upon continued protein synthesis. Although the loss of nitrate reductase activity in excised barley leaves held in the dark was prevented by cycloheximide (20), in leaves excised from maize seedlings, the inhibitor did not prevent loss of the reductase activity (14). Thus, it would appear that in maize different systems contribute to the inactivation of these two enzymes. The differential loss of nitrate reductase and PAL observed here is consistent with this concept. Recent evidence (Pan and Reid, unpublished) indicates that the level of the PAL-inactivating system is itself variable. It is possible that this variability could explain the differing rates of loss of PAL activity frequently observed (cf. Figs. 3 and 4).

The data presented in Figures 3 and 4 show that moderate water stress reduced the activity of PAL and nitrate reductase. But a further increase in the severity of the water stress from 20 to 40% or even 50% deficit caused only a negligible further decrease in activity. Any interpretation of these observations must take into account this failure of the enzymatic activities to fall completely to zero in the severely stressed tissue. It is assumed that, in maize tissue, protein synthesis and degradation are continuously proceeding and that the level of enzyme activity observed reflects the equilibrium established between the rates of synthesis and degradation. It follows that one interpretation of our observations would be that the desiccation somehow resulted in a reduced rate of protein synthesis. According to this assumption, the protein-inactivating systems would continue to inactivate nitrate reductase and PAL until they themselves also were inactivated. Once this happened, the level of activity would remain relatively constant until further drastic changes occurred, as, for example, further extreme desiccation leading to the death of the tissue or, alternatively, recovery of the protein-synthesizing capacity as a result of watering the plants.

Enhanced inactivation for a limited period is also a plausible alternative explanation of our results. Travis et al. (20) and Zucker (25) recently reported evidence which indicated that transfer of tissue from light to dark conditions promoted the activity of nitrate reductase and PAL-inactivating systems. It is possible that the stress conditions studied here also promoted inactivation. However, if this was the case, it is again necessary to postulate that the activity of the inactivating systems in time was also reduced. It is, of course, quite possible that a combination of reduced synthesis and enhanced inactivation for a transitory period may well prove to be the real explanation of the phenomena reported here.

Yet another interpretation of our data is that there are two distinct species of both enzymes, and that only one species is subject to inactivation by desiccation. There may be two species of nitrate reductase (17), but there is no convincing evidence to date for two species of PAL in maize. At this time, while this alternative may be possible, it does not seem probable.

It is also possible that the reductase and ammonia-lyase were only temporarily inactivated by the desiccation and that the hydration renatured the proteins. The fact that in tissue rehydrated for 24 hr PAL and nitrate reductase activity were only partially restored and the observation that the reductase activity did not recover overnight although the leaves became fully turgid in this time period (Figs. 1 and 2) argue against this possibility. The partial recovery of activity in 24 hr following rehydration is, however, itself significant. It means that, if reduced protein synthesis was the cause of the reduced enzyme activity, the ability to synthesize protein was not permanently destroyed in this tissue, assuming that the increase in activity was dependent upon protein synthesis. Or, alternatively, if the reduced enzyme activity was due to enhanced inactivation, then it would follow that the inactivating systems must themselves be short-lived.
The inhibition of plant growth in desiccated tissue in all likelihood could not be explained by a lower steady state level of nitrate reductase or PAL activity per se. Although moderate water stress did reduce the activity of both enzymes, sufficient activity probably remained to carry out the normal functioning of the two enzymes. Nitrate did not accumulate above the level of the controls in the desiccated tissue (Bardzik, unpublished observations), in contrast to what would be predicted had the level of nitrate reductase been limiting. This observation is in agreement with that of Younis et al. (22), who found that maize seedlings grown under conditions of suboptimal water supply contained the same or even less nitrate than did the control tissue. Mattas and Pauli (13), on the other hand, reported that a combination of high temperature and desiccation reduced nitrate reductase activity and caused nitrate to accumulate. Shading was reported to reduce the level of nitrate reductase activity in maize seedlings (7), but it had only a negligible effect on the level of nitrate in the tissue. Apparently, under most conditions the leaves of maize seedlings contain sufficient excess nitrate reductase that a large proportion of the activity can be lost without seriously impairing nitrate reduction.

PAL diverts L-phenylalanine from the free amino acid pool to the pathway of phenylpropanoid metabolism which leads to compounds considered to have a profound effect on plant metabolism and growth (8). There is no evidence, however, that reduced PAL activity would restrict growth. Rather than suggest that the reduced activity of these particular enzymes would affect growth, we view the effect of desiccation on nitrate reductase and PAL as a model of the potential effect of water stress on the activity of short-lived proteins.

LITERATURE CITED