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SHORT COMMUNICATION

Rapid determination of free proline for water-stress studies

Summary

Proline, which increases proportionately faster than other amino acids in plants under water stress, has been suggested as an evaluating parameter for irrigation scheduling and for selecting drought-resistant varieties. The necessity to analyze numerous samples from multiple replications of field grown materials prompted the development of a simple, rapid colorimetric determination of proline. The method detected proline in the 0.1 to 36.0 μmoles/g range of fresh weight leaf material.

Introduction

Severe water stress induces numerous metabolic irregularities in plants. A tremendous free-proline accumulation (up to 100 times the normal) is one of the most dramatic stress characteristics [1]; it has been used as a single parameter to measure physiological dryness[5]. The necessity to quickly sample and analyze field-grown materials prompted us to develop a simple, colorimetric determination of proline suitable for field laboratories.

Chinard [2] described an acid-ninhydrin method for proline which susbsequently was studied for the effects of various interferences [3 4 7 8]. Although several free amino acids can interfere with such proline determinations, the free amino acid levels reported in stressed plants [1 6] were low compared with proline. Color yields of interferring amino acids also were low. The techniques described by Chinard [2] and Troll and Lindsley [7] work well with purified or semi-purified proline samples, but did not work with the simple fractionation and filtration techniques we needed for rapid field analysis.

Concentration and color yield differences suggested a simplified determination of proline for field studies.

Materials and methods

Samples. Fully expanded 'sun' leaves from field-grown soybean and sorghum plants were sampled. Purified proline was used to standardize the procedure for quantifying sample values.

Reagents. Acid-ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation, until dissolved. Kept cool (stored at 4°C) the reagent remains stable 24 hours [7].

Procedure. 1) Approximately 0.5g of plant material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through

Whatman # 2 filter paper. 2) Two ml of filtrate was reacted with 2 ml acid-ninhdrin and 2 ml of glacial acetic acid in a test tube for 1 hour at 100°C, and the reaction terminated in an ice bath. 3) The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 15-20 sec. 4) The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. 5) The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:
[(μg proline/ml × ml toluene) / 115.5 μg/μmole]/[(g sample)/5] = μmoles proline/g of fresh weight material.

Results and Discussion

Field studies of water stress, requiring numerous samples from multiple replications, have been limited greatly by the absence of rapid, simple techniques for determining plant stress conditions. Proline, which increases proportionately faster than other amino acids in stressed greenhouse-grown plants [1], has been used to evaluate controlled-environment stress studies. We selected proline to evaluate similar field studies.

Practicality dictated compromising between absolute accuracy and time-consuming manipulations. We recognized that certain amino acids, notably glutamine, would increase the apparent baseline level of proline. Under stress conditions, the increase of glutamine and other interfering ninhydrin-positive compounds should be negligible in relation to the many-fold proline increase [1 6]. The color yield of glutamine, the major interference, yields less than 1.5 per cent of an equivalent amount of proline [3]. Comparisons between stressed and unstressed individuals should range slightly less than if based upon absolute proline values, but the relative values should indicate the degree of plant stress. Therefore we accepted the acid-ninhydrin reagent of Troll and Lindsley [7] as sufficiently accurate.

Preparation of free proline was simplified by using 3% sulfosalicylic acid. It is colorless, an effective protein precipitant in aqueous solution, and does not interfere with the acid-ninhydrin reaction. Additional interfering materials, which normally raised the baseline at 520 nm, were removed presumably by adsorption to the protein–sulfosalicylic acid complex.

Extraction of the proline–ninhydrin chromophore was accomplished in toluene, a less noxious and more effective solvent than the commonly used benzene. Extraction of naturally occurring free proline and of added proline was rapid and quantitative with complete conformity to Beer's Law.

The spectrum of the chromophore was determined on a Beckman DB–G spectrophotometer. An absorbance maximum at 520 nm was obtained in contrast to maxima of 515 and 517 nm reported under other conditions. The spectrophotometer was calibrated with a didynium standard.

We were able to quantify proline in a range of 0.1 to 36.0 μmoles/g of fresh weight leaf material. The rapid assay required only 2–2.5 hours per set of 20 samples.

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