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A SENSITIVE METHOD FOR THE EXTRACTION AND IDENTIFICATION OF DEOXYRIBONUCLEIC ACID IN PLANT LEAF TISSUE*

R. E. BROWN, BARBARA ZAWADZKA and D. F. MILLIKAN
Department of Horticulture, Missouri Agricultural Experiment Station, Columbia

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Abstract—Deoxyribonucleic acid from plant leaf tissue may be extracted by alkaline digestion and colorimetrically determined with *p*-nitrophenylhydrazine. The present modification differs from the Webb–Levy method in that iso-amyl acetate replaces butyl acetate for extraction of excess reagent, followed by an extraction with ethyl ether prior to the development of color in alkaline butyl alcohol. These modifications have the advantage of reducing color fading to about 1 per cent per hour. Dilute solutions containing as little as 2 micrograms of DNA per milliliter may be assayed.

INTRODUCTION

THE extraction and identification of nucleic acid components of woody plant leaf tissue have been very little examined. Consequently few methods have been proposed, and interfering substances present in many species often restrict their use. Earlier[1] we described a method for extracting total nucleic acid from cherry leaf tissue, but the absence of measurable thymine indicated that this preparation could be considered essentially as all ribonucleic acid (RNA). Later investigations indicate that all of the deoxyribonucleic acid (DNA) is not extracted without an alkaline treatment. Therefore a modification of the Schmidt–Thannhauser[2] technique was developed for complete extraction of DNA. Quantitative estimation of DNA was carried out by the *p*-nitrophenylhydrazine (PNPH) colorimetric method originally proposed by Webb and Levy,[3] but modified for plant materials to remove interfering substances and to reduce the rate of color fading.

Our early investigations on the use of the method with *Prunus* leaf extracts showed that an orange substance possessing a high absorbance at 560 mμ was present which consequently interfered with the purple color given by the PNPH reaction with deoxyribose, which is read at 580 mμ. Further studies indicated that extraction of excess reagent with iso-amyl acetate, rather than with butyl acetate[3] reduced the interference due to the orange substance. Complete separation of the purple color and the interfering orange substance was then obtained by addition of alkali and extraction with *n*-butanol, only the purple compound being extracted into the organic phase. This procedure not only eliminates the interference due to the orange substance, but also reduces the rate of fading of the purple color to about 1 per cent per hr, compared to 2 per cent per min as happens in the aqueous phase.[3] It is necessary, however, to follow the iso-amyl acetate extraction with one of ethyl ether, since traces of trichloracetic acid (TCA) which otherwise are left may cause the color to fade. Ethyl ether not only more effectively removes TCA than amyl acetate, but it will also remove residues of the latter solvent which interfere with smooth butanol extraction.

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[1] R. E. BROWN and D. F. MILLIKAN, *Plant Physiol.* **33**, 44 (1958).
[2] G. SCHMIDT and S. J. THANNHAUSER, *J. Biol. Chem.* **161**, 83 (1945).
[3] J. WEBB and H. LEVY, *J. Biol. Chem.* **213**, 107 (1955).

Estimations of the DNA content are determined by reading the optical density of the *n*-butanol layer at 580 mμ with a spectrophotometer.

ANALYTICAL PROCEDURE

Hydrolysis

Two to three hundred mg tissue is weighed into a 50 ml centrifuge tube and wetted with a small amount of ethanol. 10 ml 0·5N NaOH is added, and the suspension heated at 100° for 15–20 min. The tube is then cooled in ice, and 20 ml ethanol and 1 ml glacial acetic acid are added to precipitate the DNA. After 30 min in the ice bath, the material is centrifuged, the supernatant discarded, and the precipitate heated with 30 ml 5% TCA for 30 min at 100°. The cooled solution is centrifuged and the precipitate washed with 10 ml 5% TCA and recentrifuged. The supernatants are combined and made up to 30 ml volume, 15 ml of this being used for each determination of DNA. During heating the tubes are covered loosely with a glass ampule to retard evaporation.

A stock solution of DNA is prepared from highly polymerized material (Calbiochem A grade lot 5090493) at a concentration of 100 μg/ml in 0·05N NaOH. Dilutions of twice the required strength are made with distilled water, and hydrolyzed with an equal volume of 10% TCA to give the desired final concentration in 5% TCA.

Development of color

One half milliliter 0·5% PNPH in ethyl alcohol is added to each of the 50 ml tubes containing the DNA solution (15 ml in 5% TCA). The tubes are then loosely covered to retard evaporation and placed in a boiling water bath for 20 min.

If a reddish color develops at the end of the heating period, rather than the purple one specific for the PNPH–deoxyribose reaction, too much TCA has been destroyed during the process and all the excess PNPH will not be extracted by the iso-amyl acetate. This can be remedied by adding 1 ml 30% TCA at this point. (It can not be added earlier since a high concentration of TCA will destroy some of the deoxyribose during the heating at 100°.) The tubes are then cooled, and saturated with NaCl by adding a slight excess. Ten milliliters iso-amyl acetate are then added, the tubes stoppered and the contents well mixed on a mechanical shaker for 5 min. The layers are allowed to separate and the top one discarded. This procedure is repeated with a further 10 ml iso-amyl acetate and finally with 10 ml ethyl ether, the organic layer being discarded in each case. The tubes are then exposed to a slow current of air to evaporate any remaining ether. Five milliliters n-butanol are added, the tubes are then cooled, after adding 5 ml 50% NaOH, shaken on a shaker for 5 min, centrifuged and the optical density of the n-butanol phase read at 580 mμ in 1 cm cells.

VALIDITY OF METHOD

Recovery experiments

Early experiments by Brown[4] in which small amounts of DNA were added to *Prunus* extracts resulted in essentially complete recovery. In four additional tests recoveries amounted to 98, 99, 107 and 109 per cent. Similar results were obtained when known amounts of DNA were added to yeast RNA and to mixtures containing known amounts of the three commonly occurring pentoses (arabinose, xylose and ribose). In agreement with Webb and Levy's observations no additive effects on color intensity were found that could be attributed to these sugars or to RNA.

[4] R. E. BROWN, Unpublished data.

Sensitivity of reaction

In the procedure described additional specificity for DNA is obtained by extraction of the colored compound by n-butanol, and amounts as low as 2μg/ml can be assayed (Fig. 1).

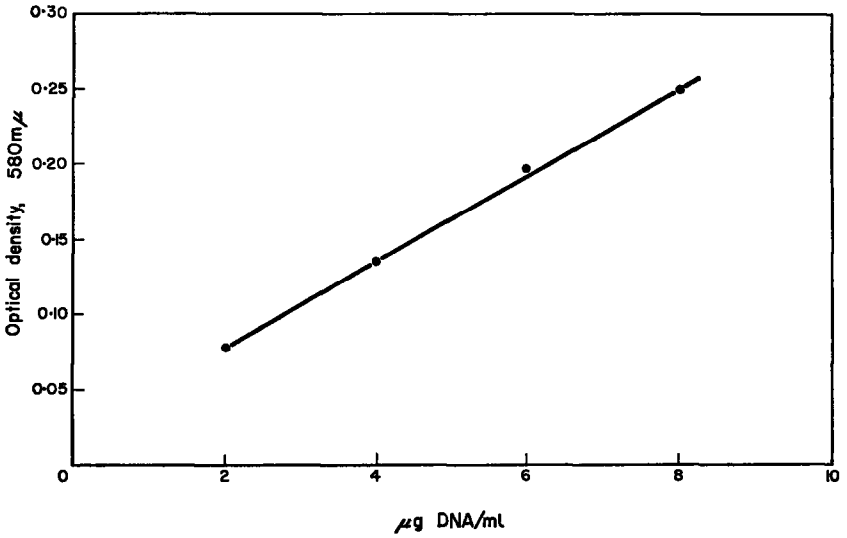

FIG. 1. SENSITIVITY OF PNPH WITH KNOWN AMOUNTS OF SALMON SPERM DNA

Comparison of PNPH and diphenylamine

Two other methods for estimating the DNA content of plant tissues have been described.[5,6] Keck proposed a modification of Ceriotti's indole test,[7] and Burton modified the diphenylamine test[8] to eliminate interfering substances. When these procedures were compared with that using PNPH, the latter proved to be the most sensitive. The indole method is more sensitive than diphenylamine, but has no particular advantage since it involves extraction techniques similar to PNPH with about one half the sensitivity. Since diphenylamine was found to be satisfactory, although less sensitive than the PNPH, comparative tests were made on *Prunus* (cherry) and *Malus* (apple) extracts. The data are listed in Table 1.

TABLE 1. COMPARISON OF PNPH WITH DIPHENYLAMINE FOR DETERMINATION OF DNA IN PLANT LEAF EXTRACTS

Sample	Plant species	μg DNA/g dry wt. PNPH*	Diphenylamine*
1	*Prunus cerasus* var. Montmorency	484·1	519·9
2	*Prunus cerasus* var. Montmorency	473·3	517·7
3	*Malus sylvestris* var. Golden Delicious	759·5	776·2

* Average of three determinations.

[5] K. KECK, *Arch. Biochem. Biophys.* **63**, 446 (1956).
[6] K. BURTON, *Biochem. J.* **62**, 315 (1956).
[7] G. CERIOTTI, *J. Biol. Chem.* **198**, 297 (1952).
[8] Z. DISCHE, *Milrochemie* **8**, 4 (1930).

As indicated in Table 1 DNA contents determined by PNPH tend to be somewhat lower than those using diphenylamine, data which supports Webb and Levy's observations on animal tissues. These workers pointed out that PNPH, as distinct from diphenylamine, does not react with protein or protein breakdown products resulting from the TCA hydrolysis. It is not known if this difference is the reason for the results obtained in Table 1, or whether the presence of the organge compound which shows obsorption at 560 mμ interferes with the diphenylamine determination. The PNPH procedure described eliminates the orange compound completely. No such studies have been carried out with the Burton method, apart from the use of acetaldehyde for eliminating impurities.

DISCUSSION

This proposed modification of the *p*-nitrophenylhydrazine colorimetric test is a useful method for detecting DNA in plant leaf extracts, particularly in woody deciduous species known to contain considerable quantities of interfering substances. Extraction of the TCA hydrolysate with iso-amyl acetate and ethyl ether, followed by addition of alkali and extraction of the purple compound characteristic of the reaction into n-butanol, not only increases the sensitivity of the test and improves the color stability but removes interfering materials. Furthermore, these investigations have indicated the necessity of an alkaline treatment prior to acid hydrolysis in order to obtain complete extraction of the DNA.

EXPERIMENTAL

Reagents

Apart from ordinary laboratory reagents, the following analytical reagent chemicals are used: n-butyl alcohol, deoxyribonucleic acid (Calibiochem),* ethyl ether, iso-amyl acetate, *p*-nitrophenylhydrazine (Eastman Kodak), trichloroacetic acid, and perchloric acid (PCA).

Preparation of leaf tissue

Either fresh leaf tissue, or lyophilized material may be used. In the case of fresh material, batches of 10–15 g are ground in a blendor with 100 ml cold ethanol. The suspension is poured on Whatman No. 3 filter paper in a buchner funnel, washed with 100 ml portions of acetone under suction, and finally dried in a vacuum dessicator. Lyophilized material may be more convenient, and is prepared according to Levitt[9] for potato tuber slices, (frozen with liquid air or dry ice, dried over alumina and ground in a Wiley Mill using 50-mesh screen). To remove interfering substances weighed samples 2–300 mg are treated on a suction filter with organic solvents in the following sequence (25 ml aliquots of each) acetone–water–methanol (80–16–4); 0·05 ml formic acid in methanol, 5% PCA in 80% ethanol;[10] 1 : 1 ethanol-ethyl ether; and ethyl ether. This material is allowed to air-dry before use.

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* Calbiochem. California Institute for Biochemical Research, 3623 Medford Street, Los Angeles 63, California.

[9] J. LEVITT, *Physiol. Plantarum* **5**, 470 (1952).
[10] T. O. DIENER and A. M. LASHEEN, *Proc. Am. Soc. Hort. Sci.* **75**, 195 (1960).