SIMPLIFIED TECHNIQUES FOR ENZYMO-HISTOCHEMICAL STUDIES OF PLANT SHOOT APEX[1]

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Abstract

Enzymo-histochemical techniques for plant shoot apex is very difficult because of its small size and soft texture. In order to get a fine preparation, freeze-drying vacuum method, freeze-drying moving dry air method, rapid paraffin embedding method and block stainning and paraffin embedding method were disigned. In freeze-drying vacuum method, tissues were dehydrated at -30°C in high vacuum, fixing the free water molecules with liquid nitrogen; in freeze-drying moving dry air method, specimens were cooled at -30°C and dried with dry air which passes through the tissues. With the former method, a 3×3×2 mm tissue required 8 to 12 hours to dry, and with the latter, the same tissue required 15 to 20 hours to dry. In studying peroxidase activity, tissues were freeze-dried and embeded in paraffin within 1 to 2 min., sectioned and brought to water and stained with benzidine. To detect succinic dehydrogenase activity tissues were trimmed by freezing microtome till near the plane wanted. The entire blocks of tissue were incubated in reaction medium, dehydrated in tertiary butyl alcohol series, paraffinized, and cut exactly parallel to the plane of reaction. Enzyme localization was based only on the first 50 μ from the reaction surface.

Introduction

Main difficulties encountered in the histochemical studies of shoot apical meristems have been the small size of the tissues and localization of unstable enzymes such as those involved in tricarboxylic acid cycle.

This paper reports a simple apparatus for freeze-drying as well as techniques for localizing the unstable enzymes designed to overcome the aforementioned difficulties.

(1) A portion of a Ph. D. dissertation submitted to Nogoya University, Japan (1969)

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DESIGN OF THE APPARATUS AND ITS APPLICATIONS

a. Designs

The frozen tissues were dehydrated at -30°C either in a high vacuum or in a stream of moving dry air.

The apparatus for vacuum method is shown in Fig. 1 and 2. It consists of a vacuum-bottle filled with liquid nitrogen (a), U-tube with desiccant (b), another U-tube with potassium hydroxide (c), voltage transformer (d), dehydration chamber (e), cooling chamber (f), air heater (g), small air compressor (h), and high vacuum pump (i).

The frozen tissues are placed on the tissue containers on the side wall of dehydration chamber (j), which was prefilled with cool alcohol to keep the

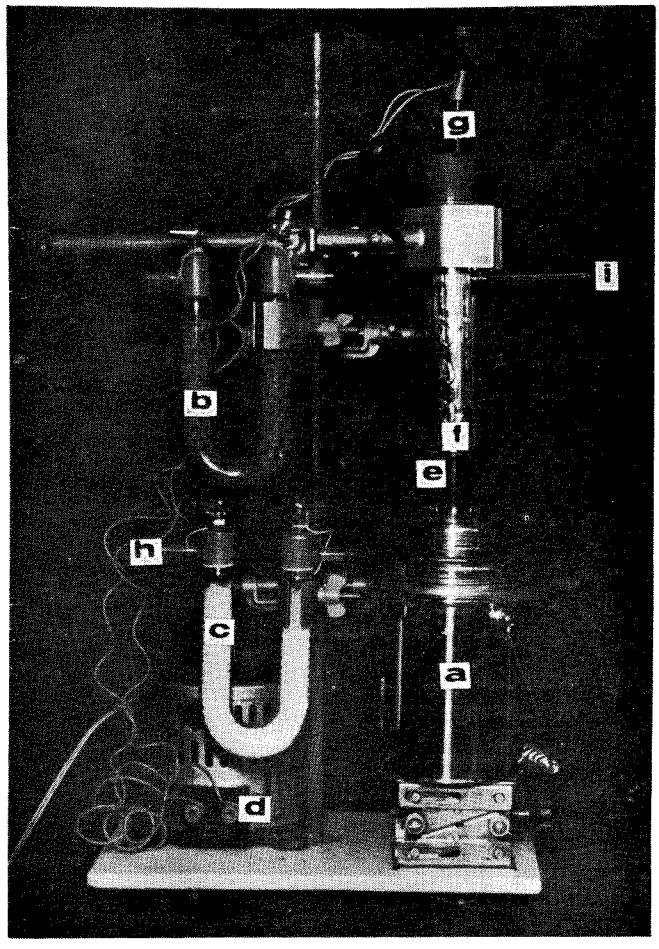

Fig. 1. An apparatus of freeze-drying, vacuum method. (a) Vacuum-bottle. (b) U-tube with desiccant. (c) U-tube with potassium hydroxide. (d) Voltage transformer. (e) Dehydration chamber. (f) Cooling chamber. (g) Air heater

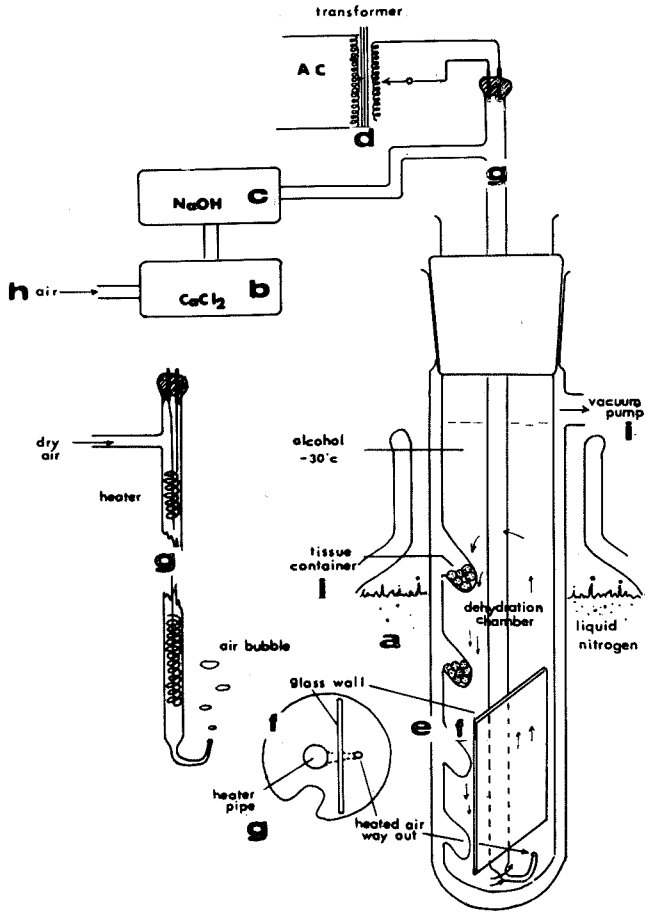

Fig. 2. Diagramatic sketching of freeze-drying apparatus, vacuum method. Legend, same as those in Fig. 1.

temparature below -50°C. The dehydration chamber is set on the vacuum chamber in such a way to keep the two walls apart for three to five mm. The whole thing is then dipped in vacuum-bottle which is filled with liquid nitrogen. Then heat the alcohol by sending hot air, keeping the temparature at -30°C by controlling the voltage of air heater with transformer. The degree of vacuum is kept between 10^{-2} to 10^{-3} mm Hg. Using this apparatus, a 3× 3×2 mm block of shoot apex can be freeze-dried in 8 to 12 hours.

The moving air method, as shown in Fig. 3, the tissues were put in small baskets made of metal wire mesh (a), the basket was hanged in a test tube (b) surrounded by a cold alcohol bath at -30°C (c). The dry air was introduced by a tube from the bottom of the wire mesh container. The dried air was precooled by liquid nitrogen (d) and when passed through the coil tube (e) dipt in -30°C alcohol, it was adjusted to that temparature. Water and carbon-

dioxcide must be removed completely before the air passes through frozen tissues. The moving air was achieved by mechanical pump or sucker. Using this apparatus, a 3×3×2 mm block of shoot tissues require 15 to 20 hours to dry.

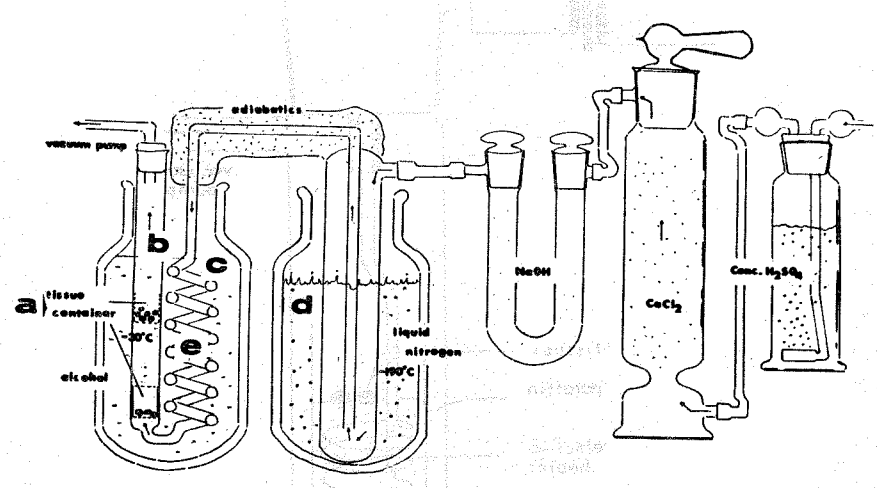

Fig. 3. Freez-drying apparatus, moving gas method. (a) A tissue container made of metal wire mesh. (b) Cooling chamber, (c) Cool alcohol at -30°C. (d) Liquid nitrogen chamber. (e) Coil tube.

b. Histochemical localization of peroxidase by freeze-drying and rappid paraffin embeding method.

In order to preserve the histological structures of shoot apex and obtain an accurate localization of the enzyme activity in it, tissues were freeze-dried and paraffin embeded in vacuum at 52°C, and then rapidly cooled to room temperature. The rappid paraffin embeding apparatus is diagrammatically shown in fig. 4. Because the moisture content of the dried tissues is necessary in the paraffin infiltration, the freez-dried tissues must be placed in a wet chamber for five hours, then transfer on the surface of solid paraffin, meanwhile the vacuum pump is put to work. After the air was partially evacuated (5 to 10 mm Hg), the electrical heater was switched on to melt the paraffin, thus the paraffin penetrate quickly into the tissues. Cool the entire chamber rapidly with cool water. This step should handle within one to two min.. The tissues were cut in 10 μ sections and affixed to glass slide with albumin. These slides were dried in moving air at room teperature, the paraffin was removed with xylene and then brought to water. The procedures therafter were the same as Isaac and winch's method (1947).

The observation and photography should be made within 10 min. after staining because the colored substance diffuses quickly. The localization of peroxidase activity is indicated by a blue to brown color.

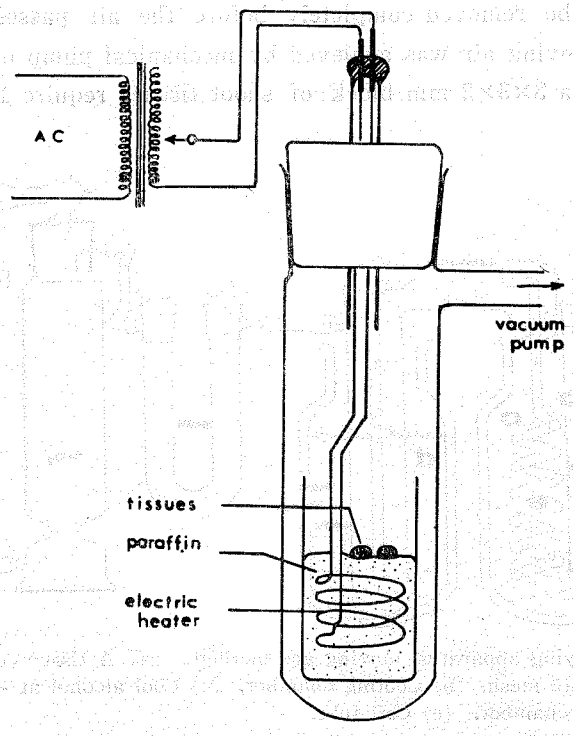

Fig. 4. An apparatus which infiltrate paraffin into freeze fried tissues.

BLOCK STAINNING TECHNIQUE FOR UNSTABLE ENZYMES

Histochemistry of succinic dehydrogenase activity—This enzyme activity in shoot apex have been reported by several investigatores (Abdul *et al* 1968, Fosket and Miksche 1966, Vanden Boun 1963). In their attempts sections were made with free-hand (Abdul *et al* 1968, Fosket and Miksche 1966) or carbon-dioxide freezing microtome (Vanden Boun 1963). In these methods, the sections were made about 50 to 80 μ thick, hence great difficulty was encountered in making a longitudinal sections just on the medial part of the apex. Furthermore, because the thickness of every section could not be cut uniformly, it would seem impossible to study the structure of the apex and the localization of enzyme activities without ambiguity.

To improve the shortcomings, block stainning technique reported by Fosket *et al* (1966) was introduced in to this study. The entire block of tissue was first put in the reaction medium and then dehydrate and brought to paraffin with an organic solvent, which do not dissolve the formazan. By this method the author found that reaction substance and the substrate penetrate into shoot apex at different rates.

The author has developed an easy technique for the preparation of specimens to localize succinic dehydrogenase activity. Hopefully, this method is also applicable for other unstable enzymes. The procedures are shown diagrammatically in Fig. 5. The shoots were trimmed with a freezing microtome to near the plane wanted. The tissues were then dipped in a reaction medium as described by Nachlas *et al* (1957), incubated at 37°C for two hours, washed in running water for 10 min. and dehydrated in tertiary butyl alcohol series. After paraffinized, sections were cut exactly paralled to the plane which was cut before incubation. Paraffin was removed with xylen and the sections mounted with canada balsam. The enzyme localization was based only on the first 50 μ from the reaction sarface, because the result obtained in this range coincided with those sectioned with cryostat or freez-dried and block stained tissues. As controls, use the reaction medium without the substrate or contain 0.2 M malonate.

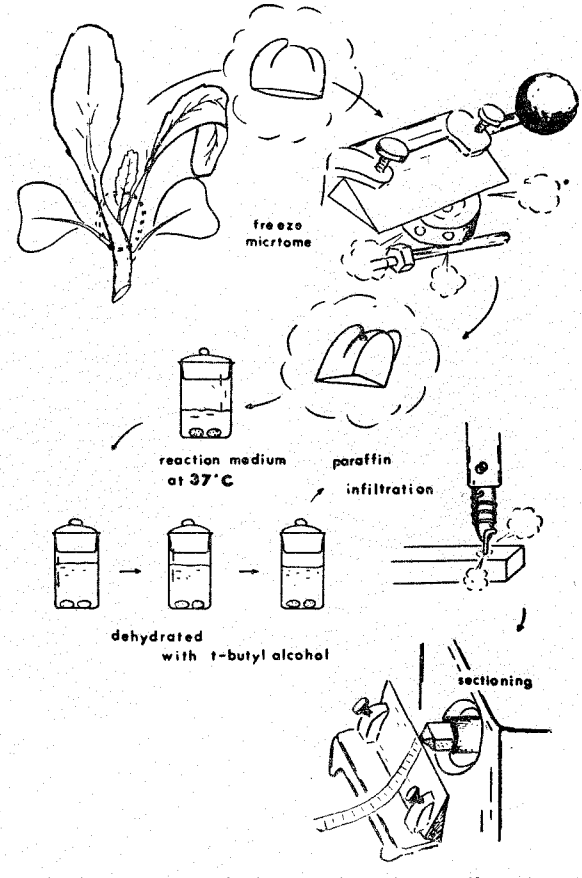

Fig. 5. A simple histochemical procedure for studing the activity of succinic dehydrogenase.

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