QUANTITATIVE ASSAY OF CHLOROGENIC ACID AND ITS PATTERN OF DISTRIBUTION WITHIN TOBACCO LEAVES[1]

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In order to relate the composition of the green tobacco leaf to the type of reactions which occur when leaves are harvested and cured, it was desirable to measure the concentration of the phenol, chlorogenic acid, in disks cut from fresh leaves. Studies on wheat (7, 10) have shown that the concentration of substances synthesized in situ such as proteins and chlorophyll is not uniform throughout the leaf but is higher at the tip than at the leaf base. A similar tip to base gradient has been found in the concentration of total alkaloids of the tobacco leaf (2), most of which enter the leaf by translocation. Ascorbic acid but not carotene concentration follows this gradient pattern in turnip leaves (5). On the other hand, no apparent gradient of ascorbic acid was found in the apple leaf (3).

Hence, analysis of the distribution of chlorogenic acid in mature tobacco leaves was undertaken so that a representative sample could be obtained without destroying the whole leaf. A quantitative colorimetric assay based on Hoepfner's original method (8) was devised for this purpose. To determine whether the definite pattern of chlorogenic acid distribution observed was a result of the pattern of translocation or represented actual physiological differences between various areas of the leaf, studies were made of the net synthesis of chlorogenic acid by various parts of the tobacco plant.

MATERIALS AND METHODS

The pattern of chlorogenic acid distribution was determined in fully expanded leaves of *Nicotiana tabacum*, var. Conn. 49, shade tobacco grown outdoors under a cheesecloth shade tent according to commercial practices (1). Leaves from the 12th and 18th nodes of three plants were harvested, washed and allowed to stand for an hour with their bases in water. Five disks, 27 mm in diameter, were punched from the lamina on each side of the midrib of the turgid leaves and weighed. The disks were taken from the tip, middle and base of each leaf at a position either near the midrib or the margin. The distance, along the midrib, of each disk from the tip of the leaf was measured on paper tracings of the sampled leaves.

CHLOROGENIC ACID EXTRACTION AND ASSAY: Each weighed disk was placed in 5 to 10 ml of boiling distilled water and held at 100° C in a water bath for 5 minutes. The boiled disk and its extracting solution then was either frozen for convenience or was transferred with water washes, after cooling, to a 15-ml glass homogenizer and ground mechanically. The suspension was boiled again for 5 minutes to insure complete extraction, cooled and diluted to 25 ml with washes from the homogenizer. This diluted sample was thoroughly mixed and filtered, and the filtrate was stored frozen until assayed.

Chlorogenic acid added to leaf tissue was recovered quantitatively when subjected to this procedure.

The Hoepfner reaction (8) is based on the fact that chlorogenic acid reacts with nitrite and acetic acid to produce a yellow color. Addition of alkali then converts the yellow complex to a bright red pigment. This reaction has commonly been employed only as a spray reagent for chlorogenic acid chromatograms (11). Our attempts to use Hoepfner's assay or various modifications of it gave erratic results initially. It was found that if chlorogenic acid was first absorbed on alumina, the assay became much more stable. Consequently the use of small columns of alumina was adopted.

Five to 10 ml of an extract prepared as described above and containing 0.1 to 1 micromole of chlorogenic acid was added to a column of chromatographic alumina previously washed with water. The chlorogenic acid, absorbed at the top as a yellow band, was washed with 5 ml of water, and a freshly mixed solution containing 2 ml of 0.5 % sodium nitrite and 2 ml of 5 % acetic acid was added. The yellow band became orange or tan as the nitrous acid mixture drained through. After washing with water again, 5 ml of 5 *N* sodium or potassium hydroxide was added to the column and the orange pigment at the top changed to bright red and moved down the column as a somewhat diffuse band. The alkaline eluate was collected and enough water was added to the column to bring the total volume of the eluate to 10 ml. After mixing, the absorbance of the red solution was measured at 525 mμ against a water blank with a Coleman model 8 colorimeter and followed the Beer-Lambert laws over the range mentioned above. The actual absorption peak of the red color occurred at 520 mμ. The column could be used again after washing it with 50 ml of water.

This reaction is given by a number of substances other than chlorogenic acid. Caffeic acid, for instance, gives one fifth the absorbance on a molar basis under the above conditions. However, as Hoepfner showed (8), caffeic acid turns red with nitrous acid alone and can be detected even in the presence of much greater quantities of chlorogenic acid. Rutin, another tobacco phenol, does not interfere with the assay.

Other cinnamic acid derivatives and some dihydroxy phenols, not usually found in tobacco, also react in the Hoepfner test (4). Consequently, application of this assay to plant extracts should be supplemented by chromatographic study.

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Dawson and Wada (6) found two substances in addition to chlorogenic acid that reacted with the Hoepfner spray reagents on paper chromatograms of Conn. 49 tobacco. They showed that these substances must be very closely related structurally to chlorogenic acid, however. Chromatography of the extracts from plants used here indicated that chlorogenic acid accounted for 80 % of the reactive material eluted from the chromatograms. The aqueous phase of the *n*-butyl acetate, acetic acid, water mixture used by Dawson and Wada was employed as a solvent.

Except where noted, plants used for the study of chlorogenic acid synthesis were grown during the winter in the greenhouse. Net synthesis was measured in tissue taken from the plants, placed in Petri dishes on moist filter paper and maintained 24 hours under 400 to 500 ft-c of constant light at 23 to 25° C. Roots were washed thoroughly before being placed in culture. Dry weights were determined on aliquots of the boiled, diluted suspensions.

Results

The fresh weights of leaf disks varied considerably with their positions in the leaf. Figure 1 shows that the weights of the disks decreased with increasing distance from the tip of the leaf. The slope of the regression line was − 0.905 mg/disk × cm and the correlation coefficient, r, was − 0.774. Thus, for leaves 40 cm long the fresh weight of disks from the tip averaged 35 mg heavier than disks from the base, a difference of 30 to 40 %.

The pattern of chlorogenic acid distribution in the leaves was similar to the fresh weight changes (fig 2). Again the highest concentrations per disk were from tip samples and the lowest from the base. The chlorogenic acid content of the tip samples was on the order of 1.2 micromoles per disk, about twice the content of basal disks. The regression of chlorogenic acid content on distance from the leaf tip yielded a line with a slope of − 0.00362 micromoles/disk × cm. The correlation coefficient was − 0.702.

The differences in chlorogenic acid content were not merely a reflection of differences in fresh weight for the ratio of chlorogenic acid to fresh weight also changed (fig 3). With increasing distance from the leaf tip the concentration of chlorogenic acid per unit fresh weight decreased significantly. The slope of the regression line was − 0.124 micromoles/g × cm and a correlation coefficient of − 0.55 was obtained. The mean chlorogenic acid concentration was 0.3 % of the fresh weight and 3 % on a dry weight basis.

Attempts were made to locate the sites of synthesis of chlorogenic acid in the plant by measuring the net change in chlorogenic acid concentration in disks of leaf lamina, 1-mm-thick stem sections, and in excised roots after 24 hours of culture. Table I shows the results of a number of such experiments. Laminar tissue from both field and greenhouse grown plants synthesized chlorogenic acid. Under the conditions of the experiments, the net amounts formed were similar even though the field grown leaves contained two to three times as much chlorogenic acid initially. Culture of disks in the dark rather than in the light did not lead to a net synthesis indicating that light is an important factor in the process. Disks maintained for more than 24 hours usually did not show any further increase in chlorogenic acid. Substitution of whole, excised leaves for leaf disks in

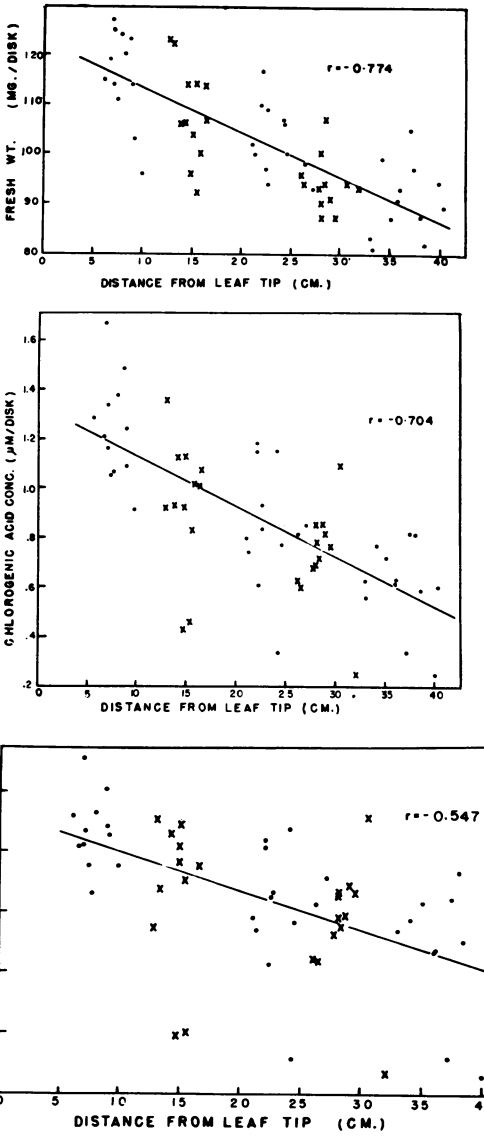

Fig. 1 (*top*). The fresh weight of 5.73 cm² disks punched from various positions in the leaf: closed circle, disks punched closer to the midrib than the margin; X, disks punched closer to the margin than the midrib of the leaf.

Fig. 2 (*middle*). The chlorogenic acid content of 5.73 cm² tobacco leaf disks. Positions of disks same as in figure 1.

Fig. 3 (*bottom*). The concentration of chlorogenic acid per mg fresh weight of leaf disks. Positions same as in figure 1.

TABLE I

NET SYNTHESIS OF CHLOROGENIC ACID IN TOBACCO TISSUE

MATERIAL	MICROMOLES OF CHLOROGENIC ACID *		MICROMOLES SYNTHESIZED
	INITIAL	24 HOURS	
	per g original fresh weight		
Leaf disks from field grown plants	12.80	13.80	1.00
Leaf disks from greenhouse plants	5.30	6.44	1.14
Leaf disks from greenhouse plants (cultured in the dark)	5.30	5.45	0.15
	per g original dry weight		
Leaf disks from greenhouse plants	52.7 (7.42)	61.8 (8.70)	9.1 (1.28)
Stem sections from greenhouse plants	12.5	21.5	9.0
Excised roots from greenhouse plants	15.6	12.9	-2.7

Figures in parentheses express the chlorogenic acid concentration on a fresh weight basis.
* Values represent data obtained from at least two plant samples.

the above experiments yielded approximately the same results.

Data in table I also demonstrate that stem tissue as well as the leaf lamina could synthesize chlorogenic acid. Roots, on the other hand, showed no net increase at all. On a dry weight basis, both stem and root tissue contained only one fourth the concentration of chlorogenic acid found in leaves.

Attempts were made to demonstrate direct physiological differences within the leaf itself by studying the synthesis of chlorogenic acid in disks from both the tip and base of greenhouse grown leaves. However, no consistent difference was found in the net amount of chlorogenic acid synthesized by disks from the two areas.

DISCUSSION

The gradient of chlorogenic acid from tip to base of the leaf correlates well with the visual gradients of change in color that occur during the air curing of tobacco leaves. The tip is the first part of the leaf to turn brown and is often the darkest after curing has ceased. These observations may be a direct result of the chlorogenic acid gradient, for this phenol appears to be involved in browning (9, 12).

Since there could be no translocation from other parts of the plant, the net increase in chlorogenic acid observed in isolated leaf disks clearly indicates that this compound can be synthesized in leaf tissue. Such findings suggest that the tip to base gradient may result from actual physiological differences between various areas of the leaf. However, since the data also show that chlorogenic acid is found in the stem and roots and can be synthesized by stem tissue as well, the possibility exists that chlorogenic acid is translocated in and out of the leaf.

Dawson and Wada (6) have reported considerably lower values for chlorogenic acid concentration in the variety of tobacco analyzed here. The differences in their values and those reported above could, in part, result from the fact that the field tobacco used in this study was grown during an exceptionally dry summer.

SUMMARY

A rapid, quantitative, colorimetric method of chlorogenic acid estimation involving the use of the Hoepfner reagents has been described. The method was used to assay chlorogenic acid in small disks of green tobacco leaves and is selective but not specific.

A distinct gradient of chlorogenic acid was found from tip to base of the tobacco leaf. The amount of chlorogenic acid in leaf disks was greatest at the leaf tip and decreased with distance toward the base.

Chlorogenic acid was found in stem and root tissue also although the concentration was one fourth that of leaf tissue. A net synthesis of chlorogenic acid occurred in leaf disks cultured in the light but not in the dark. Stem sections but not excised roots also showed net synthesis of chlorogenic acid.

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ALGAL GROWTH IN CROSSED GRADIENTS OF LIGHT INTENSITY AND TEMPERATURE[1,2]

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Two of the major factors that determine the growth responses of plants are the light intensity and the temperature. The effect of either of these variables on the growth rate is strongly influenced by the level of the other factor. A complete study of just these two variables, even for a single species, is a large-scale job.

The apparatus described here provides a visual picture of algal growth as a function of two factors varying along rectangular coordinates.

The principle is to use a thin layer of agar with algae growing on its surface. The agar layer is provided with a temperature gradient from left to right and a light intensity gradient from front to back. There is, at any given intensity, a range of temperature, and at any given temperature a range of intensities. Growth response is observed visually or photographed at intervals, thus adding the dimension of time.

Visual interpretation of algal growth response to the crossed gradients of light intensity and temperature are subjective and only roughly quantitative. Precision of the response estimate is improved if response is measured, for example, in terms of pigment formation (1, 2).

This paper gives details of construction and operation of the device which has been used to determine growth patterns of several unicellular algae (3, 4).

APPARATUS

The base of the crossed gradient culture chamber is a 12×16 inch aluminum plate $\frac{3}{4}$ inches thick. The $11\frac{3}{8} \times 12$ inch center part of the plate, the actual growth chamber, is bounded by metal edges $\frac{1}{8}$ inch high and is painted with chemically inert white Tygon. A Lucite spacer resting on the ridge surrounding the growth chamber supports the cover, a shallow Lucite box, through which warm water is circulated to prevent condensation of water. Figure 1 shows these parts in cross section.

The aluminum base plate extends 2 inches on either side of the growth chamber. On each side transverse borings carry water from the back of the plate to the front and again through the plate to a rear exit port. The left edge of the plate is kept uniformly cold and the right edge at a high temperature by continuous circulation of water from two constant temperature baths. The flow of heat through the aluminum produces a temperature gradient across the plate, which is mounted on cork for thermal insulation. The cork lined frame is supported on leveling screws.

The light comes from three 300-watt projector spot lamps run from a voltage regulator. Just below the lamps is a Lucite tank containing a 4.5-inch-deep heat filter of distilled water supplied from a reservoir with an automatic level device.

The intensity gradient on the culture surface is provided by directing the projector spot lamps toward the rear of a ground-glass plate, 4 inches above the agar surface. There is also a comb-shaped piece of translucent paper cut to appropriate shape by successive trials. The comb is on top of the ground-glass and is protected by a clear-glass plate. In addition to these arrangements for producing the intensity gradient, there is an opaque shade spaced between the ground-glass diffusing surface and the growth chamber. The arrangement of these parts is shown in figures 1 and 2.

Five percent CO_2 in air enters the growth chamber through 6 ports spaced along its front edge. Before entering these ports the gas stream, regulated by separate needle valves, bubbles through water in individual test tubes. To prevent the agar from drying out, the water is electrically heated to 80° and 90° C respectively in the tubes which saturate the gas entering the two high temperature ports.

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[2] Dedicated to Professor Richard Harder, University of Göttingen, on the occasion of his 70th birthday, March 21, 1958.

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