A RAPID TECHNIQUE FOR THE DETECTION OF NICOTINE IN DEVELOPING TOBACCO SEEDLINGS[1, 2]

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ABSTRACT

A rapid and sensitive method for detecting alkaloids, in particular nicotine, from *Nicotiana rustica* tobacco seedlings up to 2 mm in length has been developed. Growing tissue is applied (squashed) directly onto silica gel plates for thin-layer chromotographic analysis. The sensitivity of this method permits the detection of quantities of nicotine as small as 0.4 microgram.

INTRODUCTION

During the course of our study on the chemical patterns of plant growth and development (Peters *et al.*, 1972), it was necessary to use rapid procedures to analyze chemical contents of growing tissues. The present study deals with the development of a procedure for the rapid detection of the alkaloids.

The histochemical detection of alkaloids in growing tissues, as demonstrated by others (Chaze, 1932; James, 1950), is based on the reaction with iodine in potassium iodide solution. These methods are complicated by the presence of carbohydrates and proteins, which also react positively to iodine in potassium iodide solution. James (1946) was able to overcome the difficulty of liberating alkaloids from denatured proteins by blotting the tissue on filter paper prior to other treatment; however, the blotting procedure results in partial loss of cellular alkaloid content. A tedious solvent extraction procedure followed by thin-layer chromatography has been used by Speake *et al.* (1964). Other workers have used the reliable but time-consuming steam-distillation extraction technique (Solt, 1957; Brown and Byerrum, 1952). This paper describes an improved procedure whereby tobacco seedings as small as 2 mm in length are squashed directly onto thin-layer chromatographic plates for a rapid determination of alkaloid content.

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MATERIALS AND METHODS

Plant Material

Nicotiana rustica seeds are germinated for 24 hours on three layers of moistened Whatman No. 1 filter paper (9.0 cm) contained within 100 x 80 mm Pyrex petri dishes. Seedlings were planted on a daily basis and were grown in constant-environment chambers at 23°C in order to provide a continual supply of seedlings in various stages of development.

Preparation Of Material For Thin-Layer Chromatography (TLC)

A time-cource study was performed whereby 24-hour and 72-hour seedlings were harvested and placed directly onto glass microscope slides; seedlings beyond 72 hours were divided into root, hypocotyl, and cotyledon before placing on slides. All surgical procedures needed for the latter were performed on the glass microscope slide using a sharp scalpel. The TLC plates were inverted over the slides with the TLC gel in direct physical contact with either the intact seedlings or seedling components. Firm pressure was necessary to squash and release the cellular constituents. Two microliters of 0.1N HCl was used to moisten desiccated tissue prior to the squashing procedure. Any squashed tissue left on the TLC gel was removed prior to chromatographic development.

Thin-Layer Chromatography

Kodak silica gel Chromagram-plastic sheets were used for the entire study. Strips 2 x 9 cm were cut for one-dimensional chromatography, and squares 9 x 9 cm were cut for two-dimensional TLC. The following solvent systems were used: system A, 80% ethanol and 1N HCl (v/v, 5.0/0.2); system B, absolute methanol. For routine analysis one-dimensional thin-layer strips were developed in system A (4.0 ml) in a Coplin jar developing chamber. An airtight chromatographic jar was used for two-dimensional TLC: system A, (20.0 ml) was used for the first direction and system B (20.0 ml) for the second direction. Nicotine (1.0 mg/1.0 ml) was used as the standard; Dragendorff reagent (Randerath, 1964) was used for detection.

Thin-Layer Chromatographic Procedure

In one-dimensional TLC a sample of plant constituents or of the standard nicotine solution was loaded onto a TLC strip (2 x 9 cm) at a position 1.5 cm from the lower end of the strip. The solvent system was allowed to ascend to 10.0 mm from the top; the strip was dried and sprayed with alkaloid detecting agent.

In two-dimensional TLC (fig. 1A) a sample of plant constituents was loaded onto the gel layer at one corner, S, 1.5 cm from each edge of the square two-dimensional plate, 9 x 9 cm. The standard nicotine solution was applied at the two adjacent corners (N_1, N_2) at positions which are aligned with the sample origin (S) and are 7.5 mm from their respective side-edges, so that the standard nicotine could be developed simultaneously with the plant constituents in each direction. In co-chromatography (fig. 1B) the standard nicotine solution and plant constituents (S^1) were run on another square TLC plate. Sixty minutes were required for solvent A (first direction) to reach a front of 7.0 cm; for system B (second direction) about 30 minutes were required to reach the same distance. The chromatograms were thoroughly air-dried in a hood for at least 20 minutes following each run and before being sprayed with a detecting agent.

RESULTS AND DISCUSSION

The limit of the sensitivity of detecting the standard nicotine-HCl was 0.5 μg (*e.g.*, equivalent to 0.4 μg free nicotine). The Rf value of the standard solution of nicotine-HCl in system A was 0.57 and in system B 0.77. At Rf 0.30 a minor alkaloid spot originating from the nicotine standard solution was observed in the second dimension during chromatographic development with solvent system B. Apparently the minor spot represents the free form of nicotine. A single alkaloid

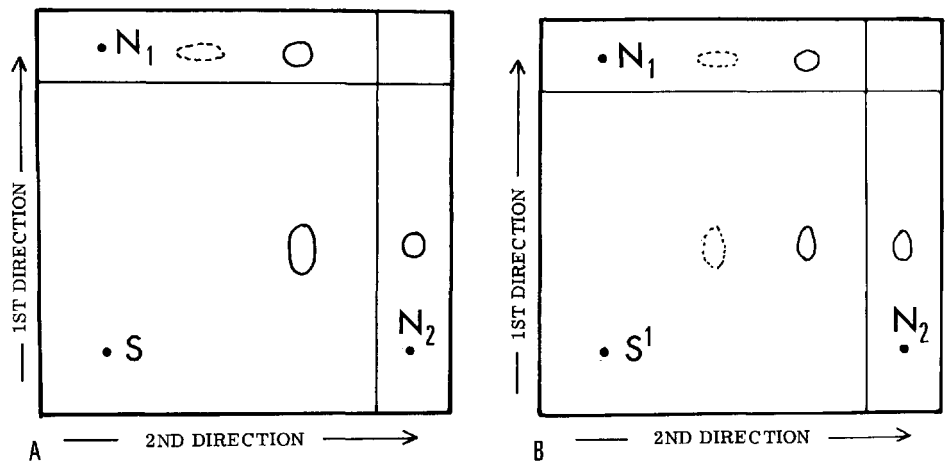

FIGURE 1. Tracings of two-dimensional chromatograms, A and B, developed in solvent system A (80% ethanol: 1N HCl, 5.0/0.2, v/v), first direction, and solvent system B (absolute methanol), second direction. N_1 and N_2 were standard nicotine solution, 1.0 mg in 1.0 ml 0.1N HCl; S was plant material spot of two 7-day-old seedlings; S^1 was plant material and standard nicotine spots combined. Reddish-orange color (solid circle) indicating alkaloid resulted from Dragendorff reagent. A lighter spot (dotted circle) was formed when standard nicotine-HCl was developed in absolute methanol.

spot having an Rf of 0.57 on the TLC strip was detected from *Nicotiana rustica*. This alkaloid spot was found identical to nicotine and confirmed the usefulness of two-dimensional TLC and co-chromatography. The use of hydrochloric acid in system A serves a dual purpose: (1) it provides a suitable solvent system for

TABLE 1

Intensity[1] *of nicotine spot (of plant origin on TLC) visualized by spraying with Dragendorff reagent*[2, 3]

Onset of germination[4]		Components of seedlings[5]		
Days	Total length seedlings (mm)	Cotyledon	Hypocotyl	Root
1	0.5	0	0	0
2	0.5– 1.0	0	0	0
3	1.0– 1.5	+	−	++−
4	6.0– 8.0	−	+	+++
5	13.0–15.0	+−	++	++
6	14.0–16.0	+++	+−	++++
7	18.0–20.0	++−	++−	+++++
8	20.0–22.0	++	+−	++++

[1]Average value of triplicates.

[2]Scoring of color intensity: very high, +++++; high, ++++; moderate, +++; low, ++; trace, +; very faint, −; no color, 0; combined + and − means slightly higher than the number of + signs.

[3]Standard nicotine scoring: 0.4 μg. ++; 1.0 μg. ++++.

[4]Onset of germination of seeds started 24 hrs after soaking in water.

[5]Three plants less than 72 hrs old or three comparable components were used.

TLC, and (2) it appears to free the alkaloid from the denatured protein and to form a stable HCl-salt.

Table 1 shows the nioctine biosynthesis of *N. rustica* seedlings. Data on nicotine content of the developing seedlings were scored semiquantitatively on the basis of the intensity of color detected with Dragendorff reagent. The alkaloid was not detected until the third day following germination; the highest concentration was found in the root sections. This pronounced concentration of nicotine in the root sections was observed consistently among geminating seedlings between days 3 and 8. This is in agreement with the work reported by other investigators (Chaze, 1932; Solt, 1957; Speake *et al.*, 1964). In all samples cotyledon and hypcotyl showed lower concentrations of nicotine than those found in root sections. It is unclear whether nicotine biosynthesis occurs *de novo* in these components or whether a nicotine gradient was established between the root and the cotyledon because of translocation. In another study with 7-day-old seedlings a high concentration of nicotine was observed in both root and cotyledon and an absence of nicotine in the hypocotyl components. This suggests that perhaps *de novo* nicotine biosynthesis was occurring in the cotyledon; this is in line with the results of tissue culture studies where alternate sites of nicotine biosynthesis have been identified (Speake *et al.*, 1964). A radiochemical time-course experiment will be required to elucidate the sites of synthesis in tobacco seedlings.

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