

CHROMATOGRAPHIC STUDIES ON THE AMINO ACID METABOLISM  
OF HEALTHY AND DISEASED LEAVES OF  
*CROTON SPARSIFLORUS*, MORONG

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INTRODUCTION

The need of accurate knowledge with regard to diverse metabolites of proteins and carbohydrates in diseased and healthy plants, has been acutely felt. Recent researches by Consden, Gordon and Martin (1944) and others have opened up new vistas for the plant physiologists. Very few workers have tried the horizontal migration method of chromatography in the study of plant physiology. Benson *et al.* (1950), Steward *et al.* (1954) and Sen and Burma (1953), however, have done substantial work using two-dimensional chromatography.

Giri (1951) used the circular paper chromatography employing single wick in the centre for the migration of the solvent. Ganguli (1954) developed an improved method by perforating his filter papers and thus separating the sample solutions from mixing up. We have tried both the methods but we could not get satisfactory picture of the chromatogram because the arcs of the adjoining sectors sometimes overlapped. We feel that the slight variation of our method described in this paper gives a considerably improved chromatogram; the *R<sub>f</sub>* values come out consistent and the amino acid bands are clear and tailless.

The work deals with the study of water-soluble amino acids and those obtained by hydrolyzing the protein content of both healthy and diseased *Croton sparsiflorus* leaves.

MATERIALS AND METHODS

(a) *Materials*.—As noted above leaves (of the same age) of *Croton sparsiflorus*, growing in wild condition, were selected for experimentation. Being a common weed it grows in fair abundance. Often the leaves have been frequently found to be infected with 'yellow-mosaic' which caused stunting of the plant and slight curling up of the leaves. It was decided to study the changes in the protein-metabolism as a result of such an infection.

(b) *Method*.—At the initial stages, Giri's method was tried. To overcome the overlapping of some of the bands, the following method was evolved, which has been used throughout the experiment. A circular piece, of diam. 40 cm. of Whatman No. 1 filter paper, was cut out. Two small circles of radii 4 cm. and 3.5 cm. were drawn from the centre. Round the periphery of the small circle 16 perforations were made. From the outer circle sixteen radiating fins 2 mm. × 15 cm. were made at equal distances. These were then clipped off to separate each radial sector, comprising in all 16 equal and separated sectors (see Fig. 1). A drop of liquid of .002 ml. was then gently placed on to the area marked for the purpose on the circle of diameter 4 cm. A single wick of size 2 cm. × 4 cm. was rolled tightly and inserted in the centre for the solvent to rise. The filter paper being rested on a petri dish of 25 cm. diameter. The whole apparatus was then covered with a trough serving as a cover for the saturation chamber (Fig. 2). This

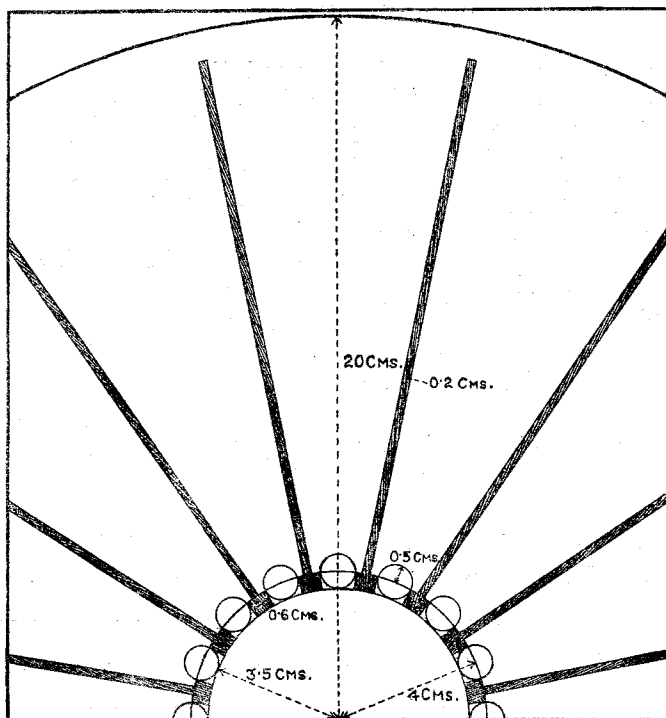


FIG. 1. Diagrammatic representation of a portion of Whatman No. 1 filter paper—40 cm. (diameter). The shaded areas are cut-off. The arrows pointing towards the centre mark the position of the wick.

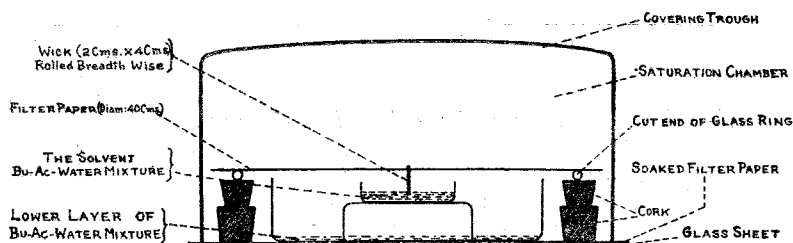


FIG. 2. Diagrammatic representation of the Chromatographic apparatus.

simple method has given quite satisfactory results and has shown distinct improvement in the clarity of the chromatogram by preventing the circular spread of the advancing liquid.

The solvent : *n*. butanol 100 c.c., glacial acetic acid 25 c.c., water 125 c.c., was used. The experiment was run for 20 hours at room temperature. Thereafter the paper was removed from the chamber, solvent front marked and the paper kept for drying. For the development of the chromatogram 0.1% nin-hydrin (in acetone) was sprayed and the chromatogram left at room temperature for 4 hours and then kept in the oven for 10 minutes at 75°C.

## EXPERIMENTS

8 gms. of leaves of each type (healthy and diseased) were plucked, weighed and dropped in distilled water, which was maintained at 60°C. to kill the enzymes. The material was crushed with its water and filtered. In this filtrate (A) tannins were precipitated and removed by lead acetate (in excess). The lead being precipitated by sodium oxalate in cold. 0.1 c.c. of chloroform was added to remove the pigments and the clear filtrate was then concentrated to 20 c.c. 5 c.c. of this was then transferred to centrifuge-tubes. The solutions were centrifuged at 2,500 revolutions per minute and the clear supernatant liquid was then transferred to 50 c.c. conical flasks. The residue being quantitatively taken in 100 c.c. conical flasks with cotton plugs, 25 c.c. of 6*N* HCl was then added and autoclaved for 2 hours at 15 lb. pressure to hydrolyze the protein content completely. The filtrate after autoclaving and filtering the content was made to 40 c.c. 0.002 ml. of each sample solution was then spotted, for the identification of amino acids, by a special micropipette.

The drops of the various solutions were kept at their respective marked places as given in the following chart (see Plate VI) :

<i>B, H and N</i>	..	Water-soluble extract of healthy leaves.
<i>C, I and O</i>	..	Water-soluble extract of diseased leaves.
<i>E and K</i>	..	Hydrolysate of healthy leaves.
<i>F and L</i>	..	Hydrolysate of diseased leaves.

\* *Known amino acids (for reference)*

<i>A</i>	..	Leucine, arginine and cystine.
<i>D</i>	..	Isoleucine, and glycine.
<i>G</i>	..	D-L valine, D-L tyrosine, and histidine.
<i>J</i>	..	Nor-leucine, L-tyrosine and threonine.
<i>M</i>	..	Methionine, aspartic acid and lysine.
<i>P</i>	..	Phenylalanine, glutamic acid and serine.

*Results.*—The following Table shows in a tabular form the result of our experiments (column 2 gives us the *R<sub>f</sub>* values of known amino acids while columns 4, 5, 7 and 8 give us the *R<sub>f</sub>* values of the unknown bands) :

\* The combination of known amino acids kept at each spot was found out after a number of chromatograms were run with single drop of only one amino acid at each smaller circle. The sequence of these appearing on the standard size of the paper was determined and from this the combination was selected.

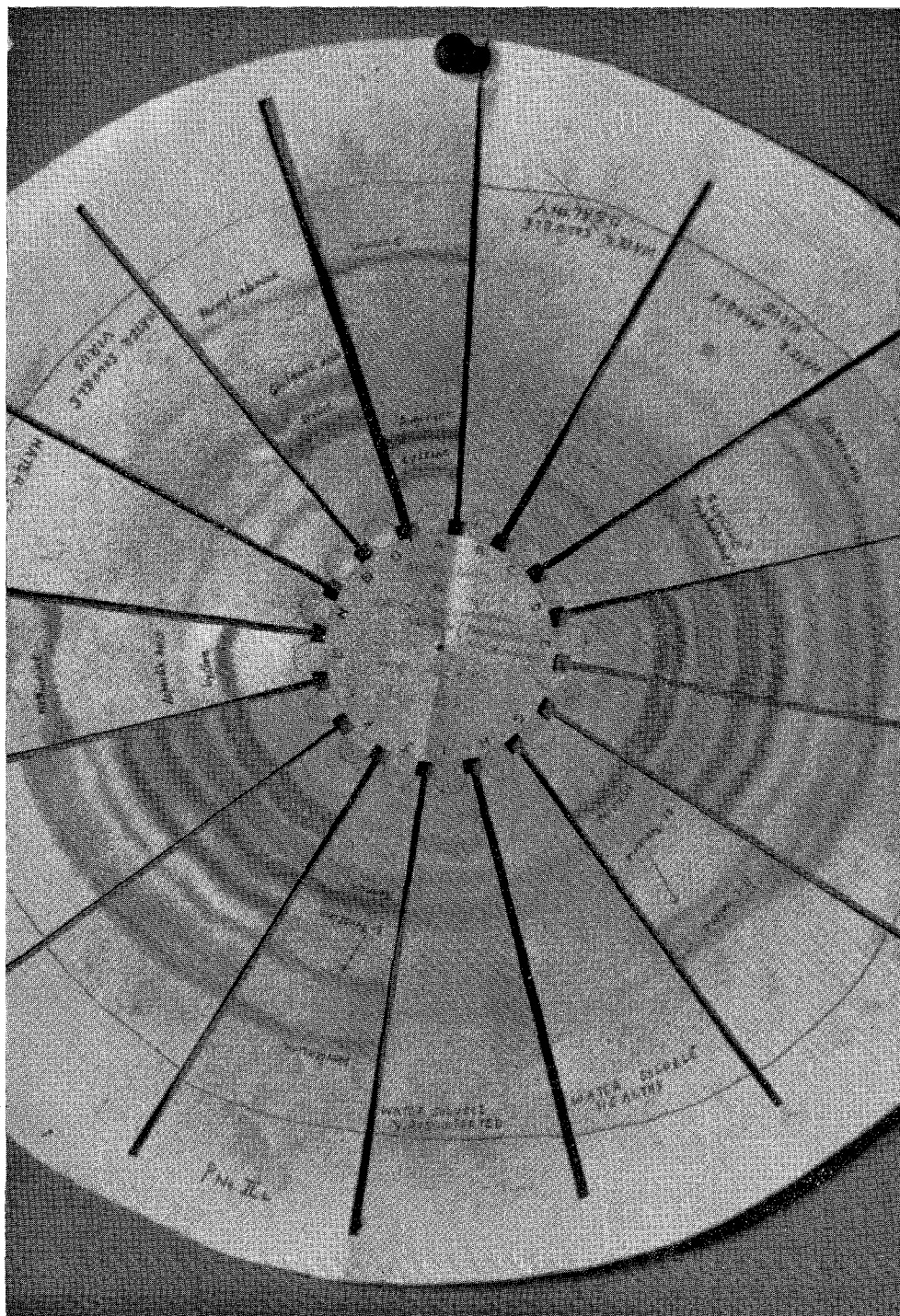


TABLE I

Known amino acid	Calculated <i>R<sub>f</sub></i> values	<i>R<sub>f</sub></i> values					
		Water-soluble			Hydrolyzed		
		Band No.	Healthy	Diseased	Band No.	Healthy	Diseased
Leucine and isoleucine.	0.78	X	X	X	I	0.78	0.78
Valine and methionine.	0.72	X	X	X	II	0.71	0.71
Phenylalanine ..	0.75	X	X	X	X	X	X
Tryptophan ..	0.69	X	X	X	X	X	X
D-L tyrosine and L-tyrosine.	0.62	I	0.62 (v. faint)	0.62	III	0.61	0.61
Proline ..		II	identified by its yellow colour and position (cf. Giri, 1952)			X	X
Alanine ..	0.53	X	X	X	IV	0.53	0.53
Glutamic acid and threonine.	0.46	III	0.46 (faint)	0.46	V	0.46	0.46
Glycine and aspartic acid.	0.40	IV	0.40 (faint)	0.40	VI	0.40	0.40
Serine ..	0.33	V	0.33 (deeper than others)	0.32	X	X	X
Arginine ..	0.25	X	X	X	VII	0.26	0.26
Histidine and lysine	0.22	VI	Absent	0.21 (Prominent)	VIII	0.23	0.23
Cystine ..	0.15	X	X	X	IX	0.15	0.15
Glutathione or cysteic acid.	..	VII	0.094 (v. faint)	0.094 (v. faint)	X	0.09	0.09

*Confirmation.*—All the amino acid bands were confirmed by adding known amino acids to the unknown solutions on separate chromatograms. The deepness in those rings as well as the constancy in the number of bands gave us the clue of all the amino acid bands identified.

In case of water-soluble extract the absence of glycine was found out by running phenol saturated with buffer pH 12 after the buffered paper (pH 12) was run on butanol: acetic: water solvent and dried.

The difference in our *R<sub>f</sub>* values with those of others can be very well explained on the basis of the difference in the pH of the solvent, temperature, degree of saturation and the distance between starting point and the solvent boundary which in our

TABLE II

Known amino acid	Known <i>Rf</i> values	<i>Rf</i> Water-extract (Healthy)	<i>Rf</i> Water-extract (Diseased)	Colour of bands
Aspartic acid ..	0.57	0.56	0.56	Violet
Threonine ..	0.57	0.56	0.57	Violet
Glycine ..	0.48	X	X	Brown
Glutamic acid ..	0.44	0.44	0.45	Violet
Serine ..	0.43	0.43	0.43	Violet

case differs with those of other authors. But our results are consistent and the keeping of reference solutions has helped us in eliminating all these defects.

In some cases the bands do not appear as complete arcs. This being explained as due to spray defect which has been eliminated in newer work.

#### DISCUSSION

(a) *Method*.—The method described above has the benefit of Ganguli's improvement: that many solutions can be estimated and at the same time the radial diffusion is also maintained. In addition, the bands come deeper because of their restricted movement, even of those amino acids which are present in low concentrations. The choice of single wick as against multiple wick gave us better separation, for in the latter case the movement of solvent was too fast and the separation was not so marked as in our case.

(b) *Material*.—Because of the fact that both the diseased (stunted) and healthy plants were growing in the same nutritional conditions, it was concluded that they had no physiological yellowing. Many of the plants showed mosaic sort of pattern at the top while the lower leaves remained healthy. This gave us the hint that the causal organism was most probably not seed transmissible. The symptomatology indicates that the causal organism of the 'yellow-mosaic' is neither due to any physiological cause nor it is due to any fungus or bacteria, for the culture of the diseased leaves showed no indication of either of the two. Considering all these it is suspected that it is some virus (the study of which is also being tried with the methods available).

(c) *Interpretation of the result*.—From a study of Table I it is clear that the following free amino acids are present in normal healthy croton leaves:

Tyrosine, proline, glutamic acid and threonine, aspartic acid, serine, and glutathione or cysteic acid. (The last two are the only two substances whose *Rf* values are below that of cystine.)

These acids are also found in common with the 'yellow-mosaic' leaves which show common *Rf* values on our chromatograms. They, however, appear more prominently in the diseased leaves. But lysine and histidine which have common *Rf* values come out very prominently in the diseased leaves only. One would like to conclude from our results that in the normal metabolic flux of proteins, lysine or histidine do not take part in the intermediate steps. The formation of this new amino acid (having *Rf* value 0.22 corresponding to the lysine and histidine band) which is present in free state in the diseased leaves goes in agreement with the suggestion of Bawden (1954) who postulates synthesis of a new range of proteins due to virus infection. Whether, this amino acid, is a constituent of the changed metabolic flux of protein in the plant due to such an infection, or it is a step in the formation of virus protein is yet to be decided.

Further work is in progress.

## SUMMARY

1. The work deals with the study of water-soluble amino acids and those obtained by hydrolyzing the protein content of the healthy and diseased leaves of *Croton sparsiflorus*.
2. Amino acid analysis made on circular papers which were divided into 16 sectors, by perforating 16 radial fins at equal distances, revealed that healthy leaves contained tyrosine, proline, glutamic acid and threonine, aspartic acid, serine and glutathione or cysteic acid.
3. 'Yellow-mosaic' leaves showed all those free acids found in common with the healthy ones but a band corresponding to lysine-histidine appears as new.
4. It is suggested that a new range of amino acid is formed due to the 'yellow-mosaic' condition of the leaves.

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## EXPLANATION OF PLATE

PLATE VI. Chromatogram showing amino-acid bands of healthy and 'diseased' leaf of *Croton sparsiflorus* along with known reference solutions.

- (1) Reference solutions: *A* .. Leucine, arginine and cystine.  
*D* .. Isoleucine and glycine.  
*G* .. D-L valine, D-L tyrosine, and histidine.  
*J* .. Norleucine, L-tyrosine, and threonine.  
*M* .. Methionine, aspartic acid and lysine.  
*P* .. Phenylalanine, glutamic acid and serine.
- (2) Plant solutions: Water-soluble extract of healthy leaves at *B*, *H* and *N* and of diseased leaves at *C*, *I*, and *O* hydrolysate of healthy leaves at *E* and *K* and of diseased leaves at *F* and *L*.