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AN ANALYSIS OF THE DIPHENYLAMINE REACTION IN SAP OF HEALTHY AND LEAF ROLL VIRUS INFECTED POTATO PLANTS[1]

Een analyse van de difenylamine-reactie in sap van gezonde en bladrolzieke aardappelplanten

BY

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INTRODUCTION

BRANDENBURG (1962) studied the nature of potato leaf roll virus (PLRV) and suggested that it is probably a free desoxyribonucleic acid (DNA) molecule. He reported further that it was possible to transmit the infectious material by rubbing the under surface of potato leaves with a phenol-water extract of infected leaves. He also suggested that the concentration of the virus in the infected potato leaves was so high that the diphenylamine reaction of DISCHE (1955) could be used as a diagnostic method.

Since it was not expected that the amount of virus could reach such a high concentration in the potato leaves we repeated the work of BRANDENBURG and analyzed the diphenylamine reaction in sap of healthy and virus infected potato plants.

MATERIALS AND METHODS

Mechanical transmission of the virus

Nucleic acid extracts were prepared from diseased potato leaves, variety 'Erdgold', and from *Physalis floridana* plants, using the method described by BRANDENBURG (1962). These extracts were applied by rubbing the under surface of healthy potato and *Physalis floridana* leaves with carborundum powder and sand paper.

Plant material

The potato variety 'Erdgold' was used throughout the experiments. The plants were raised from eyes of healthy tubers. When the young plants had two leaves and had reached a height of about 8 cm, they were infected with PLRV, using at least 20 viruliferous aphids *(Myzus persicae)* per plant. These aphids had been reared on PLRV infected *Physalis floridana* plants. The healthy plants were grown in a separate aphid-proof greenhouse. Sap was pressed from leaves which were situated in corresponding positions on healthy and diseased plants and as much as possible of the same age.

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Diphenylamine reaction

The procedure of DISCHE (1955) was used. The diphenylamine reagent was made by dissolving 1 g diphenylamine in 100 ml glacial acid and adding 2.75 ml concentrated sulfuric acid. It was found necessary to distil the glacial acetic acid. To 0.5 ml of the sap sample 2.5 ml of the diphenylamine reagent was added. This reaction mixture was heated at 100 °C for 20 minutes.

The diphenylamine reaction, modified by BURTON (1955), was also used. In this case 1.5 g diphenylamine and 1.5 ml concentrated sulfuric acid were added to 100 ml glacial acetic acid; 20 ml of this solution was mixed with 0.1 ml aqueous acetaldehyde (16 mg/ml) before using it. Two ml of this reagent was mixed with one ml of the sample. The reaction mixture was kept at 30 °C for 18 hours.

Diphenylamine was also used for the colorimetric estimation of nitrate (FEIGL, 1960), but in this case glacial acetic acid was replaced by concentrated sulfuric acid. The spectrophotometric estimations were carried out with the Unicam SP.600 spectrophotometer.

Paper chromatography

The descending technique was used with Whatman no. 1 filter-paper sheets. The solvent giving a good separation of diphenylamine positive spots consisted of a mixture of *n*-butanol (45 ml), glacial acetic acid (25 ml) and water (30 ml). Ten origins were made on one sheet, each formed by using 0.01 ml sap, clarified by centrifuging twice at 8,000 *g* for 15 minutes. The detection of the diphenylamine positive spots was carried out by dipping the strips in the diphenylamine solution of DISCHE and drying them on a plate of glass at 100 °C for 15–30 minutes. Reducing sugars and sucrose were detected by means of a *p*-anisidine spray (STANGE, 1959). Five hundred mg of *p*-anisidine were dissolved in 3 ml phosphoric acid and this solution was diluted with 100 ml 80% methanol. The filtrate was stored in darkness. The strips were sprayed and dried at 95–100 °C for 10–15 minutes. In order to detect the keto-sugars the strips were sprayed (STANGE, 1959) with a mixture of one volume 1,3-dihydroxynaphthalene solution containing 200 mg of this dye in 100 ml ethanol and one volume of a 2% aqueous trichloroacetic acid solution. The spots became visible on drying the paper at 100 °C for 5–10 minutes.

Sephadex filtration

Sephadex G 75 medium was used in the gel filtration experiments. The column dimensions were 17×1.4 cm and the flow rate was 15 ml/hour. Samples were eluted with water and fractions of one ml were collected.

Differential centrifugation

Sap, expressed from both healthy and diseased plants, was given 3 cycles of centrifugation at 8,000 *g* for 15 minutes each, followed by a centrifugation at 13,000 *g* for 30 minutes. The supernatant was given a high speed centrifugation at 100,000 *g* for two hours. DNA was extracted according to the method of OGUR & ROSEN (1950).

Results

Inoculation experiments

In our tests we were not able to transmit the virus mechanically. The extracts were inoculated on to 150 potato and 250 *Physalis floridana* plants. None of these plants became infected (the inoculated potato plants being tested for the presence of PLRV with aphids). These results are in accordance with those of Day (personal communication, 1962) and Sarkar (1962) who also failed to transmit the virus mechanically.

Absorption spectra

According to the diphenylamine reaction of Dische as well as to its modification by Burton, DNA shows in its absorption spectrum a maximum at 605 mμ (fig. 1). In the Dische reaction the colour intensity reaches its maximum after heating at 100 °C for 10 minutes. The Burton reaction, however, was found to be about 3.8 times more sensitive than the Dische reaction.

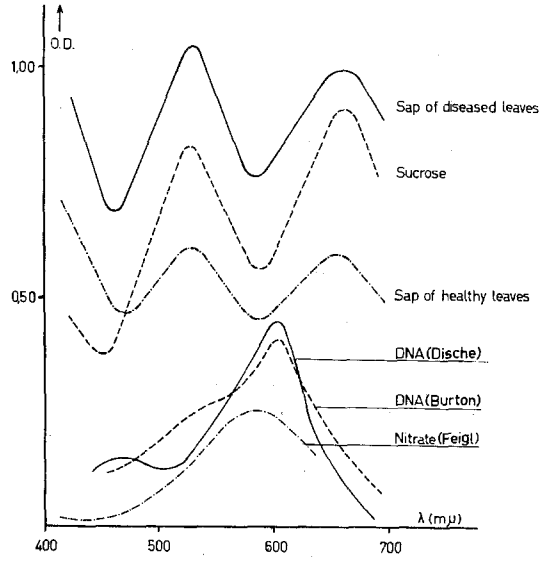

Fig. 1.
Absorption curves of diphenylamine reaction mixtures.

Sap, clarified by centrifugation at 8,000 g for 10 minutes, gave with Dische's diphenylamine reaction an absorption spectrum with two maxima, at 525 mμ, and at 660 mμ respectively. Sometimes the absorption peak at 525 mμ was higher and sometimes the peak at 660 mμ. We were not able to detect the cause of these fluctuations. No difference was found between the absorption spectra of treated sap from healthy and diseased plants, notwithstanding the fact that the reaction mixtures of sap from healthy plants often looked green or greenish-blue, whereas those of sap from diseased plants were mostly blue. Increase in optical density was found to be connected with an alteration of the colour from green to blue. Reaction mixtures with extremely high optical densities had a violet-blue colour. Sap clarified by gel filtration or differential centrifugation always had a blue colour.

Sap from healthy and diseased plants treated according to the method of BURTON developed a colour which did not differ from the untreated sap and the spectra were also identical.

In the colorimetric estimation of nitrate the diphenylamine reaction gave an absorption maximum at 580 mμ and showed a blue colour, while in the diphenylamine reaction of DISCHE, nitrate developed a yellow colour that changed to reddish-brown on cooling.

It is concluded from these experiments that the development of blue and green colours in diphenylamine reaction mixtures with healthy and diseased sap is primarily due to substances other than DNA. These substances in the sap of the healthy and diseased plants could be of the same nature. Since it is known that sugars and starch are accumulated in leaves of PLRV infected plants (THUNG, 1928; COÏC, 1945) and also that other sugars besides 2-desoxysugars react with diphenylamine, we decided to study the behaviour of some sugars in the diphenylamine reaction.

Fructose, glucose and sucrose treated with diphenylamine gave identical absorption spectra that were similar to those obtained with the saps from healthy and diseased potato plants. The reaction mixtures with these sugars developed a blue colour. A concentration of 0.4 mg/ml of these sugars in the reaction mixture with a reaction time of 20 minutes gave an optical density of 1.500 for fructose, 0.098 for glucose and 0.910 for sucrose, at 660 mμ. The colour development of these sugars increased with the reaction time. Sap from healthy and diseased plants behaved in the same manner.

Gel filtration of potato sap

In order to investigate whether or not high molecular weight substances present in saps from healthy and diseased plants react with diphenylamine, we filtered the plant sap over a Sephadex column, loading 0.5 ml sap on the column. The first fractions (1 to 17) after the void volume, having a green colour, developed no colour with diphenylamine. The fractions 18–25 gave positive reactions (fig. 2). The absorption curves of these fractions were similar to the curve obtained with the whole sap. Concentrating these fractions by lyophilization and separating the residue by paper chromatography, we found diphenylamine positive spots with Rf values of 0.292, 0.374 and 0.431. On placing samples of 25 mg fructose and 35 mg sucrose in 0.5 ml water on the Sephadex column, fructose and sucrose were found in the same fractions as the diphenylamine positive material of healthy and diseased sap. When samples of DNA were filtered on the column, fractions eluted directly after the retention volume developed a blue colour.

Analysis of sap by differential centrifugation

By means of differential centrifugation we separated the sap from leaves into several fractions in order to study the distribution of DNA in the sap. The extracts, which were made from the last two pellets and the supernatant of the high speed centrifugation, did not develop a blue colour in BURTON's reaction. In the DISCHE reaction they produced a faint blue colour with a sugar-like absorption spectrum. With all the supernatants obtained during these centrifugations colours were developed in the DISCHE reaction, which showed

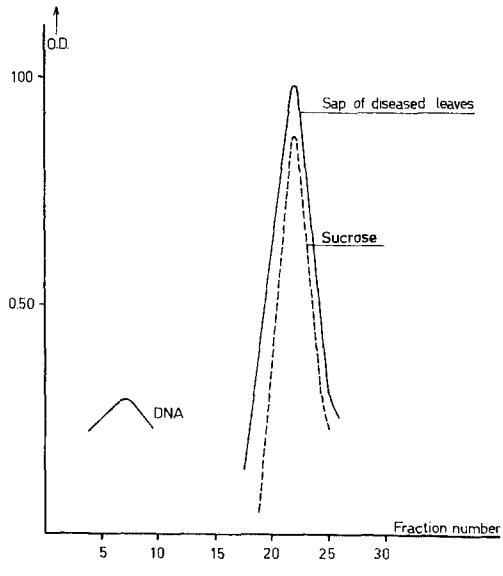

FIG. 2. Elution diagrams of DNA, sap of diseased plants and sucrose from a Sephadex G 75 column.

similar absorption spectra. The differences between the optical densities of the healthy and diseased saps and the absolute heights of these optical densities, however, were somewhat decreased during the course of centrifugation. This decrease was caused by sedimentation of coloured material in the pellets.

Paper chromatography of plant sap, sugars and DNA

Paper chromatography was employed to identify the sugars in potato sap which reacted in the diphenylamine reaction. A good separation of the diphenylamine positive material present in diseased and healthy sap was obtained in three spots with the solvent mentioned earlier. The Rf values of these spots are given in table 1. The spot with Rf value 0.370 was very faint. The origin developed no colour when stained with diphenylamine. However, a blue colour might be masked by the faint brown colour of the origin. By spraying the strips with *p*-anisidine and 1,3-dihydroxynaphthalene-trichloroacetic acid (naphtho-

TABLE 1. Rf values and colours of the spots found by staining chromatograms of potato sap, fructose, glucose, sucrose and DNA, respectively, with three different reagents. The elution solvent was a mixture of *n*-butanol (45 ml), glacial acetic acid (25 ml) and water (30 ml).

Sample	Rf value	Colours of the spots when stained with:		
		diphenylamine	*p*-anisidine	naphthoresorcine
	0.290	blue-violet	yellow-brown	red
Plant sap	0.370	blue	brown	
	0.430	blue-violet	yellow	red
Sucrose	0.300	blue-violet	yellow-brown	red
Glucose	0.378	blue	brown	
Fructose	0.433	blue-violet	yellow	red
DNA	0.0	blue		

resorcine), spots were found with the same Rf values as those in the diphenylamine staining. There were no qualitative differences between the chromatograms of sap from healthy and diseased plants. However, some quantitative differences could be found, especially for the sugars detected with diphenylamine. The eluted material from corresponding unstained spots gave the expected absorption spectrum with maxima at 525 mμ and 660 mμ on treating with diphenylamine.

Powder formed by grinding leaf material with solid carbon dioxide, and plant sap clarified by centrifugation at 100,000 *g* for two hours, gave similar results. Fructose, glucose and sucrose developed chromatographically gave spots corresponding to the sugar spots of potato sap. DNA was found to stay at the origin. The colours of the spots and their Rf values are presented in table 1. It is evident from these experiments that fructose, sucrose and glucose produced the colour in potato sap treated with diphenylamine.

Variability of colour development in sap from healthy and diseased plants

BRANDENBURG (1962) reported that when sap of old diseased plants was used the diphenylamine reaction gave a blue solution with high optical density, whereas with the sap of young diseased plants it gave a green or greenish-blue solution with low optical density. The colour intensity in the case of young diseased plants was similar to that of the reaction mixtures with sap from healthy plants. In some of our experiments, however, when sap of healthy leaves was used, the reaction mixtures developed blue colours with high optical densities. In order to learn something more about the factors influencing these variations, we analyzed the sap from leaves situated in different positions on the plant.

Sap of leaves from lower parts of the plants gave as a rule reaction mixtures with higher optical densities than sap of leaves from upper parts. Optical densities obtained with sap from the leaflets of a compound leaf showing clear leaf roll symptoms using the diphenylamine reaction are presented in table 2.

TABLE 2. Optical densities (O.D.) of diphenylamine reaction mixtures with sap from leaflets of healthy and PLRV infected leaves. The reaction mixtures are diluted eight times.

Leaflet	Healthy leaf		PLRV infected leaf	
	O.D. 525 mμ	O.D. 660 mμ	O.D. 525 mμ	O.D. 660 mμ
Top	0.172	0.169	0.760	0.760
First pair	0.159	0.154	0.652	0.556
Second pair	0.142	0.148	0.385	0.383
Third pair	0.139	0.142	0.155	0.153

These results show clearly that the highest optical density was obtained in the sap from the top leaflets. The sap from leaflet pairs of the compound leaves gave extinctions which decreased gradually according to the positions of the leaflets. No such differences in optical density were found in sap of different leaflets from healthy leaves. These findings are in agreement with data obtained by VERHOEKS (personal communication) on carbohydrates in leaves of healthy and leaf roll diseased potato plants. The differences in colour development between

healthy and diseased material were greatest in the morning and decreased during the day.

Hence we can conclude that the greatest differences in colour development between the saps of healthy and diseased plants will occur in the morning in the top leaflets of compound leaves in the lower parts of the plants. No differences in colour and optical density were found when sap extracted from plants two weeks after infection with PLRV was compared with sap extracted from healthy plants. A difference in colour was found as soon as the first symptoms were visible and it increased as the symptoms became clearer.

Discussion

It is evident from these investigations that the diphenylamine reaction in the potato sap mainly involves sugars. The difference in colour development, often found between sap of healthy and diseased potato leaves, was correlated with the development of the symptoms in the latter. This could not be caused by hydrolysis products of starch in those leaves, because the starch was sedimented by centrifugation. We could not find any DNA with differential centrifugation or gel filtration of sap from diseased leaves, and conclude that the differences in colour development are due to an increase in the concentration of sugars in the PLRV infected leaves[1].

Fructose, glucose and sucrose developed a blue colour in the diphenylamine reaction as did plant sap samples with a high optical density. However, when the optical density of the sap sample was low we found a green colour. On claryfying this sap by means of differential centrifugation or gel filtration, the colour in the reaction mixture changed from green to blue. The first-mentioned green colour probably resulted from an intermixing of the blue colour of the reaction products and some green plant material, which could be removed by further purification. When the optical densities in the reaction mixtures are high, the green plant material is too low in concentration to interfere with the developing blue colour.

In leaves with leaf roll symptoms starch is accumulated. Oortwijn Botjes (1920) and Murphy (1923) found that this accumulation begins some days before the appearance of the first external and internal symptoms and that the amount of starch increases with the development of the symptoms. We found in our investigations that the sugar concentration corresponds with the severity of the symptoms. So the starch accumulation can be correlated with the increase in the concentration of some sugars. The first difference in colour development will be found possibly at the moment starch storage begins. Therefore it is doubtful if the diphenylamine reaction according to Dische would be useful as a practical diagnostic test for the growing plant. Although we did not investigate tubers we assume that this reaction has no value as a tuber test, for appreciable differences in sugar concentrations do not occur in healthy and diseased tubers (Coïc, 1945). This opinion is in agreement with the findings of Brandenburg that no difference in colour development could be found between saps from healthy and infected tubers in the diphenylamine reaction.

[1] D. A. Govier came to similar results and conclusions (Virology 19, 1963: 561–564).

Summary

An analysis was made of a difference in colour development between saps from healthy and leaf roll virus (PLRV) infected potato plants, manifested in the diphenylamine reaction of DISCHE. The colour development was found to be caused by sugars. The differences in colour development were due to the sugar concentration which could be different in healthy and diseased leaves, and not to DNA in the latter. We could correlate the increase in sugar concentration with the severity of virus symptoms. The mechanical transmission of the PLRV, as suggested by BRANDENBURG (1962), could not be confirmed.

Samenvatting

Een verschil in kleur, dat tussen sap van gezonde en bladrolviruszieke aardappelplanten door de difenylamine-reactie van DISCHE kan optreden, werd geanalyseerd. Het bleek dat de kleurontwikkeling werd veroorzaakt door suikers. De verschillen in kleur van de reactiemengsels waren te wijten aan het feit dat de suikerconcentratie in bladeren van zieke planten een hogere waarde kan bereiken dan in bladeren van gezonde planten en niet aan een hoger desoxyribonucleïnezuurgehalte in zieke bladeren. Er bleek een duidelijke correlatie te bestaan tussen de stijging van de suikerconcentratie en de sterkte van de bladrolsymptomen. De mechanische overdracht van het bladrolvirus, zoals die door BRANDENBURG (1962) was gesuggereerd, kon niet worden bevestigd.

Acknowledgement

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