

CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*SANTALUM ALBUM*, LINN.)

Part XV. The role of plant acids in health and disease.

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It was shown in an earlier communication (*J. Indian Inst. Sci.*, 1928, **11A**, 103) that the hydrogen-ion concentration of the tissue fluid of spiked sandal was higher than that of the healthy tree. The reaction of the fluid from a partially diseased plant is, at the outset, more alkaline than that of the healthy one, but became more acid with the progress of the disease (*Ibid.*, 1930, **13A**, 295). Since the H-ion concentration of plant juice is believed to be largely due to the presence of organic acids, the present investigation was undertaken with a view to determining the nature and distribution of those acids in healthy and diseased sandal.

Materials and Methods.—The present study was carried out on leaf material derived from a number of forest areas, particularly Ragihalli and Uttarahalli in Bangalore District and Nognoor and Denkanikota in North Salem.

The leaves were minced and extracted with dilute (2.5 per cent.) hydrochloric acid. The extract was neutralised, concentrated to a small volume and treated with two volumes of 95 per cent. alcohol. After removing the precipitate, the filtrate was treated with hot saturated lead acetate. The mixture of lead salts thus obtained was suspended in water and freed from adsorbed sugars by repeated passage of carbon dioxide, washed carefully and dried. It was then treated with either dilute sulphuric acid or hydrogen sulphide to liberate the free acids. The filtrates were examined for the presence of oxalic, malic, citric, succinic and tartaric acids according to the method of Schmal-fuss and Keitel (*Z. physiol. Chem.*, 1924, **138**, 156). The method was first tested by working with small quantities of known acids both individually and in mixtures. It is possible to estimate the quantities of acids by comparison of colour intensities, but in the present case, no quantitative studies were carried out by this method owing to the small amounts of acids available.

In another set of experiments, the final solution containing the free acids was extracted with ether in an apparatus which was first

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devised in this laboratory and since independently described by Quick (*Ind. Eng. Chem. Analyt. Edn.*, 1933, 5, 76). The ether soluble acids were identified in the manner described above.

In a further set of trials the residue after extraction with ether was subjected to micro-sublimation and the sublimate obtained at various temperatures identified as their characteristic salts.

The results obtained by the three methods agreed closely with each other, and may be summarised as follows:—

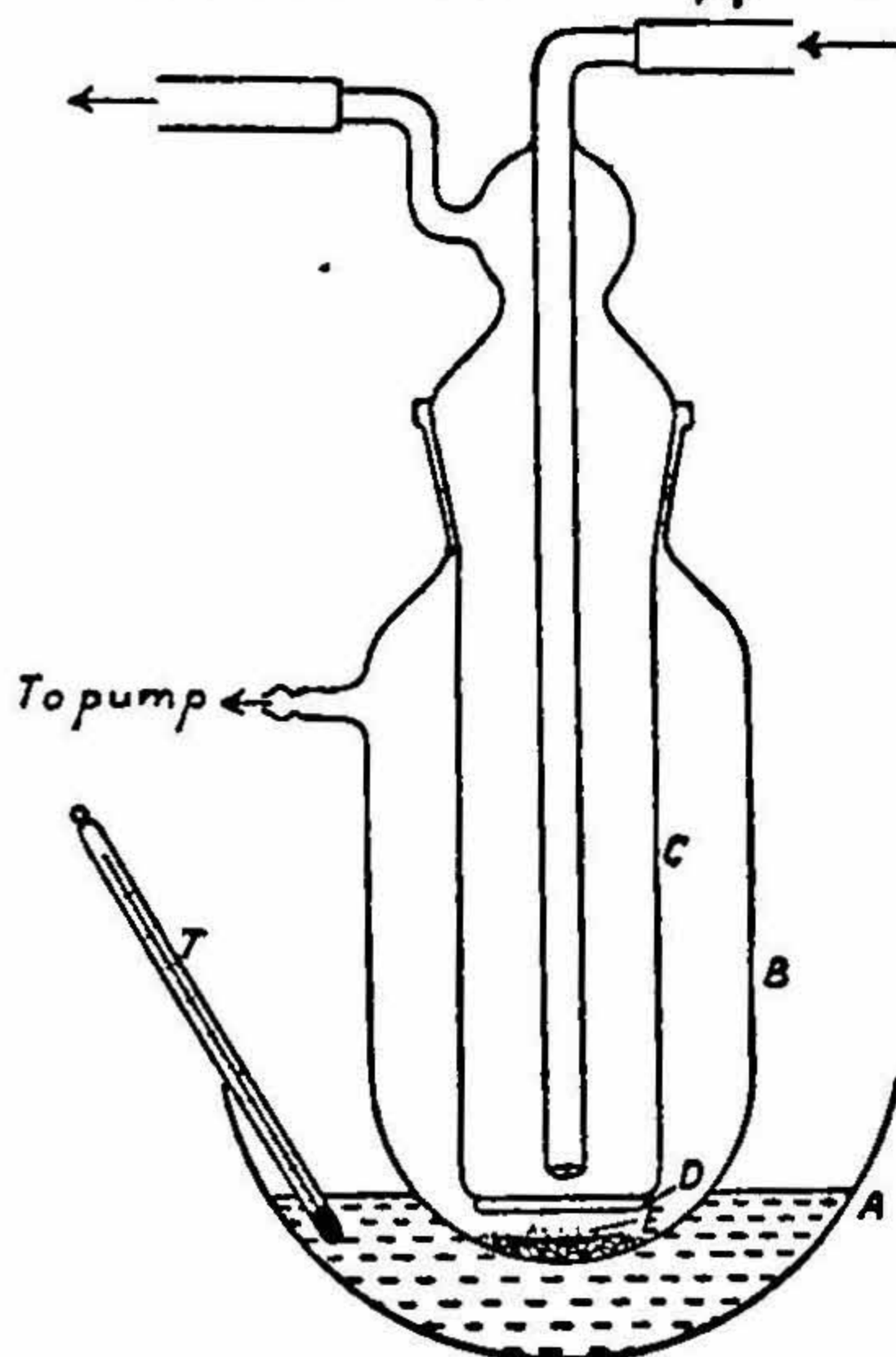
The three hydroxy acids, malic, citric and tartaric increased in quantity (from ++ in the healthy condition to +++ or ++++ in the partially spiked one) with the onset of the disease, but decreased to a much smaller amount (+) when the plant was fully spiked. On the other hand, oxalic acid, was comparatively high (+++) in the case of healthy specimens and relatively small (+ or traces) in the case of partially diseased or fully spiked plants.

‡ *Direct Examination of Acids in the tissues.*—In addition to the methods already described, the one developed by Klein and Werner (*Z. physiol. Chem.*, 1925, 143, 141) was adapted for this study. The technique as applied to sandal leaves may be described as follows:—

‡ The material under examination (0.5 cm. sq.) is placed in a circular copper piece (E) (Fig. 1) 1 cm. diameter with a special loop and dried at 60° for 10 minutes after acidification with a micro-drop of phosphoric acid. The copper piece containing the dried mixture of acids is placed in position over iron filings in the apparatus (B) fitted with a condenser (C) to the flat bottom of which is fixed a round cover slip (D). The apparatus is evacuated (2 mm. pressure) and the contents heated slowly in an oil-bath.

On attainment of the stage at which an acid is known to sublime, (this was determined beforehand for each acid) the temperature was maintained constant for 10 minutes. The pressure was then released and the cover slip replaced by a fresh one. The evacuation was resumed and the temperature raised to a further stage and again maintained at that point for 10 minutes. The cover glass with the sublimate was again removed and a fresh one placed in its stead. In this manner, the fractional sublimation was continued, and the sublimate collected on the glasses were identified as their salts. Oxalic acid was identified as the calcium or strontium salt, succinic acid as the lead salt, malic and citric acids as silver salts and tartaric acid as its anhydride. By adopting this method it was possible to establish definitely the occurrence of succinic acid which is prominent in the spiked tissue

Fig. I
Micro Sublimation apparatus



- A.—Oil bath for heating the apparatus.
 B.—The outer vessel carrying in a groundin condenser.
 C.—The groundin condenser.
 D.—The cover slip on which the sublimed acid settles on cooling.
 E.—The copper plate containing the precipitate under examination kept on iron filings.
 T.—Thermometer.

but conspicuously absent from the healthy one. This observation being rather striking, specimens from four different localities were examined and the above observation confirmed (Table I).

TABLE I.

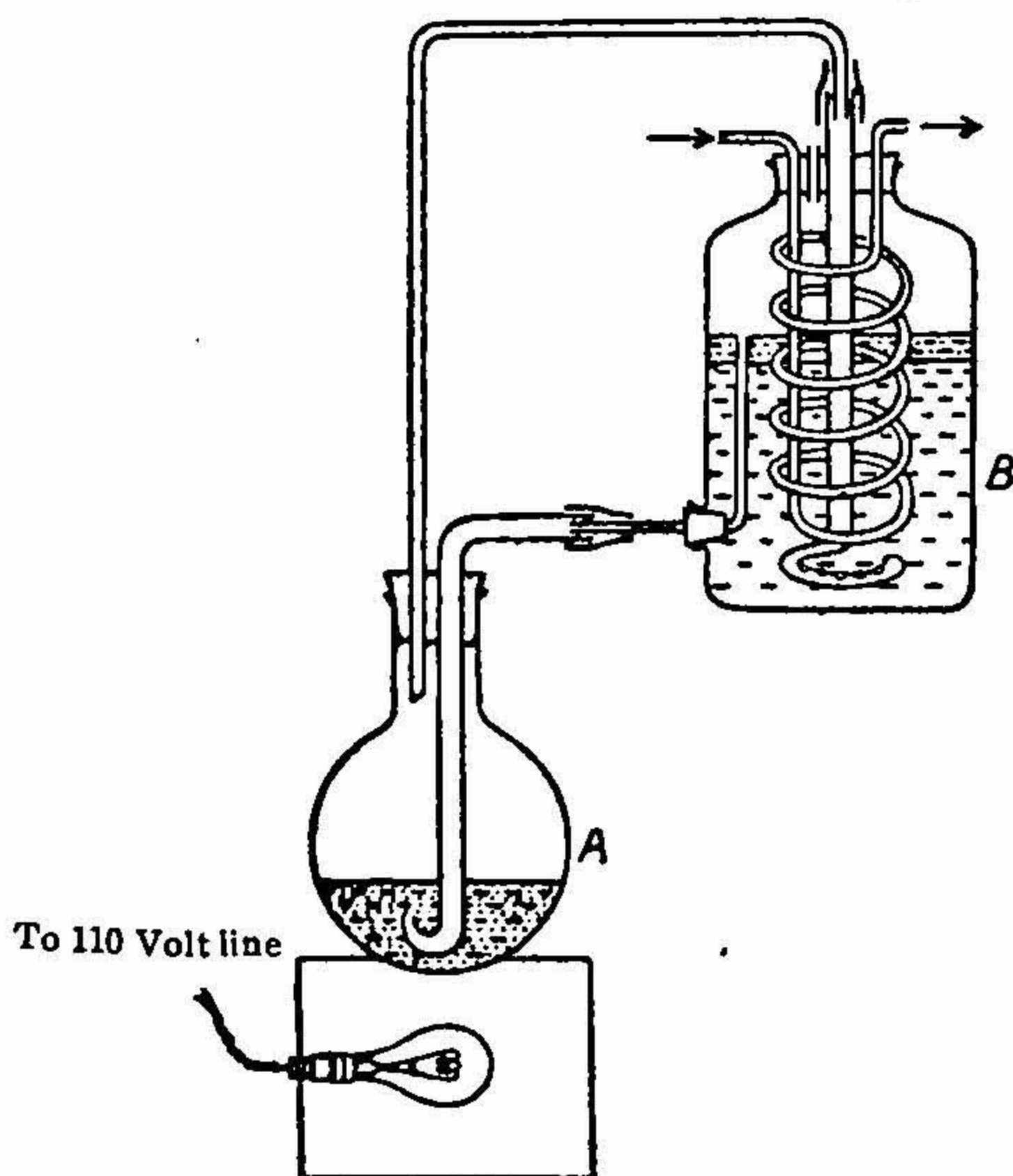
Succinic acid in leaf tissues.

Condition of tissue	Institute area	Ragihalli	Nognoor	Jawalgiri
Healthy	Absent	Absent	Trace	Absent
Spiked	++	++	++	++

Quantitative study of the distribution of organic acids.—Representative healthy and spiked trees were selected in three different localities. The leaf specimens used for each analysis were collected from single trees, the quantities examined varying between 1 and 3 Kgs. The specimens were treated according to the method of Vickery and Pucher (*J. Biol. Chem.*, 1931, **90**, 637). After removal of proteins, pectins and other interfering substances, the organic acids were precipitated as barium salts in preference to the lead ones and the free acids released by treatment with the minimum amount of sulphuric acid, 50 per cent. It was noticed that the final extracts thus obtained were usually more dark coloured in the case of diseased plants than in those of healthy.

The solution containing the free acids was extracted with ether for 320 hrs. in an apparatus specially devised for that purpose (Fig. 2). It consists of a receiver flask (A) of 1 litre capacity which contains

Fig. II
Extraction apparatus for liquids



the solvent and receives the extracted material. The vapour of the solvent passes through a tube ending in a spiral with a number of holes. It is absorbed in the container (B) a bottle of capacity 4 litres with a tube at the bottom which contains the acidulated solution to be extracted. The condensed solvent rises up in tiny bubbles extracting in its course the acids present. There is a syphon tube connecting the container to the bottom of the receiver so that as the level passes a particular point, the solvent flows down to the receiver. The condensation of the solvent was facilitated by cooling the container with ice (not shown in the figure). The condensed water was also drawn through ice so that the cooling was rendered still more efficacious. In this manner, the extraction proceeded continuously for several days without much loss of solvent. After the completion of the extraction, the combined ethereal extracts from the receiver and the container was distilled to remove the solvent and the residue esterified with acidified (2.5 per cent. HCl) absolute alcohol. After removal of the alcohol, the residue is taken up with ether, washed with water and neutralised with sodium carbonate. The sludge which then separates is again extracted with ether and the combined ethereal extracts washed, dried and the ether distilled. The residue was then subjected to fractional distillation and the quantities of organic acids represented by the different fractions determined. The results have been presented in Table II.

The various ethyl esters were further identified according to Franzen and Helwert (*Z. physiol. Chem.*, 1922, **122**, 46), as the corresponding hydrazides and the benzylidene derivatives. In all the cases the purity of the preparation was checked by the determination of mixed melting points with these derivatives of the pure acids. The respective nitrogen contents were also checked by micro as well as macro methods. The results have shown conclusively that malic and oxalic acids are present in very much larger quantities in the healthy sandal leaf than in the spiked one. On the other hand, succinic acid is prominent in the spiked tissue and either completely absent or present only in traces in the healthy one.

The residue left after extraction with water was further treated with sulphuric acid so as to liberate the organic acids from their insoluble salts. The mixture of free acids thus obtained was extracted with ether and the acids estimated in the manner already described. The results (Table II) again show that malic and oxalic acids were present as their corresponding salts to a much greater extent in the healthy sandal than in the spiked one.

TABLE II.

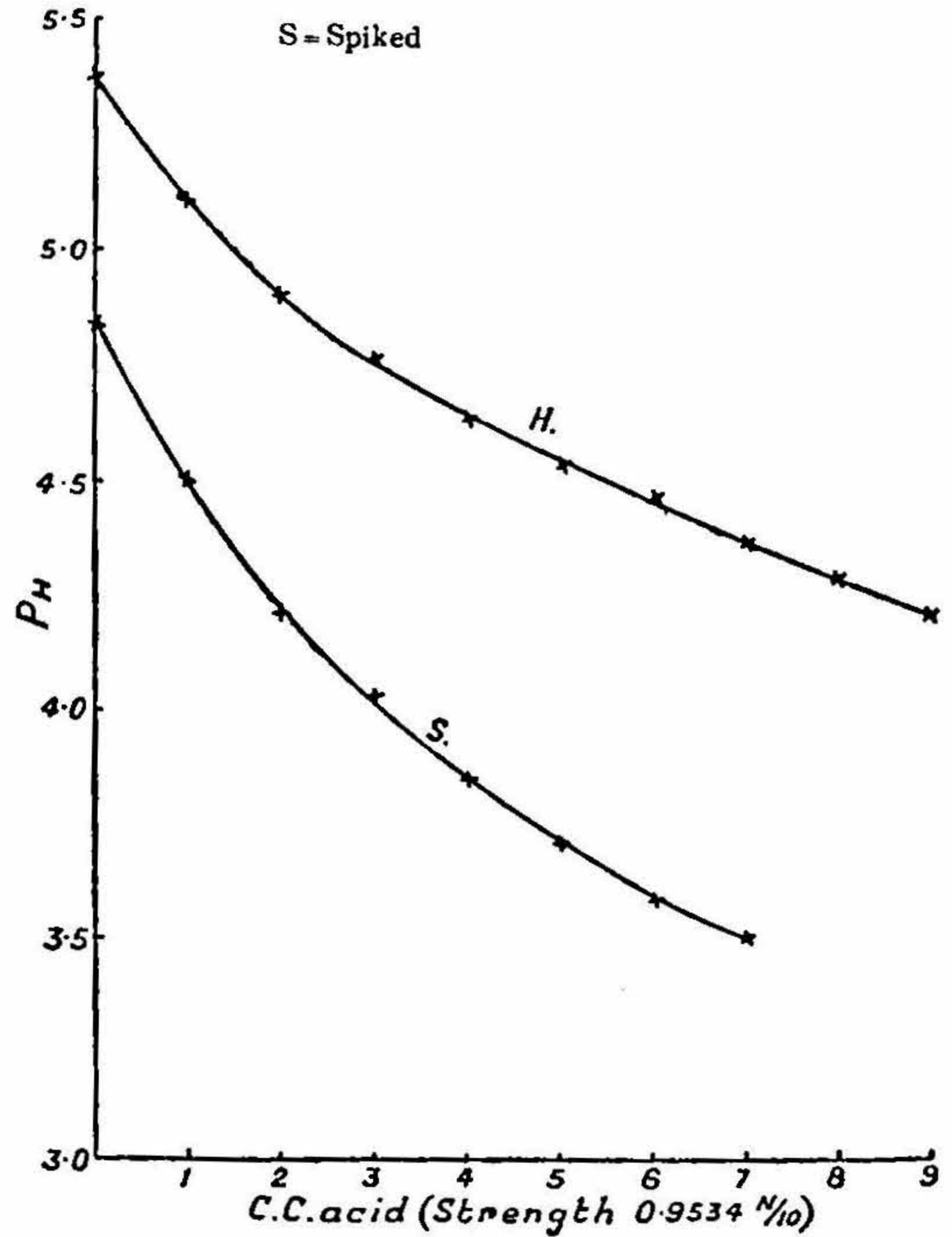
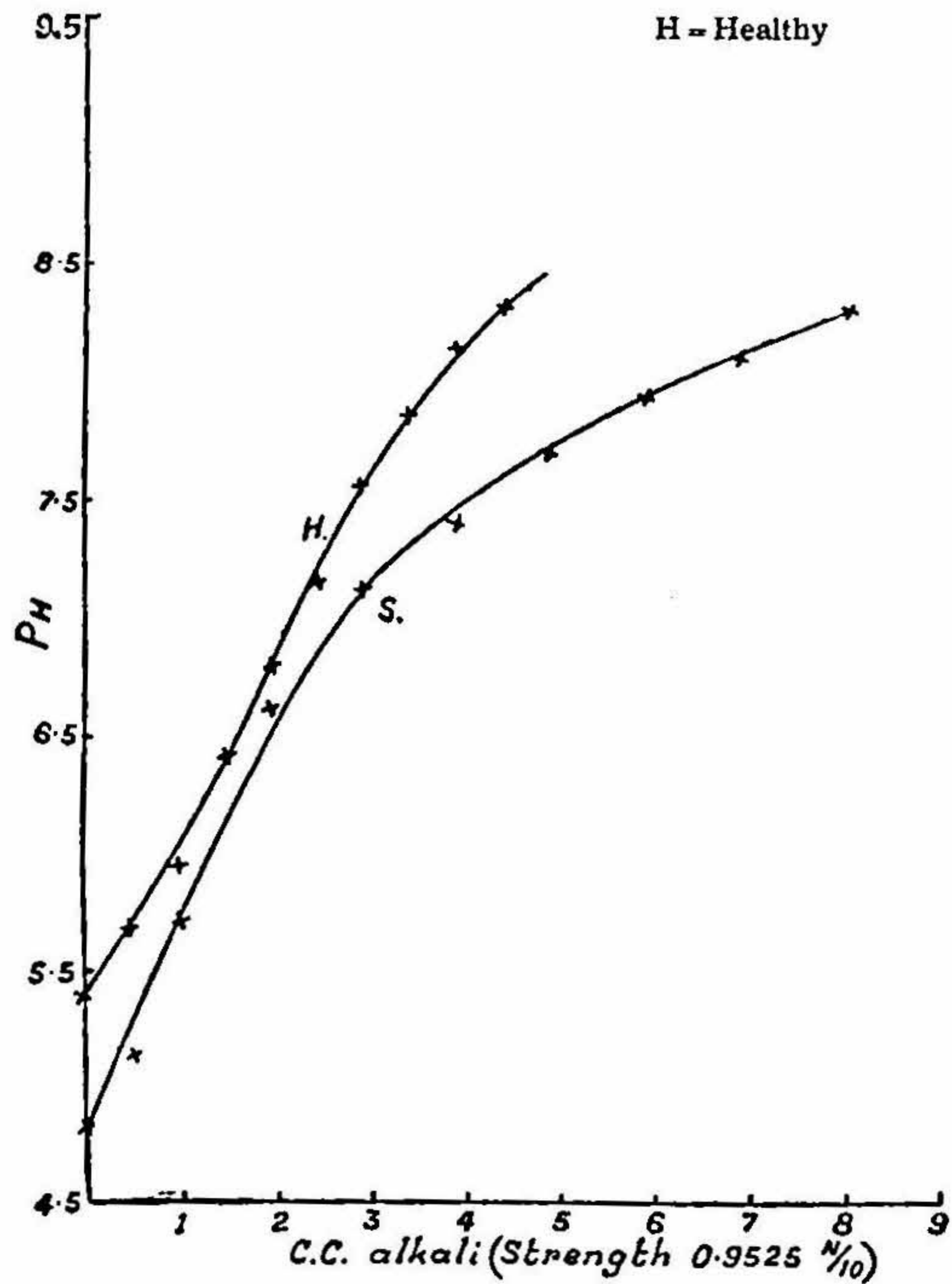
Distribution of organic acids in healthy and spiked sandal.

	SOURCES OF LEAF MATERIAL											
	RAGIHALLI				NOGNOOR				DENKANIKOTA RESERVE			
	Healthy		Spiked		Healthy		Spiked		Healthy		Spiked	
	Wt. in gm.	Per-centage on dry wt.	Wt. in gm.	Per-centage on dry wt.	Wt. in gm.	Per-centage on dry wt.	Wt. in gm.	Per-centage on dry wt.	Wt. in gm.	Per-centage on dry wt.	Wt. in gm.	Percentage on dry wt.
Fresh material taken ...	3800		1355		3400		1800		3500		1750	
Residue after water extraction ...	538		245		485		293		483		281	
<i>Acids in the water soluble portion.</i>												
Malic acid ...	2.05	0.19	0.44	0.10	2.45	0.21	1.10	0.17	2.45	0.23	0.82	0.12
Oxalic acid ...	0.38	0.04	Nil	Nil	3.38	0.29	0.13	0.02	2.24	0.21	0.30	0.04
Succinic acid .	Nil	Nil	2.40	0.53	0.06	0.005	4.0	0.62	Nil	Nil	5.06	0.75
<i>Acids in the water extracted residue.</i>												
Malic acid ...	0.51	0.05	0.21	0.04	1.17	0.10	0.45	0.07	1.49	0.14	0.48	0.07
Oxalic acid ...	5.01	0.48	1.52	0.30	6.41	0.55	1.43	0.22	6.19	0.58	2.04	0.30

Buffering capacities of tissue fluids from healthy and spiked sandal.—Since the hydrogen-ion concentration of the tissue fluid increases with the progress of the disease, some experiments were carried out with a view to determining the relative buffering capacities of the tissue fluids of sandal at various stages during the progress of the disease. Titrations were carried out against both acid and alkali and the quantities required to change the reaction of the different hydrogen-ion concentrations determined in each case. It was found (Fig. III) that though the fluids from the healthy and the spiked specimens behaved more or less alike when titrated against alkali or acid, those from the diseased showed weaker buffering capacity on the addition of increasing amounts of acid.

Fig. III

ALKALI & ACID TITRATIONS OF S.C.C. LEAF SAPS OF HEALTHY TREES AND TREES WELL ADVANCED IN SPIKE



With a view to verifying the above observation, the tissue fluids of specimens from four different localities were examined with regard to their response to the addition of acid or alkali and the quantities required for unit change of P_H on the acid or alkali side determined. The results (Table III) confirm the above observations.

TABLE III.

Titration values for healthy and diseased leaf saps (5 c.c.) against acids and alkalies.

Source of material	Initial P_H		Sulphuric acid in c.c. (N/10) required for a change of 1.25 P_H		Alkali required in c.c. (N/10) to change the reaction to P_H 8.3	
	Healthy	Spiked	Healthy	Spiked	Healthy	Spiked
Ragihalli ...	5.64	4.68	9.4	5.3	4.5	8.5
„ ...	5.49	4.72	9.8	5.5	5.6	7.5
Uttarahalli ...	5.61	5.21	10.5	6.3	5.3	4.7
Nognoor ...	5.72	4.94	8.9	6.4	3.5	5.7
„ ...	5.60	4.98	9.7	6.9	5.2	7.4
Denkanikota Reserve ...	5.63	5.05	10.1	6.5	4.3	6.6

Phosphates in health and disease.—Since these form important buffers in plant saps, the quantities present in the tissue fluids were determined according to Embden (*Z. physiol. Chem.*, 1921, **113**, 138). The results (Table IV) obtained in the case of five independent sets of healthy and spiked specimens show that the phosphate is invariably present to a greater extent in the fluid from diseased material than in that from the healthy.

TABLE IV.

Phosphoric acid contents of healthy and diseased leaf tissue fluids.

Condition of the material	P_2O_5 (as mg.) in 10 c.c. of tissue fluid				
	Specimens				
	I	II	III	IV	V
Healthy ...	7.48	13.48	5.12	8.48	10.42
Spiked ...	9.50	17.89	8.24	10.54	14.21

Organic acids and respiration.—In view of the important part played by organic acids as intermediary products in the respiration of plants, some preliminary trials were carried out estimating the evolution of carbon dioxide from healthy and spiked leaves according to the method of Dunlap (*Amer. J. Bot.*, 1930, **17**, 348) with slight modifications. The results have been presented in the following table (Table V).—

TABLE V

Material used	Duration of Experiment in hours	CO ₂ production in mg. per gram of leaf			
		Mature leaves		Young leaves	
		Healthy	Spiked	Healthy	Spiked
Cut shoots with leaves ...	13	3.7	2.2
Do. ...	15	5.4	4.7	7.9	11.6
Do. ...	22	6.6	4.1
Leaves alone ...	17	6.1	8.1	10.9	16.2

The results represent the averages of a number of determinations.

DISCUSSION.

It is well known that organic acids are formed as intermediary products of the respiratory metabolism of the plant cell. These processes serve to release the energy necessary for the growth of the plant. The substrates generally concerned in the transformations are the carbohydrates, the fats and the proteins which lead to the formation of the several acids found in plants.

The study of organic acids in plant parts has formed the subject of a number of important inquiries. The organic acid contents of fruits have been investigated by Franzen and his co-workers (*Z. physiol. Chem.*, 1921, **115**; *ibid.*, 1922, **121**; *ibid.*, 1923, **127**) and by Nelson and his co-workers (*J. Amer. Chem. Soc.*, 1925, **47**; *ibid.*, 1927, **49**). In a recent communication, Vickery and Pucher (*loc. cit.*) describe their studies on the distribution of organic acids in tobacco leaves. The relation of acidity to plant growth was investigated by Hurd (*J. Agric. Res.*, 1923, **25**, 457) who observed a parallelism between acidity and vegetative vigour in corn. In a later publication he reported an

increase in acidity in the final stages of growth in the wheat plant (*ibid.*, 1924, 27, 725).

A remarkable feature of the spike-disease of sandal is that with the onset of the disease the reaction became more alkaline than that of the one in the healthy condition. This continues only for a period after which the hydrogen-ion concentration steadily increases becoming distinctly more acid in the advanced stages of spike. Another striking transformation resulting from the diseased condition is the marked fall in malic and oxalic acid contents together with a distinct increase in succinic acid which is practically absent from the tissues and the tissue fluids of the healthy plant (Table II). Oxalic acid is present mostly in the insoluble form as the calcium salt and since the spiked plant is always deficient in calcium as shown by the author (*loc. cit.*), the deficiency in this acid may be traceable to the diminished quantity of calcium.

The foregoing studies have also brought out the importance of the micro-sublimation technique. It has been of much assistance in the identification and estimation of the different organic acids. It has helped to show conclusively the presence of succinic acid in the spiked plant (Table I). With the extension of this technique, it should be possible to devise a series of quantitative methods suitable for the identification and the handling of substances of biological products which are present only in the minutest quantities.

The abnormal change in reaction characteristic of the onset of the disease is supported by similar observations by other workers. Wagner observed a similar condition in the case of plants infected with a variety of pathological organisms (*Zentr. f. Bakt. Abt. II*, 1916, 44, 708). He observed an increase in P_H values immediately after the infection. The reaction tended to become acid with the progress of the disease. In case of recovery, the reaction of the infected plants became normal, but with the persistence of infection, there was a temporary rise in the P_H value which dropped suddenly at the stage prior to the death of the plant. Hurd (*loc. cit.*) observed an abnormal increase in the hydrogen-ion concentration in wheat plant attacked by mildew. The variation in resistance could not, however, be correlated with either the initial hydrogen-ion concentration or the titratable acidity. Furthermore, the acid reaction does not appear to hinder the attack of stem rust; nor does a low acidity predispose the plants to disease. Again Hurd and Karrer (*Amer. J. Bot.*, 1925, 12, 259) could not trace any correlation in hydrogen-ion concentration or titratable acidity and resistance of wheat to infection by *Tilleti Tritici*: in a like manner the differences in hydrogen-ion concentration were not, in any way,

correlated with immunity to wart disease in potato (Weiss and Harvey, *J. Agric. Res.*, 1921, 21, 590). In the case of spike, on the other hand, a distinct formation of succinic acid and the diminution in the hydroxy acids (Table II) might be in some way related to the increased hydrogen-ion concentration particularly in the advanced stages of the disease, while the presence of the corresponding mono and dihydroxy acids as also the neutralized oxalic acid in the tissue and the tissue fluids of healthy sandal and in the diseased specimens from partially spiked plants, may be considered to have imparted a less acid reaction.

The titration values for the tissue fluids of healthy and spiked sandal (Table II) bring out certain important features of the disease. The alkali required to neutralize the tissue fluid in the partially spiked plant is less than that of the healthy. With the progress of the disease the amount of alkali required increases in quantity in the advanced stages of the disease. On the other hand the acid required to bring about unit change shift of P_H in the diseased condition is less than in the normal. It can be seen from the titration curves presented in Fig. III, that the character of the curve is steeper in the affected specimens. In the case of acid titration this indicates a greater P_H change on the addition of small quantities. It appears from this that the spiked leaf sap is less buffered than the healthy one. The significantly low initial acidity may explain the increased alkali requirement in such cases. In this connection it may be mentioned that sulphuric acid was specially chosen for the titrations. The application of poorly ionised acids like acetic or oxalic acid would not only require large quantities of these acids for bringing about even small changes in the hydrogen-ion concentration, but would also introduce error due to high dilution. On the other hand, the employment of a highly ionised acid such as hydrochloric would bring about sharp changes through the addition of even small quantities, thus rendering it difficult to notice subtle differences between the different specimens.

The phosphate content of the tissue fluids does not provide sufficient information regarding the nature of the factors leading to increased acidity of the fluid from the spiked material. The presence of consistently larger quantities of phosphates in the spiked material than in the healthy, is rather significant (Table IV). It is probable that phosphorus exists as the acid salts of alkalies or alkaline earths or even as free phosphoric acid and this may explain to some extent the increased alkali requirement of the diseased leaf sap. Some preliminary experiments on the estimation of phosphorus present in organic form were carried out by extracting leaf powders with different solvents. The results showed that more phosphorus was present in that

form in the healthy tissue than in the spiked one. The relation of this observation to buffering capacity is, however, still obscure and further work is in progress to establish the rôle of phosphates in conditions of health and disease.

The respiration studies show that the spiked leaf gives off per unit weight more CO_2 than the healthy. On the other hand the shoots bearing the leaves show a distinctly reduced respiratory activity in the case of spiked specimens than that of the healthy (Table V). This would indicate that portions of the shoot other than the leaves do not show any appreciable respiratory activity in the case of diseased specimens.

Observations on the respiratory activity of sandal in health and disease are supported by a number of previous investigators. Weimer and Harter (*J. Agric. Res.*, 1921, **21**, 627) observed that in the case of sweet potatoes attacked by *Rhizopus tritici*, the diseased portion gave off 6.3-7.8 times as much CO_2 per unit weight as the healthy one. Davis (*Bot. Gaz.*, 1926, **81**, 323) noticed a striking increase in CO_2 production in the case of potato tubers with the onset of Blackheart. Dunlap (*loc. cit.*) observed a decrease in the CO_2 production in plants suffering from yellows and mosaics, but in the case of young tobacco leaves affected by mosaics his conclusions correspond to those in the case of young sandal leaves, in that the respiratory activity is high.

It would appear that in many of the previous studies particularly with those on fungus disease the apparent increased respiratory activity would be accounted for by the respiration of the parasite itself. Since the nature of the parasite (or the infective principle) in the case of spike is still obscure it is not possible to state at this stage as to what extent the previous observations can be applied to the present study. The relation of the nature and the distribution of organic acids to the respiratory activity is still comparatively obscure. The predominance of succinic acid in the case of spiked leaf as compared with those of oxalic and malic acids in the healthy one would suggest that some internal reduction takes place in the diseased condition. The increased CO_2 production may, however, be related to the larger quantities of soluble carbohydrates in the spiked leaf, as has been previously shown by the author (*J. Indian Inst. Sci.*, 1928, **11A**, 93). The above explanation would however require further experimental support.

It is generally believed that organic acids arise through incomplete oxidation of hexoses in the respiratory processes of the cell. The investigations of Ruhland and Wetzel (*Planta*, 1926, **1**, 558) and other workers would suggest a close correlation between the formation of organic acids and transformations of plant proteins. Kostychev

(*Plant Respiration*, edited by Lyons, 1927) and Onslow (*Principles of Biochemistry*, part I, 1931) have discussed the possibilities of several methods of formation of organic acids. Kostychev holds the view that acids arise as either transformation products of amino-acids or as intermediate products in the course of transformation of sugars to amino-acids in the synthesis of proteins. The researches of a number of previous workers (e.g., Neubauer and Fromherz, *Z. f. physiol. Chem.*, 1911, **70**, 326; Neuberg and Ringer, *Biochem. Zeit.*, 1918, **91**, 131) have shown that succinic acid is formed by the yeast from glutamic acid by a process of oxidative deamination. In a similar manner the presence of oxalic and malic acids would be traceable to aspartic acid. The extent to which the increased malic and oxalic acids in the healthy condition and the exclusive presence of succinic acid in the spiked tissues are traceable to the above two amino acids is still obscure. Further researches are in progress to throw light on this aspect of the problem.

One of the main objects of the present study was to trace the resistance offered by sandal occurring in certain localities or in association with certain hosts to the hydrogen-ion concentration and the buffering principles present in the tissue fluids. The factors which determine the resistance offered by plants to disease has been the subject of a number of important inquiries by Nobecourt (*Contribution à l'étude de l'immunité chez les Végétaux*, 1928) and Carbone and Arnaudi (*L'Immunita Nelle Piante*, 1930). According to these authors the defensive mechanism of plants against parasitic attack is traceable to deficient chemical entities such as hydrogen-ion concentration, sugars, organic acids and tannins etc. Comes (*Reale Istituto d'Incoraggiamenti di Napole*, 1916) first showed that acidity was a very important factor determining immunity of plants. Mumford studied the relation of hydrogen-ion concentration of tissue fluids of sugar beet in relation to 'curly top' disease, but could not find any significant difference between those from healthy and diseased materials or from resistant and susceptible varieties (*Ann. Appl. Biol.*, 1930, **17**, 35). The more recent observations of Walker and his co-workers (*J. Biol. Chem.*, 1929, **81**, 369; *ibid.*, 1929, **84**, 719) have shown however that the resistance in certain varieties of onions to the smut disease is traceable to the presence of protocatechuic acid which is present in the scales of red and yellow varieties which are known to be resistant but absent from the white ones which are susceptible to the disease. The present study gives no clear indication of the relation of organic acids to the resistance offered by sandal to spike-disease. The extent to which the presence of tartaric, malic and oxalic acid, contribute towards resistance to spike and that of succinic acid to one of susceptibility is still obscure. These and related problems would form subjects of a later communication.

SUMMARY.

1. Leaves from healthy and diseased sandal from different localities were examined by micro and macro chemical methods for the several organic acids present in them. It was observed that the tissues and the tissue fluids from the former contain more malic and much larger quantities of oxalic acid than the latter. On the other hand, succinic acid was prominent in the spiked tissues and tissue fluids while being completely absent from or present only in traces in the healthy material.

2. An apparatus for extracting large quantities of solutions with light solvents has been described.

3. Titrations against acid or alkali showed that the disease brings about a disturbance in the buffering capacity of the tissue fluid.

4. The tissue fluids from spiked sandal contain more phosphates than those of the healthy.

5. Carbon dioxide production is more intense in the case of spiked leaf tissue than that of the healthy. On the other hand cut shoots (with leaves) from the spiked plant gives out less carbon dioxide per unit weight than the corresponding healthy specimens.

In conclusion the author wishes to express his thanks to Prof. V. Subrahmanyan for his valuable and helpful criticisms.

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