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

## Part III. Physico-Chemical Study of the Leaf-Sap.

*By A. V. Varadaraja Iyengar.*

Spike disease of sandal is characterised by marked changes in the concentration of certain chemical constituents in the plant tissues. In view of the very definite physiological disturbances induced by the disease, it is of considerable interest to make a study of the sap and compare the composition of this material in healthy and diseased trees. Since the first visible symptoms of the disease are exhibited by the leaves and since the leaves are the seat of active metabolism, leaf-sap might be expected to yield the most valuable information and has been used in the experiments now to be described. In Part I of the series (*This Journal*, 1928, 11A, 23), the diastatic activity of the sap has been examined; and the present paper contains the results of a physico-chemical investigation of the centrifuged sap from diseased and healthy trees. The constants determined are (*a*) Hydrogen-ion concentration, (*b*) Osmotic concentration, (*c*) Titratable acidity and (*d*) Electrical conductivity. The figures thus obtained might be expected to give material information regarding the nature and quantity of the various sap constituents.

### Collection of Samples: Extraction of Sap.

The samples were obtained from two areas in the neighbourhood of Bangalore, namely Ragihalli and Uttarahalli. Specimens from healthy and diseased trees in the same area were collected at the same time of the morning. Changes in the samples preceding examination in the laboratory were minimised by keeping the specimens in an ice-box from the time of collection until they reached the laboratory.

It is obviously of the first importance to utilise a method of sap-extraction which will give an average sample of sap from any one specimen and without disruption of the cells. The various factors involved have been fully discussed by Dixon and Atkins (*Proc. Roy. Dublin Soc.*, 1913, **13**, (N. S.) 422), by Gortner and Harris (*Plant World*, 1914, **17**, 53) and by Gortner, Lawrence and Harris (*Biochem. Bull.*, 1916, **5**, 139). These authors are agreed that freezing methods

are the most reliable for rendering the cell walls permeable to the cell contents and our own observations support this view. Both liquid air and solid $CO_2$ are effective ; the former has certain obvious advantages, however, since no chemical factor can be involved and being freely available has been used throughout the present investigation. There is every reason to believe that the sap is obtained unchanged by such treatment and only moderate pressures are needed to express it.

Details of extraction are presented in Part I of the series and the sap so obtained was centrifuged to remove any suspended matter that might have come out while the pressure was being applied. The conditions were maintained constant for all the samples and the differences observed may therefore, we think, be taken as representing real differences in the sap constituents.

## Experimental Methods.

The freezing-point determinations were made by the thermo-electric method developed by Dixon and Atkins (*Proc. Roy. Dublin Soc.*, 1910, **12**, (N. S.) 275). This method is essentially a differential and comparative one in which a thermo-couple is used consisting of a Eureka wire and two fine copper wires, the ends of the former being soldered to one end of each of the copper wires. The other two free ends of the copper leads are connected to a galvanometer through a liquid junction by means of a reversing key. The current due to the potential difference set up when the two ends of the thermo-couple are at different temperatures is measured by means of a suitable galvanometer. An instrument of the Ayrton-Mather type has been found very suitable with the following constants: resistance 30 ohms; deflection at 1 meter for 1 microvolt, 6 mm. and for 1 micro-amp., 180 mm. The apparatus is first calibrated using sodium chloride solutions of known concentration. The method has been found convenient and reliable, 2 c.c. of sap being sufficient for a determination. The apparatus is shown in Fig. 1.

An observation is made as follows: The freezing-bath is filled with a mixture of ice and salt. The two small test tubes are removed from the small cork; one of them is filled with about 2 c.c. of solution and the other with 2 c.c. of distilled water; they are cooled in a freezing-bath. When it is judged that the liquids have reached their freezing point, a little hoar frost from the outside of the freezing bath is transferred to the distilled water by a cooled platinum needle. Ice crystals are formed and some adhere to the needle which is immediately used to inoculate the solution. Crystallisation takes place in this and the needle is withdrawn. The test tubes are placed in the smaller

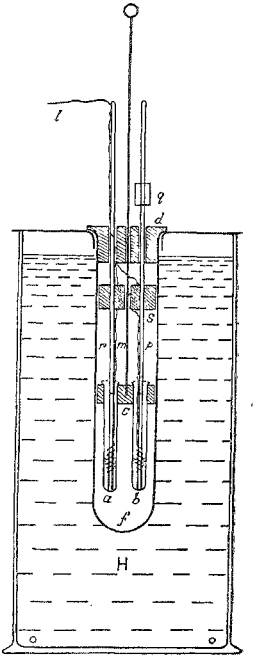



Fig 1.

*a*, test-tube containing solution *b*, " test-tube containing distilled water; *r* and *p*, pine rods supporting the thermo junctions; *c*, cork with two holes to hold the test-tubes and with a wire *m* fixed into it. *S* is a cork connecting piece which rigidly connects *r* and *p* and supports the eureka wire of the couple wound upon it. The wire *m* works loosely in *S* but is fixed in the cork bung *c*, not tightly closing the freezing-chamber *f*. The two pine rods work loosely in the perforation in *d*, *g* is merely a stop to prevent the rods from slipping out when the cork *c* is removed; *l* — the copper leads from the freezing-chamber, to the galvanometer and *H* is the freezing-bath.

Reproduced from - Dixon & Atkins, Sci Proc. Roy. Dub Soc., 1911, 13 (N.S.) 51.

cork which is fixed to the lower end of the wire handle and which has been removed from the chamber for a short time. The thermo-junctions from the pine rods are immersed in the freezing liquids in the test tubes.

The whole system is then arranged as shown in the figure. Stirring of the handle is immediately started by moving the pine rods up and down and as they are held rigid, the two solutions will be subjected to precisely the same conditions. The freezing-bath also is kept stirred. The galvanometer is now put in circuit by means of a clip on support in the petroleum key (a reversing key). After about a minute, the spot of light moves from zero and reaches a steady position. The stirring is actively kept up to avoid supercooling round the lower layers of the junction. A reading is taken and the reverse connection is made with the reversing key, and a reading is taken. This first observation is generally a preliminary one, but can be depended upon provided much ice has not been present.

The test-tubes are raised from the freezing-chamber and the one containing the solution momentarily touched by the finger to give it a little heat to melt the ice. The upper cork is readjusted and stirring commenced, allowing the solution to cool and the connection is taken by removing the clip.

At about the time when radiation should have cooled the solution, the connection is again made. The movement of the spot of light is noted. If it goes indefinitely beyond its previous position, the solution is inoculated with a little hoar frost. The stirring is actively maintained. Readings are again taken as before, before and after reversing. A third reading also can be taken easily. Generally the readings agree fairly closely.

A sample reading is given showing the accuracy of the arrangement. The scale reads continuously from left to right. 250 mm. marks its middle point.

SOLUTION OF 1 PER CENT. SODIUM CHLORIDE (PURE) RECRYSTALLISED.

| | 1st position | 2nd position | Zero | Deflection | Mean deflection |
|---|---|---|---|---|---|
| Observation I | 323·1 | 184·1 | 253·0 | 69·5 | 69·70 |
| ,, II | 322·8 | 182·6 | 252·1 | 70·1 | |
| ,, III | 323·5 | 184·5 | 252·8 | 69·5 | |

The deflection is shown by subtracting the second from the first and halving the difference. The readings do not vary much from the

mean. According to Raoult, 1 per cent. sodium chloride lowers the freezing point of water by 0·596°.

The osmotic concentration was calculated in terms of pressure in atmospheres from the freezing-point depression, by means of the formula of Nernst,

$$\Delta \times 12{\cdot}03 = P,$$

where P is the osmotic pressure in atmosphere at 0°, and $\Delta$ is the depression in the freezing-point.

The specific electrical conductivity was determined at 30° in a cell consisting of a small test-tube in which the two rectangular platinum electrodes were maintained at a fixed distance apart. The cell was immersed in an electrically controlled thermostat, and a bridge of the Leeds and Northrup type employed. The conductivity was calculated from the formula,

$$K = \frac{1000-l}{l} \times \frac{N}{R},$$

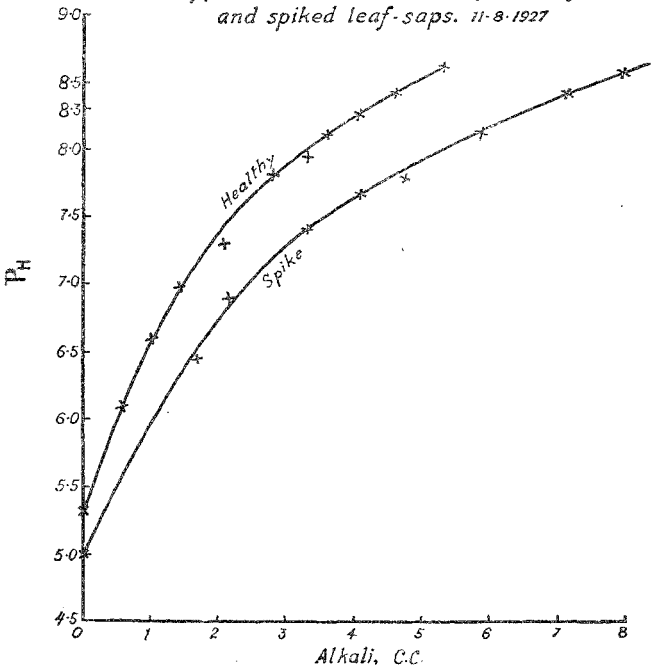
where $l$ is the bridge wire-reading, N the cell-constant, R the resistance in ohms introduced into the circuit, and K the specific electrical conductivity. The cell was standardised using $N/50$ potassium chloride. Each time a measurement was made, the cell-constant also was determined.

The hydrogen-ion determinations were made electrically by means of the usual gas-chain method. A saturated potassium chloride-calomel cell was employed; a Leeds and Northrup type K potentiometer, a Western standard cell, a battery, a galvanometer and a Bunker type of hydrogen electrode completed the equipment. Hydrogen electrolytically prepared was used for the experiments. The Bunker electrode was adapted for measuring both the $P_H$ and the total acidity, by electrometric titration.

Palladinised platinum electrodes were employed and no difficulty was experienced in obtaining reliable readings. The amount of sap required for each determination was 4 c.c.

The initial $P_H$ was first determined and then successive quantities of decinormal alkali added, the $P_H$ being re-determined after each addition. The results so obtained were plotted and the amount of alkali required to bring the sap to a $P_H$ of 8·3 was read from the curve so obtained. The figures quoted therefore represent the titration value of 4 c.c. sap with phenolphthalein as indicator. Fig. 2 is a graphical representation of the titration carried out for one set. Invariably the determinations were carried out on the same day of bringing the samples.

Fig. 2
*Typical titration curves of healthy and spiked leaf-saps. 11-8-1927*



## TABLE I.

*Initial $P_H$ titration value and specific electrical conductivity of saps from healthy and spiked sandal leaf tissue.*

| Date in 1927 | Locality | Initial $P_H$ | | Alkali (c.c. $N/10$) for 4 c.c. sap | | Specific electrical conductivity K × $10^5$ | |
|---|---|---|---|---|---|---|---|
| | | Healthy | Diseased | Healthy | Diseased | Healthy | Diseased |
| July 15 | Uttarahalli... | 5·36 | 4·76 | 3·32 | 4·17 | 1658 | 1614 |
| | | 5·15* | | 3·1* | | 1491* | |
| ,, 29 | Ragihalli .. | 5·2 | 4·69 | 8·17 | 7·25 | 1535 | 1182 |
| August 5 | Uttarahalli... | 5·27 | 4·70 | 1·82 | 2·45 | 2046 | 1986 |
| ,, 11 | Ragihalli ... | 5·29 | 4·98 | 4·2 | 6·5 | 1313 | 1277 |
| ,, 18 | Uttarahalli... | 5·68 | 4·95 | 3·53 | 3·7 | 1782 | 1747 |
| September 8 | Ragihalli ... | 4·69 | 4·99 | 5·35 | 6·23 | 1696 | 1179 |
| ,, 13 | ,, ... | 5·22 | 4·88 | 4·80 | 6·52 | 1341 | 1284 |
| ,, 1 | Uttarahalli... | 5·71 | 4·77 | 3·75 | 4·15 | 2014 | 1840 |

* Sample from a flowering healthy tree.

## TABLE II.

*Depression in freezing-point* ($\Delta$) *and osmotic pressure in the leaves of spiked and healthy sandal: relation between* $\Delta$ *and* $K \times 10^3$.

| Date in 1927 | Locality | Depression in freezing point, Δ | | Osmotic pressure in atmospheres | | Ratio K × $10^3/\Delta$ | |
|---|---|---|---|---|---|---|---|
| | | Healthy | Diseased | Healthy | Diseased | Healthy | Diseased |
| August 18 | Uttarahalli... | 1·568 | 1·691 | 18·88 | 20·34 | 11·37 | 10·33 |
| September 8 | Ragihalli ... | 1·433 | 1·540 | 17·24 | 18·52 | 11·84 | 7·67 |
| ,, 15 | Uttarahalli... | 1·428 | 1·542 | 17·18 | 18·55 | 14·31 | 11·92 |

## DISCUSSION.

There are few references in the literature to determinations of $P_H$ in cases of plant disease. Harvey (*J. Biol. Chem.*, 1920, **42**, 397) and Boas (*Zeits. Pflanzenkrank.*, 1919, **29**, 171) have, however, studied this phenomenon in the case of Mosaic Disease of tobacco, and Leaf Roll Disease of potato. It will be seen from Table I that the $P_H$

values of the healthy saps lie between 5·15 and 5·71. In the spiked samples, however, the limits are 4·69 and 4·99. In other words, sap from spiked leaves has a distinctly more acid reaction than that from healthy leaves. The titratable value of the spiked samples is similarly greater than the healthy ones. The specific electrical conductivity, on the other hand, is lower in the spiked specimens. These figures are of interest in view of the results described in Parts I and II of this series where it was shown that the spiked tissue has a much lower calcium-content than the healthy material, but a higher diastatic activity. The $P_H$ being a measure of the dissociated acids, the spike-sap either contains more readily dissociable acids or some factor leading to a greater ionisation of these acids. In this connection it may be noted that Leuthardt (*Koll. Chem. Beihefte*, 1927, **25**, 1) has shown that the addition of dextrose, glycerol, or sucrose to a buffered solution of organic acids such as malic or tartaric acid brings about an increase in the hydrogen-ion concentration. Spiked leaves have been shown (Part II of this series) usually to contain a higher sugar-content. On the other hand the $P_H$ of the spiked sap is very close to the optimum reaction for vegetable diastases and may therefore be the cause and not one of the results of the increased diastatic activity of the spiked sap.

The total acid in the spiked sap is, however, generally greater than in the normal sap, the titratable acidity being higher in the former case. It is clear that a fuller knowledge of the acids contained in the sap is required and this point is being investigated.

In connection with the sap titrations, it was noticed that in the samples from the healthy leaves a precipitation, presumably of protein material, occurred at a $P_H$ of 6·5–6·7. In the spiked samples, however, similar precipitation was observed at a more acid stage of the titration, when the $P_H$ was about 6–6·1. A separation of the protein of the saps and a determination of their iso-electric points might be of interest.

Table II shows that the freezing-point depression and the osmotic pressure values are higher in spiked sap than in the normal fluid. The depression in the freezing-point has been assumed to be a measure of the total solutes while the specific conductivity represents the electrolyte content.

Chandler (*Mo. Agric. Exp. Stn. Research Bull.*, 1914, **14**, 489), Dixon and Atkins, (*Proc. Roy. Soc. Dublin*, 1915, **14**, 445) and Mason (*Proc. Roy. Soc. Dublin*, 1919, **15**, (N. S.), 651) have endeavoured to differentiate between the electrolytes and non-electrolytes by expressing the conductivity of the sap in terms of the osmotic concentration which would be produced by a solution of potassium chloride having the

same conductivity. The results of Dixon and Atkins on this basis have been questioned by Haynes (*Biochem. J.*, 1919, **13**, 111) but we may perhaps assume that the ratio of the specific conductivity to the depression in the freezing-point as shown in Table II indicates the relative concentration of dissociated ions and total solutes. It will be noticed that this figure is lower in the spiked samples than in the healthy sap. Dixon and Atkins consider that an increase in the osmotic concentration in the sap is generally due to dissolved sugars and in the present case, as has already been shown, the quantity of reducing sugars is greater in spiked specimens than in healthy ones. The greater depression in the freezing-point in the spiked samples must be due to an increased content of soluble matter in such saps, and this is actually found to be the case. In potato affected with Mosaic, an increase in the osmotic pressure of the leaves compared with that of the healthy ones, has been recorded.

## SUMMARY.

1. The $P_H$ of healthy sandal leaf-sap has been found to lie between 5·15 and 5·71, while in the case of spiked trees from the same area the figure was between 4·69–4·99.

2. The titratable acidity of the spiked samples is greater than that of the normal samples.

3. The osmotic concentration is higher in the diseased samples.

4. The saps of both healthy and spiked leaves have a high specific conductivity, but this is always smaller in the spiked samples.

5. The ratio of the specific conductivity to the depression in the freezing-point is lower in spiked sap than in normal material.

The author's thanks are due to Professor R. V. Norris at whose suggestion this work was undertaken, and to Mr. Sreenivasaya for supplying him with the samples of the sap.

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[Accepted, 1-3-28.]

PRINTED AND PUBLISHED
BY GEORGE KENNETH AT THE DIOCESAN PRESS
POST BOX 455, MADRAS—1928. C5295