CONTRIBUTIONS TO THE STUDY OF SPIKE-DISEASE OF SANDAL (*Santalum Album*, LINN).

PART XVII.—HYDROGEN-ION CONCENTRATION AND BUFFERING CAPACITY AS FACTORS OF DISEASE RESISTANCE.

By M. Srinivasan and M. Sreenivasaya.

Disease resistance in plants is a problem of great economic value since it offers the most rational and effective method of combating the diseases affecting them. The factors imparting disease resistance are both mechanical and physiological. Cuticular structure of the host (Valleau, W. D., *J. Agr. Res.*, 1915, **5**, 365; Willamann, J. J., Pervier N. C., and Triebold, H. O., *Bot. Gaz.*, 1925, **80**, 121; Lutman, B. F., *Vt. Agr. Exp. Sta. Bull.*, 1919, 215), waxy coatings (Howitt, J. E., *Rept. Que. Soc. Prot. Plants*, 1924, **16**, 9) and hairy growth of the epidermis (Blackmann, V. H., *B.A.A.S.*, 1924, Sec. K, 1-14) are some of the known types of defensive mechanism.

When the host is infected despite the mechanical barriers, physiological factors of resistance come into play. Among such factors may be mentioned a highly toxic or agglutinating medium obtaining in the host (Berridge, E. M., *Ann. Appl. Biol.*, 1929, **16**, 567) and the presence therein of tannins (Cook, M. T., and Taubenhaus, J. J., *Delaware Agr. Exp. Sta. Bull.*, 1911, 91; Cook, M. T., and Wilson, G. W., *N. J. Agr. Exp. Sta. Bull.*, 1916, 291; Graves, A. H., *Phytopath.*, 1926, **16**, 615; Hawley, L. F., Fleck, L. C., and Richards, C. A., *Ind. Eng. Chem.*, 1924, **16**, 699), alkaloids and glucosides (Reynolds, E. S., *Plant Physiol.*, 1926, **1**, 151; Brierley, W. B., *Rept. Internat. Potato Confer.*, London, 1921, 93), phenols (Newton, R., and Anderson, J. A., *Canadian J. Res.*, 1929, **1**, 86) and sulphur compounds (Tims, E. C., *J. Agr. Res.*, 1926, **32**, 183), all of which have been shown to exert an inhibitory influence on the growth of parasites.

Free hydrogen ions in the cell sap of the host as a determining factor in disease resistance is a recent idea. So far, no correlation between them has been recorded (Hawkins, L. A., and Harvey, R. B., *J. Agr. Res.*, 1919, **18**, 275; Weiss, F., and Harvey, R. B., *J. Agr. Res.*, 1921, **21**, 589; Hurd, A. M., *J. Agr. Res.*, 1923, **23**, 373; *ibid.*, 1924, **27**, 725; Hurd-Karrer, A. M., *Amer. J. Bot.*, 1925, **12**, 359; Mumford, E. P., *Ann. Appl. Biol.*, 1930, **17**, 28; Newton, R., Lehmann, J. V., and Clarke, A. E., *Canadian J. Res.*, 1929, **1**, 5),

though Comes (*Reale Istituto d' Incoraggiamento di Napoli*, 1916, 1; *Bull. Agr., Intelligence*, **7**, 1205) argued that acidity was a very important factor determining immunity in plants. Laurent (*Ann. de l'Inst. Pasteur*, 1899, **13**, 1) and Lepoutre (*Ann. de l'Inst. Pasteur*, 1902, **16**, 304) observed that reduction in the acidity of the cell sap did in some way facilitate the attack of bacteria on plant tissues. However, all these authors only tried to correlate disease resistance with initial acidity of the host tissue fluid, but not with its buffering capacity. Only one attempt till now seems to have been made in this direction without definite conclusions (Wille, F., *Zentr. Bakt., Abt. II*, 1933, **87**, 340).

In the course of our investigations on sandal, we observed that sandal growing in combination with certain species of host plants withstood the attack of spike-disease even through grafting (*Investigations on the Spike-Disease of Sandal—Working Committee Report*, 3, 1931). That immunity in sandal is largely controlled by associated host plants even under sylvicultural conditions has been shown by Sreenivasaya and Rangaswami (*J. Indian Inst. Sci.*, 1931, **14A**, 59).

The present communication is a study of hydrogen-ion concentration and buffering capacities of the tissue fluids of sandal in their relation to disease resistance. For this purpose, sandal plants in combination with *Ruta graveolens*, *Murraya kœnigii*, *Melia azadirachta* and *Toddalia aculeata*—types of hosts imparting resistance—and those in combination with *Acacia farnesiana*,—a type rendering sandal susceptible to disease—have been studied, together with spiked plants and those growing without a host.

EXPERIMENTAL.

Sandal plants for the investigation were reared as follows :—Seedlings were transplanted into pots containing suitable make-up of soil and manure, as also the desired host plants. In one instance no host was supplied. The plants were watered daily. For receiving fresh supplies of manure, the growing plants along with their hosts were transplanted into bigger pots from where they were removed at the desired stages for experimental purposes. Together with healthy sandal, spiked ones of the same age and feeding on the same hosts (*Acacia farnesiana*) as the healthy were selected. By thus choosing plants of the same age and grown under equal environmental conditions, inherent differences, due to host combinations or disease, would not be masked in respect of initial acidity and buffering capacity.

For the experiments, the plants were pulled out of the pots, adhering soil washed off in running tap water, then with distilled water and dried by pressing between folds of filter paper. They were then divided into (1) leaf, (2) bark, (3) wood and (4) root.

The samples, collected at 9 A.M. each day, were treated with toluene (after shredding in case of wood and root), in wide-mouthed

bottles, stoppered and left in ice-room. After 24 hrs., sap was expressed from the tissues as described previously (Sreenivasaya, M., and Sastri, B. N., *J. Indian Inst. Sci.*, 1928, **11A,** 23; *ibid.*, 1929, **12A,** 239). The saps obtained with root and wood tissues were so small that centrifuging was not possible, so these, as well as the other experimental tissue fluids, were all filtered prior to electrometric titrations. Filtering does not detract from the accuracy of the results, for Harvey (*J. Biol. Chem.*, 1920, **42,** 397) showed that in a buffered plant juice, minor differences in concentration did not affect hydrogen-ion measurements appreciably. Hurd (*loc. cit.*) did not centrifuge the sap in her experiments on varietal disease of wheat rust, but strained it through cloth. Similarly, Martin (*Protoplasma*, 1928, **3,** 273) used filtering as a method of clarifying saps in her study of the buffers of sun-flower stem and root, while Haas (*Soil Science*, 1920, **9,** 341) in determining acidity and buffering capacity of red clover, used its tissue fluid as such for the purpose without filtering or centrifuging.

Hydrogen-ion concentration and buffering capacities were determined electrometrically as described elsewhere (Iyengar, A. V. V., *J. Indian Inst. Sci.*, 1928, **11A,** 103). The quantity of sap used was throughout 2 c.c. It usually took 30 mins. for the hydrogen electrode to attain equilibrium.

Results.—The first experiment was to find out the effect of keeping, in the laboratory as well as in ice-room, on the initial acidity and buffering capacities of sandal tissue fluids. Results, taken at random from a large number of determinations, are recorded in Tables I and II.

TABLE I.

Effect of keeping on the initial acidity of sandal saps.

Tissue fluids from	Initial pH	pH after keeping			
		in the laboratory for			in the ice-room for 24 hrs.
		2 hrs.	4 hrs.	8 hrs.	
Healthy leaf—					
Sample I ..	5·45	5·45	5·45	5·45	5·45
Sample II ..	5·50	5·50	..	5·50	5·50
Sample III ..	5·40	..	..	5·40	5·30 (after 48 hrs.)
Spiked leaf ..	5·00	5·00	5·00	5·00	5·00
Healthy root ..	5·85	..	..	5·85	..

TABLE II.

Effect of keeping on the buffering capacities of sandal tissue fluids.

A. HEALTHY LEAF SAP (2 c.c.)			B. SPIKED ROOT SAP (2 c.c.)		
	pH			pH	
Alkali c.c.	immediately after pressing	4 hrs. after pressing	Acid c.c.	immediately after pressing	4 hrs. after pressing
0·0	5·50	5·50	0·0	5·15	5·15
0·5	5·70	5·65	0·2	4·85	4·85
1·5	6·30	6·30	0·5	4·60	4·60
2·5	6·85	6·80	1·0	4·45	4·40
3·5	7·20	7·25	2·0	4·20	4·30
3·5	7·50	7·55	3·0	4·10	4·10
5·5	7·80	7.80	5·0	4·00	4·05
6·5	8·05	8·00			

It is clear from the above that the expressed juice of sandal, healthy or spiked, does not change in pH on keeping in the laboratory for about 8 hrs., or in ice-room for 24 hrs. Other workers (Haas, A. R. C., *loc. cit.* ; McLenden, J. F., and Sharp, P. F., *J. Boil. Chem.*, 1919, **38**, 531), however, found that reactions of plant tissue fluids changed on keeping. The buffering capacities remained fairly unaltered for 4 hrs. in the laboratory. This also shows the close agreement between duplicate determinations.

Sulphuric acid and acetic acid as titres.—At first, sulphuric acid, acetic acid and sodium hydroxide were used as titres. The results of these titrations are given graphically in Fig. 1. The use of sulphuric acid was discontinued later, when it was found that working with root and wood saps, even small additions of this titre gave large pH shifts, rendering their measurements difficult. Subsequently, therefore, acetic acid was used instead, since large quantities of this titre would have to be added to the saps to bring about significant shifts in their pH, while the errors due to the resulting dilution would also be negligible (Clark, *Determination of Hydrogen Ions*, 1928).

Further, as organic acids are among the metabolic products of the spike-disease of sandal, one way of simulating the condition of disease in the healthy plant is by addition of known quantities of one

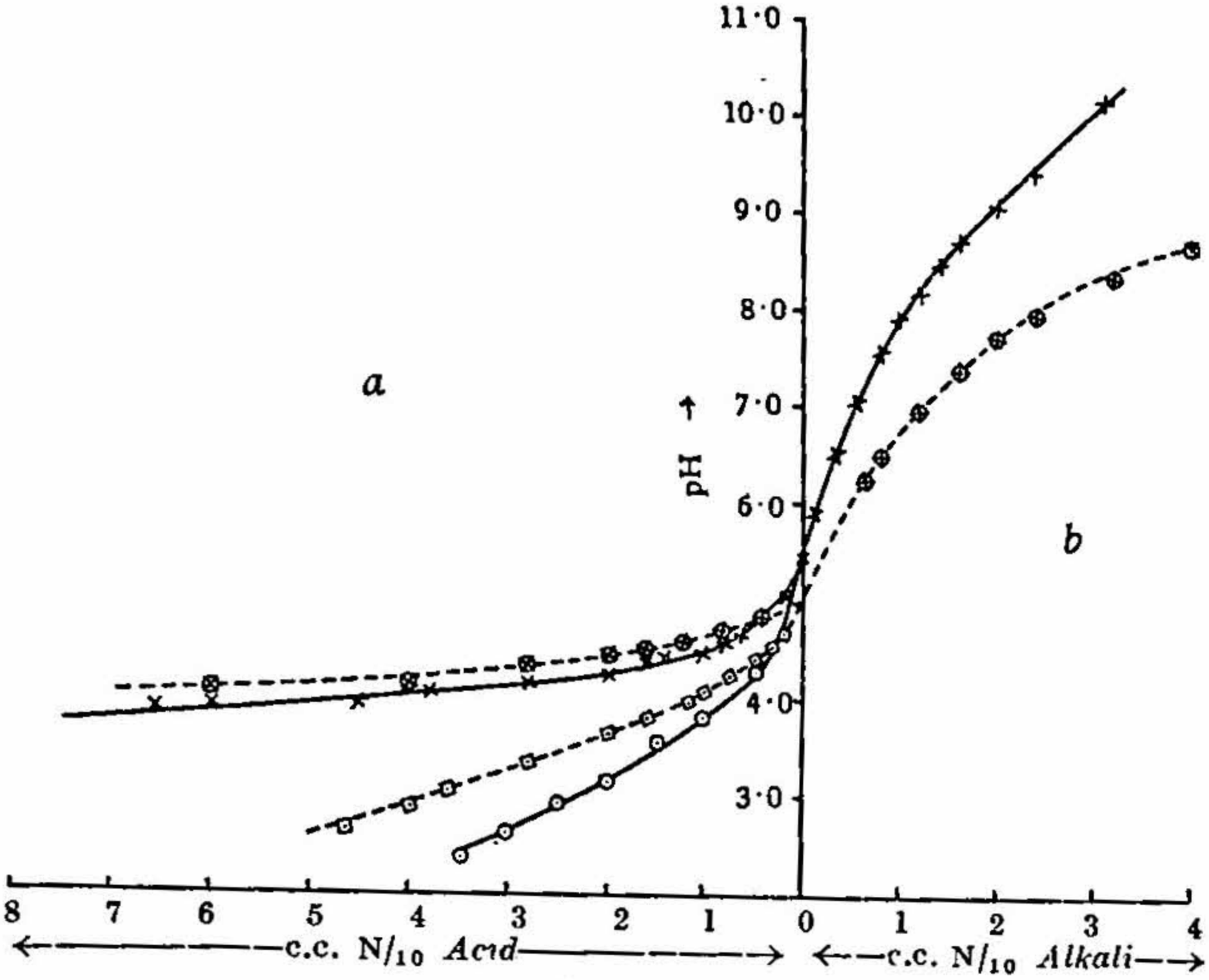

Fig. 1.

Titration curves of healthy and spiked leaf tissue fluids.

a Spiked, Healthy } with CH_3COOH.
Spiked, Healthy } with H_2SO_4.

b Spiked, Healthy } with NaOH.

of the metabolic organic acids to healthy tissue fluid till its pH shifts to that of the spiked. Such acid requirements or buffering capacities of healthy tissue fluids would reasonably be a measure of resistance offered by that plant towards disease, greater buffering capacity (greater acid titre) necessarily implying greater resistance. Hence, in measurements of buffering capacity in relation to disease resistance in plants (where with the onset of disease the tissues usually get more acidic), only organic acids should be chosen as titres, and titrations continued till the pH of healthy tissue fluid has shifted just beyond that of the diseased. So, in our studies mostly acetic acid was used as the titre.

The results of the electrometric titrations of the leaf, bark, wood and root tissue fluids of sandal with the different host combinations are given graphically in Figs. 2, 3, 4 and 5. These include for comparison also sandal grown without a host plant and spiked sandal. In all these cases, titrations (only against acetic acid) were made till the initial pH shifted beyond that of the spiked (*i.e.*, by about 0·5 of a unit). From these graphs are calculated the quantities of titre required to shift the pH by 0·5 of a unit from the initial value (Table III). These values are taken as a measure of the buffering capacities of the various tissue fluids.

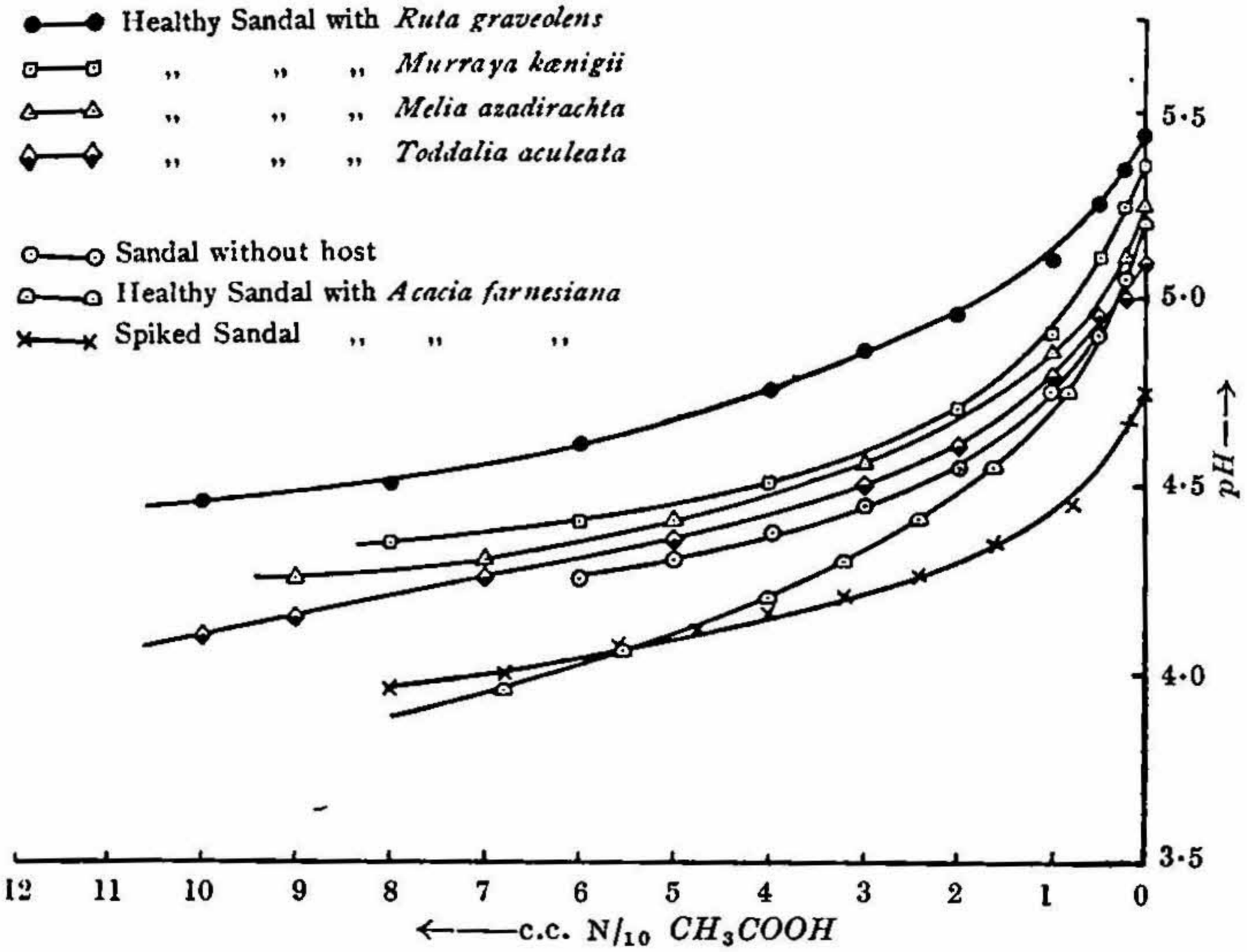

Fig. 2.

Titration curves of tissue fluids of Sandal-Leaf.

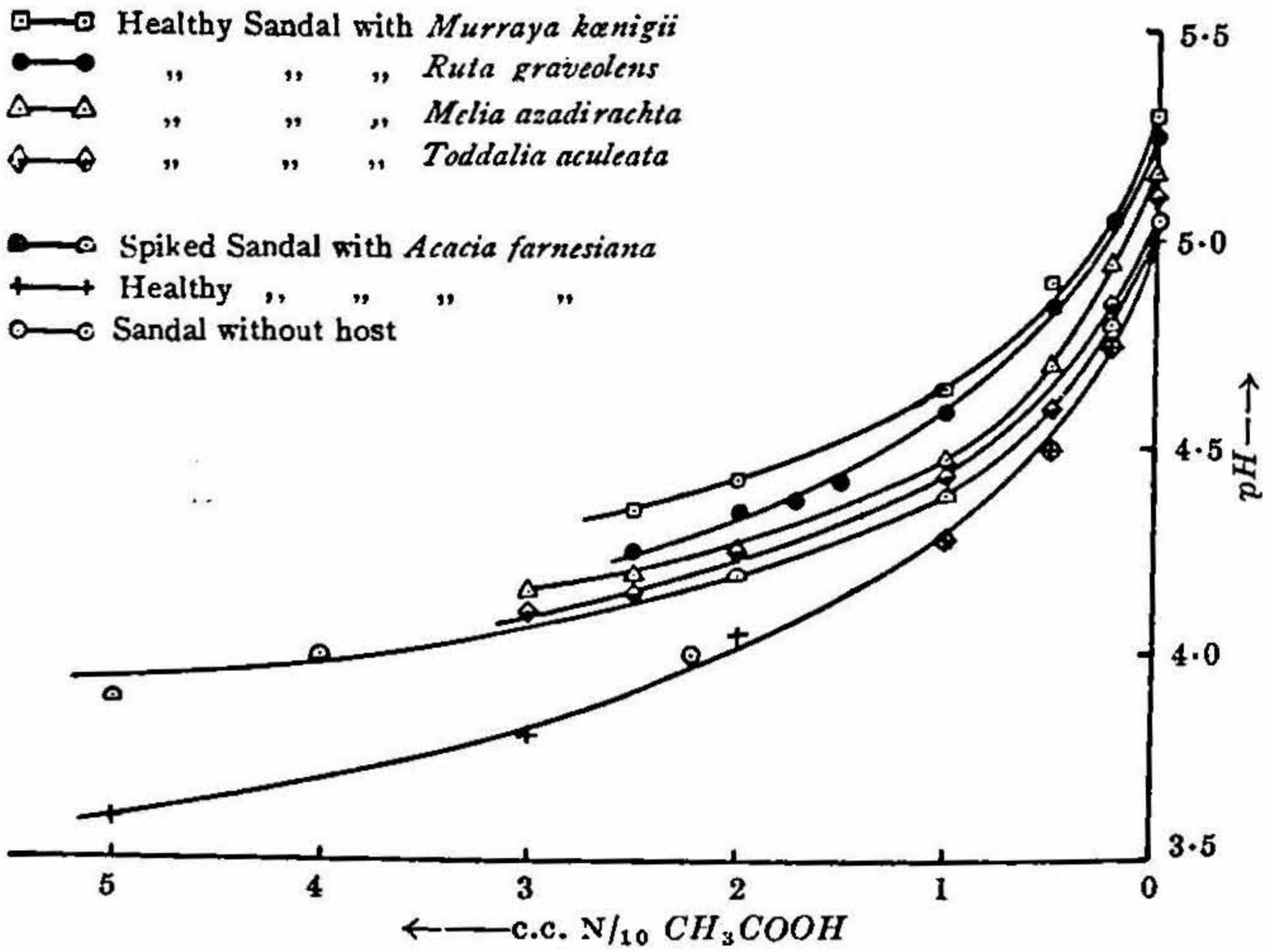

Fig. 3.

Titration curves of tissue fluids of Sandal-Bark.

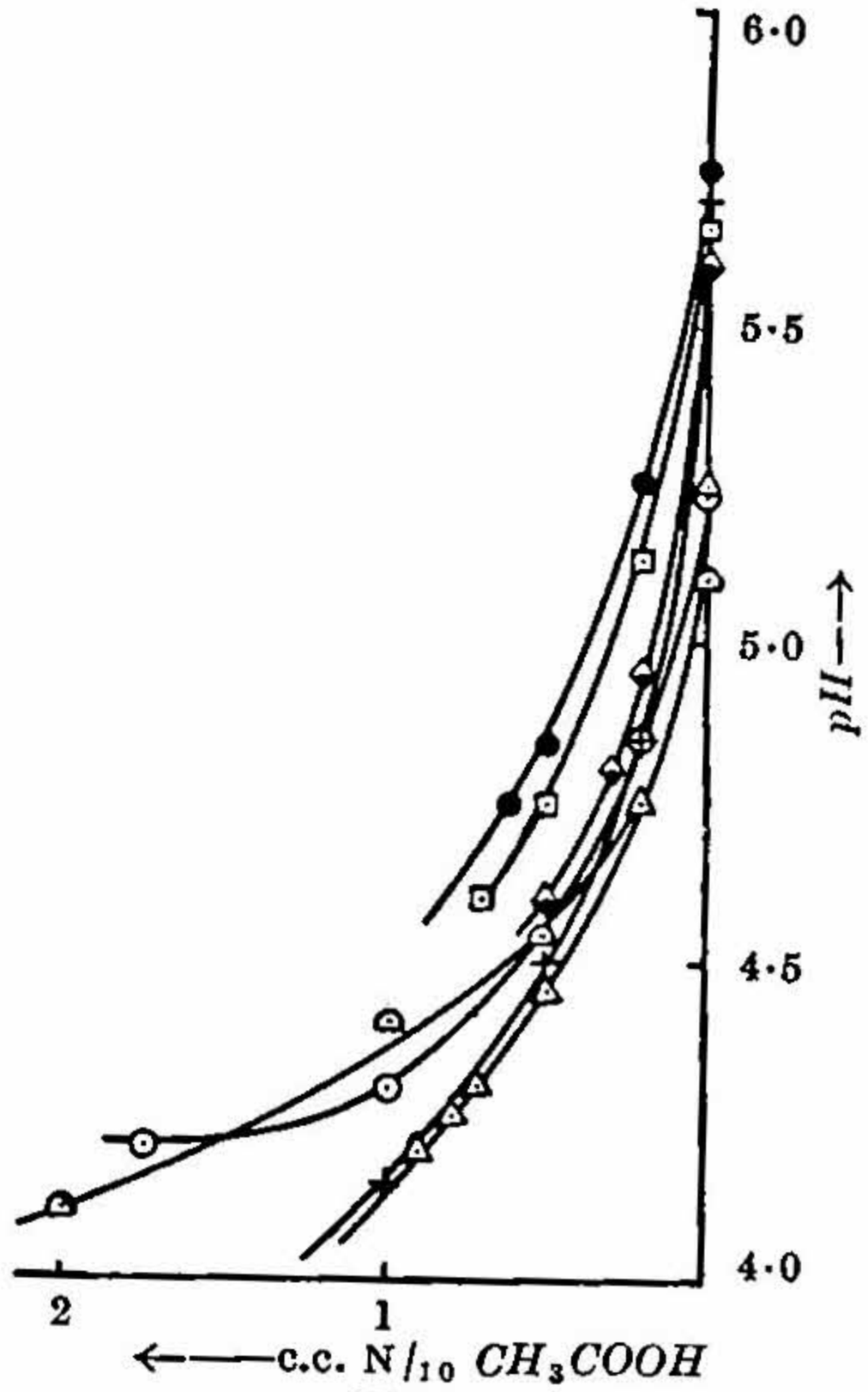

Fig. 4.

Titration curves of tissue fluids of Sandal-Wood. Legends as in Fig. 3.

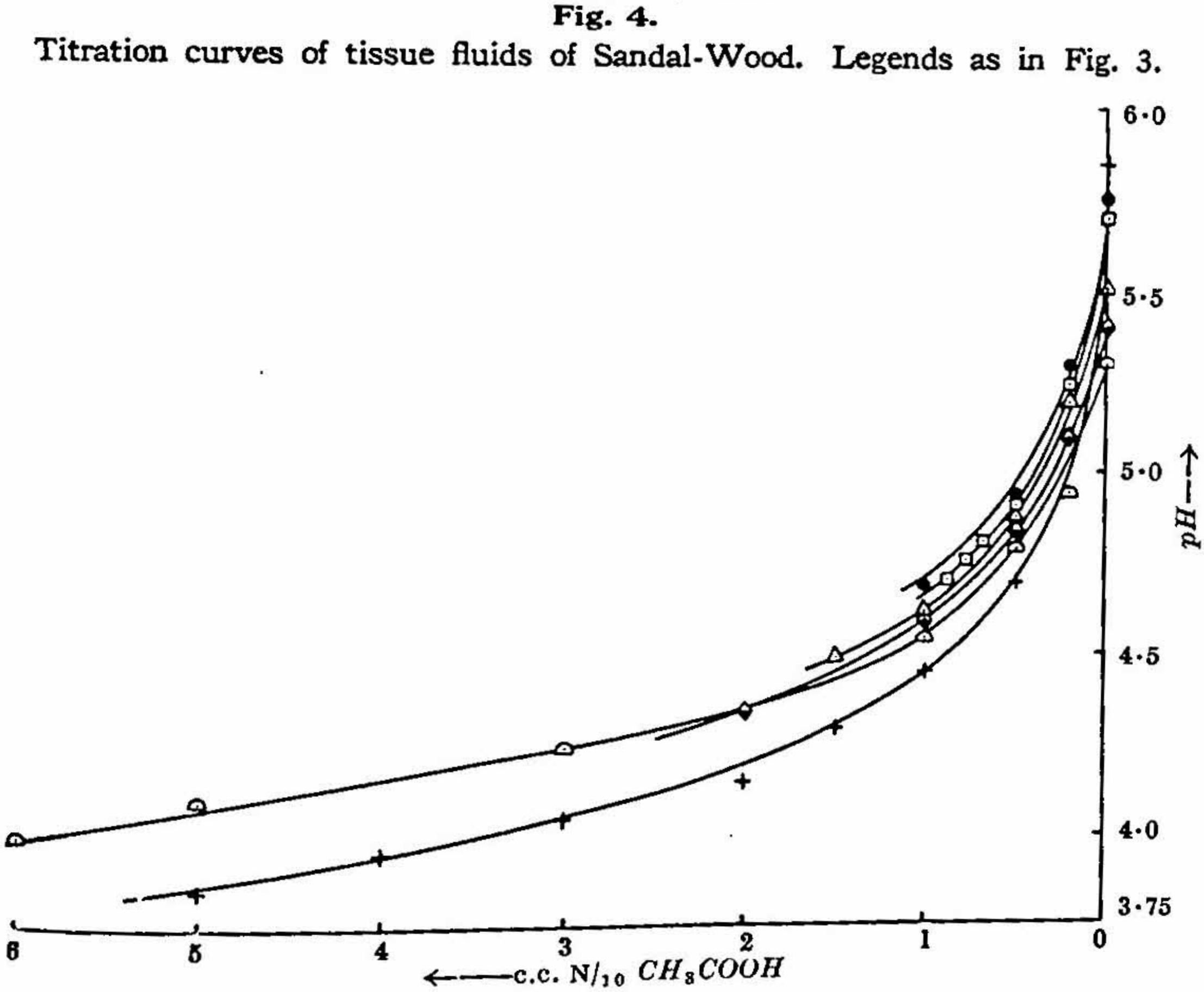

Fig. 5.

Titration curves of tissue fluids of Sandal-Root. Legends as in Fig. 3.

TABLE III.

Buffering capacities of tissue fluids of sandal grown with different host plants.

Sandal grown in combination with	c.c. of 0·1 N acetic acid needed for a shift in pH by 0·5 of a unit from the initial value of the tissue fluids of			
	Leaf	Bark	Wood	Root
Ruta graveolens ..	1·70 (5·50)	0·65 (5·25)	0·20 (5·75)	0·20 (5·75)
Murraya kœnigii ..	1·25 (5·35)	0·60 (5·30)	0·20 (5·65)	0·20 (5·70)
Melia azadirachta ..	1·50 (5·25)	0·55 (5·15)	0·20 (5·25)	0·40 (5·50)
Toddalia aculeata ..	2·00 (5·10)	0·60 (5·10)	0·15 (5·60)	0·40 (5·40)
Acacia farnesiana ..	0·45 (5·40)	0·55 (5·00)	0·05 (5·70)	0·08 (5·85)
Without host ..	1·00 (5·25)	0·45 (5·05)	0·30 (5·25)	....

The figures in brackets in the above table refer to the initial pH of the various tissue fluids.

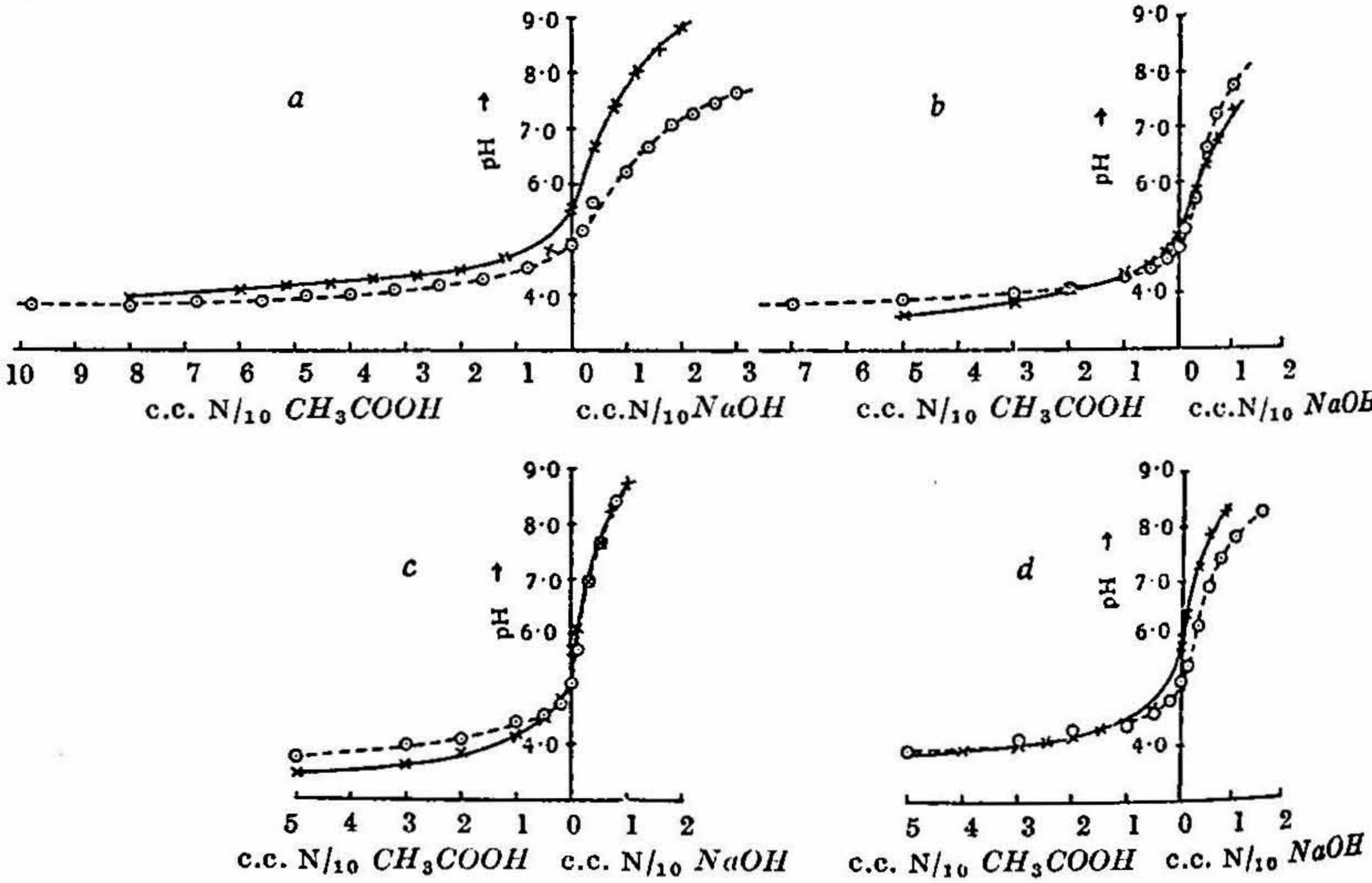

Fig. 6.

Titration curves of healthy and spiked Sandal tissue fluids.

a Leaf. *b* Bark. *c* Wood. *d* Root. ×——× Healthy ⊙- - -⊙ Spiked

Fig. 6 gives the general form of curves obtained with leaf, wood, bark and root of healthy and spiked sandal tissue fluids as titrated against acetic acid and sodium hydroxide. These titrations were performed with tissue fluids from six healthy and four spiked sandal plants. The corresponding titration curves were constructed and from these the buffer index = calculated according to van Slyke (*J. Biol. Chem.*, 1922, **52**, 525). These values are compiled in the following table.—

TABLE IV.

Buffer index of healthy and spiked sandal tissue fluids.

Tissue fluid from	Condition of sandal	Sample	BUFFER INDEX pH Initial	5—4	5·5—4·5	5·5—6·5	6—7	6·5—7·5
Leaf	Healthy ..	1	5·45	..	..	..	..	..
	(From Fig. 1 with sulphuric acid)	..	..	(0·014)	..	..	..	..
		..	..	0·250	..	0·0144	0·0144	0·0137
		2	5·50	0·343	0·094	0·0137	0·0175	0·0198
		3	5·45	0·410	..	0·0274	0·0327	0·0388
		4	5·50	0·435	0·174	0·0281	0·0319	0·0314
		5	5·30	0·383	..	0·0350	0·0395	0·0440
		6	5·30	0·393	..	0·0198	0·0228	0·0258
		7	5·35	0·410	..	0·0213	0·0228	0·0274
	Average ..		5·40	0·368	0·134	0·0228	0·0259	0·0287
	Spiked ..	1	5·00	..	..	..	..	..
	(From Fig. 1 with sulphuric acid)	..	..	(0·052)	..	0·0230	0·0290	0·0350
		2	5·00	..	..	0·0426	0·0547	0·0722
		3	4·95	..	..	0·0289	0·0380	0·0570
	Average ..		5·00	..	..	0·0315	0·0406	0·0547
Bark	Healthy ..	1	5·00	0·1525	..	0·0133	0·0114	0·0171
		2	5·00	0·1150	..	0·0133	0·0152	0·0247
		3	5·00	0·1525	..	0·0152	0·0152	..
	Average ..		5·00	0·1400	..	0·0139	0·0139	0·0209

TABLE IV.—(*Contd.*)

Tissue fluid from	Condition of sandal	Sample	BUFFER INDEX pH Initial	5—4	5·5—4·5	5·5—6·5	6—7	6·5—7·5
Bark	Spiked ..	1	4·85	0·1525	..	0·0114	0·0114	0·0133
		2	5·00	0·1900	..	0·0114	0·0133	0·0152
	Average ..		4·90	0·1713	..	0·0114	0·0124	0·0143
Wood	Healthy ..	1	5·80	0·0800	0·0250	..	0·0095	0·0076
		2	5·70	0·0675	0·0200	..	0·0095	0·0076
		3	5·45	0·0950	..	0·0133	0·0114	0·0095
	Average ..		5·65	0·0808	0·0225	0·0133	0·0101	0·0082
	Spiked ..	1	5·10	0·1425	..	0·0076	0·0076	0·0076
		2	5·35	0·1700	..	0·0076	0·0076	0·0114
	Average ..		5·20	0·1562	..	0·0076	0·0076	0·0095
Root	Healthy ..	1	5·85	0·1500	0·0450	..	0·0095	0·0114
		2	5·85	0·2750	0·0725	..	0·0114	0·0114
		3	6·30	0·1850	0·0450	..	..	0·0114
		4	5·90	0·2180	0·0650	..	0·0114	0·0152
		5	6·05	0·1850	0·0450	..	0·0114	0·0152
	Average ..		6·00	0·2026	0·0545	..	0·0109	0·0129
	Spiked ..	1	5.15	0·0180	..	0·0076	0·0133	0·0152
		2	5·45	0·3300	0·0750	0·0095	0·0114	0·0114
		3	5·30	0·3430	..	0·0095	0·0152	0·0190
	Average ..		5.30	0·2303	0·0750	0·0089	0·0133	0·0152

DISCUSSION.

From Tables III and IV, it is clear that, in all the cases studied, initial acidity is highest with leaf and lowest with the root, bark and wood having corresponding intermediate values. Such gradient reactions have been recorded previously in other plant tissue fluids as well (Haas, A. R. C., *loc. cit.*; Gustafson, F. G., *Amer. J. Bot.*, 1924, **11**, 1). The acidity, however, of the sap from any tissue of the

spiked plant is higher than that of the corresponding tissue from healthy sandal. This is in keeping with the general finding that the acidity of the tissue fluids of diseased plants is higher than that of the corresponding healthy ones (Hurd, A. M., *loc. cit* ; Wagner, R. J., *Zentr. Bakt. Parasitenk., Abt. II.*, 1916. **44**, 708; Laurent, M. E., *loc. cit.*; Robertson, I. M., and Smith, A. M., *Biochem. J.*, 1931, **25**, 768).

Leaf, again, has the highest buffering capacity, irrespective of host combinations or the physiological condition of the sandal plant. Comparing spiked and healthy tissue fluids, spiked leaf tissue fluid is more buffered than healthy both against acid (acetic or sulphuric) and alkali (graphs 1 and 6; Table IV). Especially against acetic acid is this buffering so high that additions of this titre to spiked leaf sap (2 c.c.) even in quantities far in excess of those added to the other experimental tissue fluids did not shift its pH beyond 0·5 of a unit from the initial value. This explains the absence of the values for the buffer index of spiked leaf sap against acetic acid in Table IV. The high buffering of spiked leaf tissue fluid against acid is contrary to the findings of Iyengar (*J. Indian Inst. Sci.*, 1933, **16A**, 91) *viz.*, that it is less buffered than healthy in the acid region. This discrepancy might be attributed to uncontrolled sylvicultural conditions under which the healthy and spiked plants grew and to the undefined stage of the disease.

Referring to Table III, leaf, bark, wood and root tissue fluids of sandal grown in combination with *Ruta graveolens*, *Murraya kœnigii*, *Melia azadirachta* and *Toddalia aculeata* as hosts are more buffered than those in combination with *Acacia farnesiana*. As the previous four hosts are known to impart relative immunity to sandal, while the last one renders it susceptible, it may be concluded that sandal, nourished by host plants imparting disease resistance, is more buffered than the one fed on a host rendering it susceptible, indicating that buffering capacity is a factor in controlling disease. In support of this view appears also the low buffering capacity (Table III) of the tissue fluids of leaf and bark of sandal grown without a host, which is known to easily succumb to the disease. Further, Sreenivasa Rau (*J. Indian Inst. Sci.*, 1933, **16A**, 167) observed that of the sandal plants nourished by *Pongamia glabra* and *Acacia farnesiana*, the one more buffered (*Acacia farnesiana*) is less susceptible to spike disease than the one less buffered (*Pongamia glabra*).

While there appears to be thus a significant correlation between disease resistance and buffering capacities of the tissue fluids of sandal grown with the different host plants, the possibility of other factors like the associated glucosides and alkaloids controlling resistance in sandal should not be ignored.

SUMMARY.

1. Hydrogen-ion concentration and buffering capacities of the tissue fluids of sandal, both healthy and spiked, have been studied. Healthy sandal included sandal grown with different host combinations.

2. The initial acidity and buffering capacities of the spiked tissue fluids are higher than those of healthy.

3. The following is the gradient in reaction in decreasing order, irrespective of host combinations or physiological condition of sandal plant—leaf, bark, wood and root.

4. Tissue fluids of sandal grown in combination with *Ruta graveolens*, *Murraya kœnigii*, *Melia azadirachta* and *Toddalia aculeata*—hosts imparting relative immunity to sandal—are more buffered than tissue fluids from sandal nourished on *Acacia farnesiana*, or sandal without a host, both of which are known to render sandal particularly susceptible to disease.

The possible relationships, arising out of these observations, between buffering capacity and disease resistance are discussed.

Our thanks are due to Dr. V. Subrahmanyan for the interest he has taken in this investigation.

Department of Biochemistry,
Indian Institute of Science,
Bangalore.

[Accepted 8–9–1934.]

1579-34.—Printed at The Bangalore Press, Mysore Road, Bangalore City, by T. Subramania Aiyar, Superintendent.