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

Part VI.—Nitrogen Metabolism in Healthy and Spiked Sandal Leaves.

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The nitrogen metabolism of sandal is one of fundamental interest, both physiological and pathological. Pot-culture experiments (unpublished) have shown the indispensability of providing hosts, more especially leguminous, for the healthy and vigorous growth of sandal. Without a host, sandal continues to have a struggling existence and one of the essential limiting factors appears to be nitrogen. When a suitable host is given to the starving sandal, the pale yellow leaves characteristic of nitrogen deficiency gradually become deep green, and the sandal plant as a whole begins to show visible growth.

Analyses have proved that with the onset of spike the total nitrogen of diseased tissues, whether leaves, stem or root is generally higher than that of the corresponding tissues of healthy sandal. We have seen that the vigorously growing sandal draws a large proportion of nitrogen from its host plant and it is curious to find that there is a further increase in the total nitrogen in the diseased state.

Two problems arise in this connection :—(*a*) The ultimate source of the extra nitrogen accumulating in pathological sandal tissues, and (*b*) The nature of the nitrogenous constituents in the diseased tissues. The present communication is a comparative study of the various forms of nitrogen in the healthy and the diseased leaves of sandal.

MATERIAL.

The leaves employed in this investigation were collected from two areas, Uttarahalli and Ragihalli, the former lying 6 miles south-west of Bangalore and the latter lying 13 miles south of Bangalore : the samples were collected at about 9 a.m. The characteristic external symptoms were the guiding factors in the collection of the diseased and healthy samples. The healthy leaves measured on an average 6·1 cm. in length and 2·6 cm. in breadth, the diseased leaves being 2·4 cm. long and 0·5 cm. broad. The samples were conveyed

in stoppered bottles by motor to the laboratory immediately after collection. The leaves were separated from twigs, spread on trays, and rapidly dried in a vacuum oven under reduced pressure, over fused calcium chloride between 35° and 40°. These precautions were taken to minimise as far as possible the biochemical changes *in vitro* affecting the various nitrogenous constituents. The samples, after 48 hours' drying, were ground in a mortar, passed through a 30-mesh sieve and preserved in covered jars.

Moisture was estimated by drying in a steam oven at 95° to constant weight. At each weighing of a sample for analysis, a re-determination of moisture was necessary, the leaves having been highly desiccated during the above process of drying; these moisture-values have been employed in calculating results on the moisture-free basis.

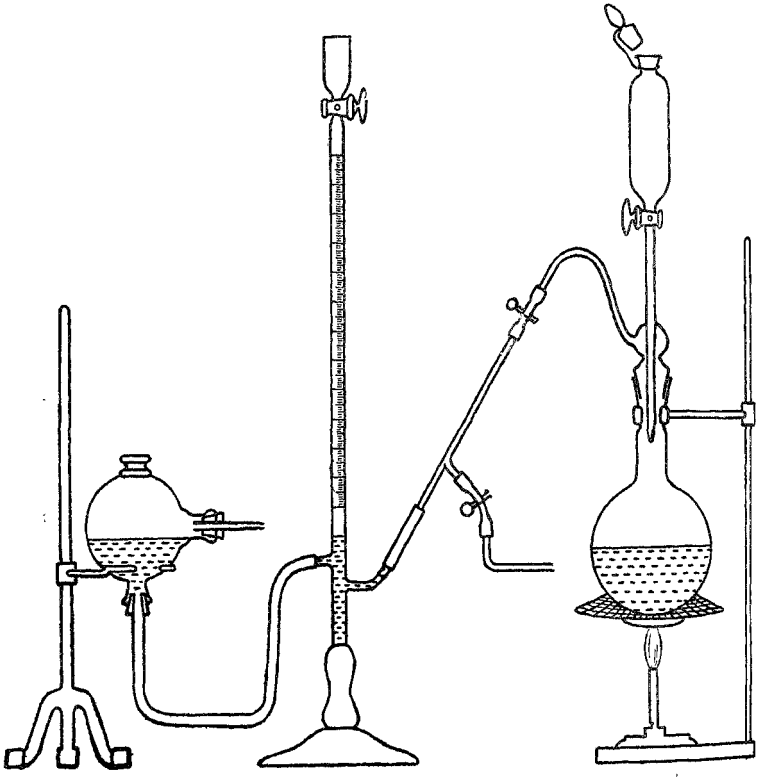
The two sets of samples employed for the investigation were so chosen as to represent extreme types. The Ragihalli samples represent the usual type, the healthy leaves possessing a lower nitrogen value, while in the other set of samples from the Uttarahalli area, the healthy leaves have a higher percentage of total nitrogen than the spiked leaves.

METHODS.

Total Nitrogen.—Total nitrogen was determined by Gunning's modification of Kjeldhal's method to include nitrate nitrogen (A.O.A.C., Methods of Analysis). The data represented in Table I are the averages of two or more individual analyses.

Protein Nitrogen.—Protein nitrogen was estimated by a method proposed by Ritthausen and Heinrich (*Die Eiweisskörper der Getreidearten, Hulsenfrüchte und Olsamen*, p. 252; Bonn, 1872) and perfected by Stutzer (*J. Land.*, 1880, **28**, 103). The leaf powder (1 g.) was treated with 100 c.c. of water, heated to boiling and kept on the steam bath for about 10 minutes; 2 c.c. of a saturated potassium-alum solution was added followed by 30 c.c. of Stutzer's solution (corresponding to 0·45 g. of copper hydroxide), and the whole was well stirred. On cooling, the insoluble residue was filtered, washed with water, and the nitrogen estimated according to Kjeldahl's method. Nitrogen in the filtrate was also estimated as a check. The average values are recorded in Table IV.

Nitrate Nitrogen.—Nitrate nitrogen in the sandal leaves was estimated according to the method of Schulze and Tiemann (Abderhalden's *Biochemische Arbeitsmethoden*, 1912, 6312). The leaf powder (10 g.) was repeatedly extracted with 80 per cent. alcohol, of which



about 400 c.c. was used in all. The extract was made alkaline with lime and evaporated to dryness in vacuum at 40–45°, the residue being dissolved in hot water, transferred to a 1000 c.c. measuring flask, treated with lead acetate and made up to 1000 c.c. After the precipitate had settled, the solution was decanted into a filter and 200 c.c. portions of the perfectly clear filtrate used each time for the estimation of nitrate nitrogen. The apparatus (see figure) included a 250 c.c. ground stoppered wash bottle in which the long tube was cut about 6 cm. below the neck and drawn to a capillary opening, a 50 c.c. separating funnel being fused on to the other end. The delivery tube was connected to a nitrometer through a T-piece by means of thick-walled rubber tubing; pinchcocks were used at the places indicated. The joint at the neck of the reaction flask escapes attack by the concentrated hydrochloric acid, and was ground with emery to make it perfectly air-tight; the length of the ground contact being about 5 cm., the pressure developed by steam during manipulation does not push up the stopper. The nitrometer was filled with 50 per cent. caustic alkali. The solution to be analysed for nitrate was concentrated in the flask to about 25 c.c., air in the apparatus being thus displaced by steam. After cooling, 25 c.c. of ferrous chloride solution (prepared by treating 400 g. of iron nails with a litre of concentrated hydrochloric acid) followed by 25 c.c. of concentrated hydrochloric acid was sucked through the separating funnel. The liberated nitric oxide was transferred to the nitrometer by alternate boiling and cooling of the mixture in the flask. The volume of nitric oxide collected in the nitrometer was measured at room temperature and pressure. Blank determinations were made with reagents alone. The blank value which came up to 0·05 c.c. after correcting the volume to N.T.P. was subtracted from the N.T.P. value of the nitrometer reading, and this figure was taken to represent the actual volume of nitric oxide evolved. The nitrate content in 0·2 per cent. potassium nitrate solution was also estimated, the results agreeing within 1 per cent. of the theoretical value.

The Ragihalli samples contained very small quantities of nitrate nitrogen, and accordingly the colorimetric method of estimation was used. The clear filtrate prepared as above (100 c.c.) was evaporated to dryness in a porcelain dish, treated with 1 c.c. of phenoldisulphonic acid, leached with water and then transferred to a Nessler's tube; 1 c.c. of 4 *N* potassium hydroxide was then added, and the volume made up to 50 c.c. The yellow tint was compared with that produced by a standard solution of potassium nitrate containing 0·01 mg. of nitrate nitrogen per c.c. of the solution. The data represented in Table IV (series 3) are the mean of more than one individual determination.

Nitrite Nitrogen.—The clear solution prepared for the nitrate determination (50 c.c.) was placed in a Nessler's tube to which was

added 1 c.c. of 1 per cent. sulphanilic acid and 1 c.c. of α-napthylamine acetate. The volume was made up to 100 c.c. and the pink colour compared with that produced by a standard solution of sodium nitrite containing 0·01 mg. of nitrite nitrogen per c.c. The results are represented in Table IV (series 4).

Ammonia Nitrogen.—Ammonia nitrogen in the sandal leaves was estimated according to Grafe's method (Grafe and Erich, *Zeits. Physiol. Chem.*, 1906, **48**, 300). The leaf powder (10 g.) was placed in a 500 c.c. Claisen flask and washed down with 25 c.c. of ammonia-free distilled water, 25 c.c. of saturated sodium chloride solution and 12·5 c.c. of alcohol. The flask was then connected to a one litre filter-flask containing 40 c.c. of 0·05 *N* sulphuric acid to absorb the ammonia, and cooled in ice: saturated sodium carbonate solution (12·5 c.c.) was then added and the mixture distilled under reduced pressure (15 mm.) on a water bath at 42–44°. After 6 hours of distillation, the acid in the filter flask was back titrated against standard alkali. The ammonia found by this method was taken to represent the free ammonia in the aqueous leaf extract, and the values are represented in Table IV (series 6).

Water-soluble Nitrogen.—The determination of the distribution of water-soluble nitrogen was made according to Hausmann's method (Hausmann and Walther, *Zeits. Physiol. Chem.*, 1899, **27**, 95; Osborne and Harris, *J. Amer. Chem. Soc.*, 1903, **25**, 323) and as applied to the spinach materials by Jodidi and co-workers (Jodidi, Kellogg and True, *J. Agric. Research*, 1918, **15**, 385). The method of extracting the water-soluble nitrogen as well as the methods of determining the various nitrogenous constituents (amide, humin, basic, etc.,) were strictly those detailed in the above paper, and a description of the methods will therefore not be attempted here. The amino-nitrogen in the water-soluble extract, however, as well as the mono-amino-nitrogen and the peptide nitrogen were determined according to the method of Van Slyke (*J. Biol. Chem.*, 1911, **9**, 185 and 1912, **12**, 275).

The results obtained by these methods are summarised in Table II, and are expressed as percentages on the weight of the moisture-free material. Table III expresses the same results as percentages of the total water-soluble nitrogen. Column I (Table II) gives the total water-soluble nitrogen as obtained by the digestion of 20 c.c. of the water-extract with sulphuric acid according to the method of Kjeldahl; and therefore the value of the total water-soluble nitrogen is possibly less by the amount of nitrate and nitrite nitrogen. Column 6 (Table II) gives the results of the mono-amino-nitrogen determinations made

according to the method of Van Slyke on 10 c.c. aliquots of the 100 c.c. of non-basic extract. The filtrate, after removing the phosphotungstic acid precipitate of basic nitrogen, was freed from excess of phosphotungstic acid by means of baryta, excess of which was removed by means of carbon dioxide. The filtrate was then concentrated, made up to 100 c.c. and was characterised as the non-basic extract. Column 7 (Table II) gives the difference between the total non-basic nitrogen (as determined by the Kjeldahl's method on 20 c.c. portions of the non-basic extract) and the mono-amino-nitrogen in column 6: and this is characterised as the non-amino-nitrogen in the non-basic extract. The difference between the amino-nitrogen found in 10 c.c. of the 100 c.c. of peptide extract and in a similar aliquot of the water-soluble extract gives the peptide nitrogen; the values are recorded in column 8 (Table II). The peptide extract was obtained by adding concentrated hydrochloric acid (20 per cent.) to 100 c.c. of the water extract and then hydrolysing the mixture for 8 hours. After removing the amide and humin nitrogen, the filtrate after concentration was made up to 100 c.c. and represented the peptide extract.

TABLE I.

Percentage total nitrogen in the sandal leaves on the moisture-free basis.

No.	Sample	Healthy	Diseased
1	Ragihalli, 15-8-28 ...	1·733	2·052
2	Uttarahalli, 12-9-28 ...	3·318	3·030
3	Ragihalli, 15-2-29 ...	1·416	2·261

DISCUSSION OF RESULTS.

Table I shows that in the three sets of samples analysed, the total nitrogen of diseased leaves from Ragihalli is higher than that of the healthy leaves from the same area. The healthy leaves from the Uttarahalli area have a higher percentage of total nitrogen, obviously due to the association of a richly leguminous host with the sandal from which the samples were derived. This is an observation which has been confirmed by numerous analyses and is one of vital importance in the choice of samples for analysis. Uttarahalli samples always show a higher nitrogen content than the Ragihalli samples, which, as already shown, is attributable to the fact that, in the Uttarahalli area,

TABLE II.

Nitrogen distribution in the water-soluble portion expressed in percentages of the oven-dried sandal leaves (on the moisture-free basis).

No.	Material and date of collection	Treatment HCl per cent.	1 Total water-sol. N	2 Ammonia N	3 Amide N	4 Humin N	5 Basic N	6 Mono-amino N	7 Non-amino in non-basic extract	8 Peptide N
1	Uttarahalli, 12-9-28 Healthy ...	20 for ½ hr.	0·935	0·013	0·073	0·121	0·236	0·163	0·467	...
2	,, ,, ,, ...	4 for 2 hrs.	0·935	0·013	?	0·118	0·214	0·110	0·501	...
3	,, ,, ,, ...	20 for 8 hrs.	0·935	0·013	0·066	0·114	...	...	..	0·008
4	,, ,, Diseased ...	20 for ½ hr.	1·167	0·015	0·033	0·160	0·291	0·158	0·520	...
5	,, ,, ,, ...	4 for 2 hrs.	1·167	0·015	0·030	0·167	0·306	0·111	0·586	...
6	,, ,, ,, ...	20 for 8 hrs.	1·167	0·015	0·043	0·167	...	..	...	0·000
7	Ragihalli, 15-2-29 Healthy ...	20 for ½ hr.	0·460	0·005	0·022	0·211	0·048	0·043	0·124	...
8	,, ,, ,, ...	4 for 2 hrs.	0·460	0·005	0·020	0·166	0·046	0·042	0·177	...
9	,, ,, ,, ...	20 for 8 hrs.	0·460	0·005	0·021	0·209	...	...	...	0·002
10	,, ,, Diseased ...	20 for ½ hr.	0·988	0·009	0·057	0·200	0·172	0·044	0·521	...
11	,, ,, ,, ...	4 for 2 hrs.	0·988	0·009	0·055	0·177	0·179	0·034	0·593	...
12	,, ,, ,, ...	20 for 8 hrs.	0·988	0·009	0·056	0·209	...	...	...	0·000

TABLE III.

Distribution of water-soluble nitrogen expressed in percentage of the total soluble nitrogen.

No.	Material and date of collection	Treatment : HCl per cent.	1	2	3	4	5	6	7	8
			Total water-sol. N	Ammonia N	Amide N	Humin N	Basic N	Mono-amino N	Non-amino in non-basic extract	Peptide N
1	Uttarahalli, 12-9-28. Healthy ...	20 for ½ hr.	100	1·390	7·81	12·9	25·2	17·4	49·9	...
2	,, ,, ,,	4 for 2 hrs.	100	1·390	?	12·6	22·9	11·8	53 6	...
3	,, ,, ,,	20 for 8 hrs.	100	1·390	7·06	12·2	..	...	...	0·856
4	,, ,, Diseased.	20 for ½ hr.	100	1·290	2·83	13·7	24·9	13·5	44·6	...
5	,, ,, ,,	4 for 2 hrs.	100	1·290	2·57	14·3	26·2	9·51	50·2	...
6	,, ,, ,,	20 for 8 hrs.	100	1·290	3·68	14·3	...	..	..	0 000
7	Ragihalli, 15-2-29. Healthy ...	20 for ½ hr.	100	1·090	4·78	45 9	10·4	9·35	27·0	...
8	,, ,, ,,	4 for 2 hrs.	100	1·090	4·35	36·1	10·0	9·13	38·5	...
9	,, ,, ,,	20 for 8 hrs.	100	1·090	4·57	45·4	...	...	...	0·435
10	,, ,, Diseased.	20 for ½ hr.	100	0·911	5·77	20·2	17·4	4·45	52·7	...
11	,, ,, ,,	4 for 2 hrs.	100	0·911	5·57	17·9	18·1	3·44	60·0	...
12	,, ,, ,,	20 for 8 hrs.	100	0·911	5·67	21·2	...	...	...	0·000

TABLE IV.

Nitrogen distribution in the sandal leaves.

No.	Form of nitrogen	Uttarahalli Samples, 12-9-28				Ragihalli Samples, 15-2-1929			
		Percentage of the oven dry material (on the moisture-free basis)		Percentage of total nitrogen		Percentage of the oven dry material (on the moisture-free basis)		Percentage of total nitrogen	
		Healthy	Diseased	Healthy	Diseased	Healthy	Diseased	Healthy	Diseased
1	Total ..	3·318	3·030	100	100	1·416	2·261	100	100
2	Protein ...	2·038	1·690	61·42	55·78	0·981	1·235	69·28	55·89
3	Nitrate ...	0·234	0·056	7·053	1·848	0·004	0·001	0·283	0·044
4	Nitrite ...	0·003	0·001	0·090	0·035	0·0001	0·000	0·007	0·000
5	Total water-soluble ...	0·935	1·167	28·18	38·52	0·460	0·988	32·49	43·70
6	Ammonia ...	0·013	0·015	0·392	0·495	0·005	0·009	0·353	0·358
7	Amide ...	0·070	0·032	2·11	1·056	0·021	0·056	1·483	2 477
8	Total amino ...	0·275	0·377	8·288	12·44	0·076	0·143	5·367	6·325
9	Humin ...	0·118	0·165	3·556	5·446	0·210	0·205	14·830	9·067
10	Basic ...	0·225	0·299	6·781	9·868	0·047	0·176	3·319	7·784
11	Peptide ...	0·008	0·000	0·241	0·000	0·002	0·000	0·141	0·000

sandals are to be found in the midst of luxuriant and leguminous hosts aided by cultivation. The Ragihalli sandals do not have these advantages, the soil is barren and rocky, leguminous hosts are rare, and there is no cultivation. It is pertinent to reiterate that the composition of the parasitic sandal will vary with the nature of the associated host-plants and that the results of one area cannot be compared with those of another whose ecological environment is widely different.

However, the higher nitrogen-content generally found in the diseased tissues of sandal is in strange contrast with the usually lower percentages of nitrogen found in the diseased tissues of spinach blight, tobacco mosaic, curly top of sugar-beet, etc. The accumulation of the extra nitrogen in the leaves with the onset of spike disease is possibly due to one of the following factors:—(1) Abnormal distribution of the constituents due to unbalanced translocation, (2) Selective absorption of nitrogenous components from the host or soil or both by the sandal in its pathological state, and (3) The infective principle responsible for causing the disease being possibly a nitrogen-fixer. ?

An examination of Table IV (series 2) reveals that the protein content (as determined by Stutzer's method) is higher in the healthy than in the diseased leaves for both the areas when calculated as percentage of the total nitrogen. There seems to be a remarkable tendency in the plant to maintain an equilibrium between the total nitrogen and the protein nitrogen, an increase in the total nitrogen being followed by a corresponding increase in the protein nitrogen. In the diseased leaves also, the same phenomenon is noticeable, although the ratio of protein nitrogen to total nitrogen is less than that in the case of the healthy plants, due probably to the degradation of the proteins. The higher proteoclastic activity exhibited by the diseased tissues and the higher content of the total water-soluble nitrogen also testify to the above assumption. This higher content of the water-soluble nitrogen and the ratio of the protein to the water-soluble nitrogen given below, may well be marked as characteristics of the disease.

Ratio of the protein to the water-soluble nitrogen of leaves.

		Healthy	Spiked
Uttarahalli	...	2·18	1·44
Ragihalli	...	2·13	1·19

Reviewing the distribution of water-soluble nitrogen, we find that the humin nitrogen calculated as percentage of the dry-weight material (Table III, column 4) is not significant.

The nitrate-content of spiked leaves is strikingly low and possibly points to the poor absorption of the nitrates from the host or the soil, in view of the fact that in the diseased condition sandal loses its haustorial connections and root-ends.

Basic nitrogen is present in greater quantities in the diseased than in healthy samples for both areas, when calculated both in terms of water-soluble nitrogen and of moisture-free material. This seems to be a distinguishing characteristic of the disease.

Examination of Table II (column 6) shows that the value of mono-amino-nitrogen fluctuates according to the concentration of hydrochloric acid used in hydrolysing the water-soluble extract. The solution treated with 4 per cent. hydrochloric acid for 2 hours contains always a lower percentage of mono-amino-nitrogen than that treated with 20 per cent. hydrochloric acid for ½ hour. This fact goes hand in hand with the observation that when the solution is treated with 4 per cent. hydrochloric acid for 2 hours there is present in the non-basic portion a greater quantity of non-amino-nitrogen than when the solution is treated with 20 per cent. hydrochloric acid for ½ hour. The values represented in Table II (column 6) cannot, however, be taken to represent the true amount of mono-amino-nitrogen in the samples, because it has been proved that a part at least of the humin nitrogen is formed at the expense of the mono-amino-acids while boiling with hydrochloric acid (Hart and Bentley, *J. Biol. Chem.*, 1915, **22**, 477; Roxas, *J. Biol. Chem.*, 1916, **27**, 71).

An examination of Table IV reveals that the total amino-nitrogen is greater in the spiked than in the diseased plant for both areas, whether expressed as percentage of the dry-weight material or as percentage of the total nitrogen. This increase in amino-nitrogen seems to be another characteristic of the disease, indicating proteoclastic degradation of proteins in the leaf.

SUMMARY.

1. The total nitrogen in spiked leaves is generally greater than in healthy leaves, and the total nitrogen-content varies with the locality.

2. As physiological characteristics of the disease there may be put forward an increase in (1) total water-soluble nitrogen, (2) basic nitrogen, and (3) total amino-nitrogen, and a decrease in the nitrate nitrogen in the diseased leaves, both in reference to the dry-weight of the leaves and in reference to the total nitrogen.

3. The lower content of protein nitrogen (with reference to the total nitrogen) taken along with the lower content of the polypeptide nitrogen and increase in amino- and water-soluble nitrogen seems strongly to indicate a proteoclastic degradation of the proteins proceeding in the diseased leaves.

4. A detailed investigation of the basic constituents has been undertaken and the work will be extended to an examination of the stems and roots of sandal.

In conclusion, we wish to express our grateful thanks to Professor Roland V. Norris for his many helpful criticisms and the keen interest he has evinced throughout this work.

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