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著者	FUJIWARA Akio, TSUTSUMI Michio
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BIOCHEMICAL STUDIES OF MICROELEMENTS IN GREEN PLANTS

III. ON THE COMPOSITION OF NITROGENOUS CONSTITUENTS OF THE ZINC DEFICIENT BARLEY LEAVES AND THE MATERIAL EXUDATED FROM THEM

By

Akio FUJIWARA and Michio TSUTSUMI

Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University, Sendai, Japan

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It has been reported (1) that the barley plants grown on the cultural solution deficient in zinc but with ammonium salts added together with nitrates as the nitrogen source showed retardation in growth and a white and granular material about 0.5—1.0 mm in diameter was observed to be exudated from their leaves new and old at the tip of the blade. This material seemed to be water-soluble and exudated accompaning to nocturnal guttation, since it emerged after the evaporation of gutta. The similar phenomenon has subsequently been observed in the barley plants deficient in sulphur, magnesium, and iron respectively and sometimes in the normal plants also, though in these plants the exudation was not so intensive as in the case of zinc deficiency. In the case of manganese, copper, and molybdenum deficiency, the barley plants did not exhibit such phenomenon at all. Therefore, it may be regarded as a characteristic of zinc deficient plants.

A similar phenomenon was reported by Greenhill and Chibnall (3), who described that under certain conditions the perenial rye-grass when treated with ammonium sulphate produced a white exudation on the upper half of the blades which consisted almost entirely of glutamine. The problem arises as to what sorts of substances are the constituents of this material. This phenomenon may perhaps be caused by the disturbance of metabolism within the plants due to the deficiency of zinc. Therefore, it is considered that the elucidation of the chemical nature of this material may provide a key to the function of zinc within the plants. For this reason the material was collected and chemically examined. The results obtained revealed it to agree partially with that described by Greenhill and Chibnall, and glutamine was shown to be a dominant constituent, but contrary to their finding the coexistence of asparagine and certain amino-acids was also recognized. Since the status of nitrogenous constituents of the leaf-tissue itself which was exudating such material raised interest, the estimation of them was also carried out.

Materials and Methods

The two-week old barley seedlings which were brought up on distilled water were transplanted on the zinc defcient cultural solution of the following composition; $(NH_4)_2SO_4$, 0,0012M; $NH_4H_2PO_4$, 0,0006M; KNO_3, 0,0015M; $Ca(NO_3)_2$, 0,00075M; $MgSO_4$, 0,001M; B, 0,5 ppm; Fe, 2 ppm; Mn, 0,5 ppm; Cu, 0,02 ppm; Mo, 0,01 ppm; Thus it contained an equivalent amount of ammonium- and nitrate-nitrogen. In addition 0,05 ppm of Zn was given to the solution for the complete nutrition as a control. They were grown in a green-house under the natural condition of autumn.

The exudate of zinc deficient barley leaves was taken every day during the period of intensive exudation which begun on about the 20th day after the transplantation and continued for several weeks. All exudate-granules taken were collected into a mass and mixed, then used for the chemical examinations. The sample of leaf-tissue was taken after five weeks of culture, when the zinc-deficient plants were exudating the material yet intensively. One portion of the fresh tissue was immediately macerated in a mortar and pestle after washing with water and suspended into 80 percent ethanol over night. This ethanol-extract was condensed *in vacuo* after filtration and used for the detection of free amino acids in the leaf-tissue by means of paperchromatography. The other portion was dried in an oven at about 70°C and ground, then used for the fractionation of nitrogenous constituents.

The paperchromatography of amino acids in the exudate and in the leaf-tissue-extract was made on the filter-paper of Toyo-Roshi No. 50 by the two-dimensional ascending method. The solvent used was first 80 percent phenol then the mixture of water, acetic acid, and n-butanol in 2:1:4. Spots of amino acids were developed with ninhydrine.

In order to estimate the nitrogenous fractions, the sample was first suspended in the sodium-tungstate solution and allowed to stand overnight then filtered. The nitrogen-content of the residue was determined as protein-nitrogen by the Kjeldahl method, and the filtrate was further fractionted into amino-, amide-, nitrate-, ammonium-, and residual undetermined nitrogen according to Schlenker's method.

Results

The exudate of the zinc deficient barley was not taken completely, so the exact yield of it remained unknown, but it approximate several miligrams per plant in total throughout the sampling period. This material was found to be dissolved rather readily in water.

In view of the resemblance of the phenomenon to that observed by Greenhill and Chibnall, the exudate was first tested with ninhydrine for the possiblity of glutamine. The positive reaction was observed. Consequently, the paperchromatography was carried out and it revealed especially large ninhidrine-positive spots corresponding to glutamine and asparagine respectively together with several other spots indicating either amino acids or peptides. For the purpose to ascertain these two amides more decisively, the exudate was boiled with 0,6N hydrochloric acid for two hours to be hydrolysed and the hydrolysate was again chromatographed. On the other hand, to see whether peptides were present, the exudate was dissolved with 1:1 hydrochloric acid and enclosed in a glass-tube, then hydrolysed at 105°C over night and also chromatographed. Both chromatograms showed a pattern identical with each other. Moreover, they revealed no difference from that of the original exudate except that the spots corresponding to glutamine and asparagine shown in the latter disappeared and instead of them the spots corresponding to glutamic acid and aspartic acid became striking in the formers.

The same procedures were applied to the leaf-tissue-extract. The species of amino acids including amides thus detected are shown in Table 1. From these results, it is found that the species of amino acids present in the exudate coincide with those in the extract of zinc deficient leaves and furthermore

Table 1. Free amino acids detected in normal and zinc deficient barley leaves and exudate from the latter.

Amino acid	Normal leaves	Deficient leaves	Exudate
Alanine	+	+	+
Arginine	(+)	(+)	(+)
Asparagine	+	++	+
Aspartic acid	+	+	+
Cysteine	−	−	−
Cystine	−	−	−
Glutamic acid	+	+	+
Glutamine	+	+++	+++
Glycine	(+)	(+)	(+)
Histidine	−	−	−
Hydroxyproline	−	−	−
Leucines	(+)	(+)	(+)
Lysine	(+)	(+)	(+)
Methionine	(+)	(+)	(+)
Phenylalanine	+	+	+
Proline	+	+	+
Serine	+	+	+
Threonine	(+)	(+)	(+)
Tyrosine	−	−	−
Tryptophan	−	−	−
Valine	(+)	(+)	(+)

+ : Detected, − : Undetected, (+) : Unidentified they are identical with those in the normal leaves' extract.

That the hydrolysate of exudate with 1:1 hydrochloric acid gave the same chromatogram as that of the original exudate except for the disappearance of two amides in the former suggests the absence of peptides in the latter. This was confirmed through the lacking of biuret reaction with it.

The results obtained by fractionation of nitrogenous constituents within the leaf-tissue and also exudate are given in Table 2. In the deficient plants,

Table 2. The composition of nitrogenous constituents in normal and zinc deficient barley leaves and exudate from the latter.

Sample	Total-N	Protein-N	Amino-N	Amide-N	NH_4-N	NO_3-N	Residual-N
Normal leaves	3.43	4.52	0.12	0.18	0.008	0.10	0.50
Deficient 〃	5.06	1.61	0.56	1.86	0.188	0.09	0.75
Exudate	10.2	—	3.3	6.9	—	—	—

the total-nitrogen had abnormally high value and contrary the protein level was strikingly low. This status can be regarded to accord with that found by Hoagland (4) and by Steinberg (6) in other plants. Consequently the total soluble nitrogen content of deficient leaves was very high. Among its fractions, the concentration of ammonium-nitrogen relative to that in the normal was highest and amide-nitrogen was next, amino-nitrogen was also represented by a fairly high value.

The fractionation of nitrogenous constituents of exudate revealed that about one half of the total nitrogen was made up by amide-nitrogen and the other half by amino-nitrogen. The ash content of exudate was about 13 percent and the sugar content did not exceed 1 percent, although the Molisch reaction was shown to be positive.

Discussion

As regards the composition of nitrogenous constituents in zinc deficient barley leaves, the low level of protein and accumulation of soluble nitrogen can be considered to be characteristic. As a component of soluble nitrogen-fraction, amino acids were also markedly accumlated together with amides or ammonium, furthermore the species of them were entirely similar with those in normal plants as revealed dy chromatography. Therefore, the synthesis of amino acids in general appears not to be restricted even in the zinc deficient plants and it seems that the step which is restricted is the synthesis of protein from amino acids as pointed out by Hoagland (4). It is said that when particular amino acids which are essential units of protein are lacking, the synthesis of protein is limited even if the total amino acid-level is high. Tsui (7) on tomato, and Nason *et al* (5) on *neurospora*, respectively found that the activity of tryptophan-synthesizing enzyme dropped by the deficiency of zinc. Accordingly, although in both normal and deficient plants trytophan, which can be regarded as the essential unit of plant-protein, could not be detected as free amino acid paperchromatographycally, it is conceivable that the tryptophan synthesizing activity might be decreased in the zinc deficient barley leaves and consequently the protein-synthesis is restricted. However, Nason *et al* (5) described that since the profound decrease in protein concentration in mycelia of the zinc dificient *neurospora* was not changed by supplmenting it with a full complement of known amino acids, purines, pyrimidines, and vitamins, it could be seem that the basic defect in zinc deficiency lies not in the synthesis of these simple units, but in their subsequent metabolism. This concept should also be kept in mind for the case of barley plants.

The exudate was shown not to be consisted of an unusual substance but of the usual constituents of leaf tissue. Since these substances exist in abnormally high concentration within the zinc deficient leaves and the species coincide between the two, this exudation-phenomenon seems to result from the excess accumulation of these substances in the leaves. The accumulation of these substances may be raised in order to convert the excess ammonium, which was once absorbed but did not be utilized for building up to protein, to innocuous form by the deficient plants. When nitrates only were used as the nitrogen source, the zinc deficiency did not cause such exudation-phenomenon.

Although the soluble inorganic substance is contained in the exudate, this phenomenon does not appear to be due to a confused excretion of the soluble substance in the cell caused by, for instance, the damage of the cell membrane, for the sugar content is very little. If it is due to a confused excretion of all the soluble substances, the sugar content of the exudate could be expected to be higher, because the concentration of soluble sugar, of which the molecular weight matches that of the amides or amino acids, is much higher than the concentration of amide- and amino-nitrogen within the zinc deficient leaves.

In the case of sulphur, iron, and magnesium deficiencies the exudation of similar material was observed. In sulphur deficiency the synthesis of sulphur-containing amino acids is necessarily restricted and consequently the synthesis of protein also must be limited. In iron deficient barley leaves the amide content was shown to increase as already reported (2). Thus the chemical nature and presumably the mechanism of excretion may be similar with those in the case of zinc deficiency.

Summary

1. When the barley plants were grown on the cultural solution deficient

in zinc and containing an equivalent amount of ammonium- and nitrate-nitrogen, the white, granular material was observed to be exudated by then at the stip of the blade. This material was chemically examined and proved to be mainly composed of glutamine, asparagine, and certain amino acids, and to contain ash of about 13 percent.

2. The protein level of the zinc deficient leaves was markedly low but the concentration of the soluble nitrogen including amide- and amino-nitrogen was abnormally high. Therefore, it may be presumed that this accumulation of soluble nitrogen eaused the excretion of such material.

3. The species of amino acids present in the exudate, in the soluble nitrogen-fraction of the zinc deficient plants and in that of the normal plants were found to coincide among the three.

4. Since the sugar content of the exudate was very low it was suggested that this phenomenon was not due to a confused excretion of the soluble substance of the cell.

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