ACCUMULATION OF FREE AMINO ACIDS AS A CHEMICAL BASIS FOR MORPHOLOGICAL SYMPTOMS IN TOBACCO MANIFESTING FRENCHING AND MINERAL DEFICIENCY SYMPTOMS[1]

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Received October 8, 1949

Introduction

The chemical mechanism whereby plants respond to modifications in environment resulting in alterations in morphology has on the whole remained obscure. However, whatever the cause, it is clear that each change in environment may result in the appearance of a whole aggregate of symptoms characteristically specific for each condition. No change in environment can be said to cause only a single symptom. Though individual symptoms may to some extent resemble each other with various environmental variations, the aggregate of symptoms is usually characteristic of each particular environment and plant in question.

The work discussed herein consists of studies on the chemical mechanism of symptom production in frenching and mineral deficiency of tobacco. The well defined aggregate of symptoms characteristic of these particular environments afford very favorable material for a study of the chemical mechanisms underlying morphological change.

The condition of tobacco known as frenching is a well known deformity. Growth of the terminal bud of the plant slows or stops and the slowly expanding new leaves develop a network type of chlorosis. In an extreme form the newly developed leaves become strap shaped because of the failure of the lamina to expand. Growth of axillary buds then starts but results only in the formation of similarly strapped leaves. Stem elongation whether of the main axis or of axillary branches is greatly inhibited in an extreme case.

STEINBERG (**12**) found that diffusates from numerous bacterial strains ordinarily present in soil are capable of causing changes in morphology of tobacco seedlings grown in aseptic culture. These alterations were in some cases suggestive of, but were not identical with, frenching in the field. Other experiments with amino acids in aseptic culture (**11**) (**13**) revealed that slightly excessive quantities of these and other natural metabolic compounds each caused the production of specific symptom complexes in tobacco seedlings. The conclusion naturally drawn was that release of excessive quantities of free amino acids into the tissues was a probable causative chemical factor in the production of morphological symptoms in the plant.

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[1] Cooperative investigation by the Division of Tobacco, Medicinal and Special Crops, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, U. S. Department of Agriculture and the Maryland Agricultural Experiment Station.

Though none of the plants obtained with metabolites were identical with those showing frenching or mineral deficiency, they were, with respect to individual symptoms, often quite suggestive in appearance. Isoleucine particularly caused as close an approximation to frenching in the field as was to be expected in very young seedlings under the environmental conditions of flask culture in low intensity artificial light.

The suggestion (**11**) was therefore made that the mechanism of symptom production in mineral deficiencies might also depend on the same chemical mechanism as in frenching: accumulation of free amino acids. McMURTREY (**4**) has described the symptom-aggregates characteristic of mineral deficiencies in tobacco. However, while there are a great deal of analytical data on tobacco available, none are in a form suitable for deciding the accuracy of this assumption. Values for the individual free amino acids present in the normal and abnormal plants would be desirable.

It was considered probable, moreover, that if toxic concentrations of free amino acids are responsible in part or whole for the morphological alterations that appear, these would be reflected by a rise in total free amino acids in the plant. Since some of the amino acids are effective at dilutions far beyond the range of the Van Slyke method for determining a-amino nitrogen the accuracy of this assumption could only be determined by trial. There were some data available to bear out this belief, however, in the report of NIGHTINGALE, SCHERMERHORN, and ROBBINS (**9**) that sulphur deficiency in tomato caused an increase in amino acid nitrogen of the plant. RICHARDS and TEMPLEMAN (**10**) also found barley plants showing symptoms of phosphorus deficiency contained a "pronounced accumulation of amino nitrogen" as did those showing symptoms of potassium deficiency. On the other hand NIGHTINGALE, SCHERMERHORN, and ROBBINS (**8**) did not note any increase in amino nitrogen with potassium deficiency, nor did NIGHTINGALE, ADDOMS, ROBBINS, and SCHERMERHORN with calcium deficiency (**7**).

The work herein reported therefore consists of analytical determinations of free a-amino nitrogen in field plants showing symptoms of frenching, and mineral deficiencies of the following elements: K, Mg, Ca, P, B, and N. Growth experiments had already demonstrated the ability of externally applied natural metabolites to cause the appearance of manifold morphological changes. However this did not furnish definite proof of an excess of symptom-producing compounds in the plant tissues when the growth differences occurred.

Experimental methods

The tobacco plants used in the study of the effects of mineral deficiency on free amino acid content were grown at the Tobacco Experiment Farm at Upper Marlboro, Maryland. McMURTREY (**5**) has already described the experimental techniques, and the extent of the deficiencies attained. Samples from plants infected with tobacco mosaic were obtained from this locality also through the courtesy of Dr. E. E. Clayton. Samples of frenching soil and plants used in this work were obtained from the George Merrick and Deshield farms in Prince Georges County, Maryland.

Individual leaves (25–100) from as many plants as conditions would allow were selected in sampling. The midribs were removed and the lamina quickly dried in a forced draft cabinet at 60–65° C. In the selection of samples both the position of the leaf on the plant and the age of the plant were taken into consideration, the age being based on time of flowering. In preparation for analysis the oven dried material was ground to pass a 40-mesh sieve and preserved in tightly stoppered containers. The method outlined by GARNER, BACON, BOWLING, and BROWN (**2**) was used for the determination of amino acid nitrogen. A correction for the content of moisture and soil material in the prepared sample was applied to the analytical data.

In barest essentials the analytical method consisted of the coagulation and removal of protein nitrogen with dilute acetic acid and heat, removal of amide and ammonia nitrogen by hydrolysis with 1.08 N-H_2SO_4 followed by distillation with 10% NaOH and determination of a-amino nitrogen by the Van Slyke nitrite method. The value for a-amino nitrogen in the residual solution so obtained corresponds to the number of amino acids present except that lysine gives a two-fold value, and proline and hydroxyproline fail to react.

Total a-amino nitrogen was determined in a similar manner except that 6.0 N-H_2SO_4 was used for hydrolysis instead of 1.08 N-H_2SO_4 in order to hydrolyze peptide, peptone, and proteose (**17**). Total a-amino nitrogen might also be called non-protein, a-amino nitrogen. Presumably it is an approximate measure of the sum total of units used in the synthesis and break-down of proteins present at the time of sampling.

FREE AMINO ACIDS IN FIELD PLANTS SHOWING SYMPTOMS OF FRENCHING OR MINERAL DEFICIENCY

The analytical data for field plants has been arranged according to the crop year. The samples dated as 1936, 1940, and 1945 (table I) were obtained for the study of other factors than those here under consideration (**1**), and prior to the time this study was begun. It will be noted that of the samples collected in 1936 and 1940, the plants were grown with a wide range in quantity of a potash fertilizer application. Definite symptoms of potash deficiency were noted where no potash was applied. Symptoms in 1940 were apparently more severe than in 1936 but seem to have been confined to the leaf tips and adjacent margins. Total free amino acid nitrogen did not vary from the controls in either year according to these data—a result which will be discussed in connection with the figures for later analyses.

The other values afford a comparison of normal and slightly frenched plants grown in 1945. It is seen that the leaves of frenched plants contained approximately double the free a-amino acid content of that in normal plants. Amide and ammonia nitrogen varied in a similar manner. Through the courtesy of Dr. J. L. Stokes of Merck & Co., Rahway, N. J., it was possible to have the natural isomer of isoleucine estimated with *Streptococcus faecalis* (**15**). The isoleucine content, it can be computed, increased in the frenched plants by 50.0% in one case and by 49.6% in the other. It has been estimated that this is equivalent to an increase in the isoleucine content of 100 to 150 p.p.m., or about the same concentration of natural isoleucine (100 p.p.m.) in aseptic agar culture required to cause a similar morphological alteration artificially. The interpretation of the analogous increase in amide and ammonia of frenched plants requires additional data.

TABLE I

THE FREE A-AMINO NITROGEN CONTENT, IN DRY LEAF LAMINA OF MARYLAND MEDIUM BROADLEAF TOBACCO GROWN AT UPPER MARLBORO, MARYLAND, AS AFFECTED BY FRENCHING AND MINERAL DEFICIENCIES AT TIME OF FLOWERING*

CROP YEAR	TREATMENT	APPEARANCE OF PLANTS	NITROGEN CONSTITUENTS IN MOISTURE-FREE LEAF LAMINA			
			FREE a-AMINO NITROGEN			
			CONTENT	RELATIVE CONTENT	AMIDE & AMMONIA NITROGEN	FREE 1(+) – iso LEUCINE
			%	%	%	%
1936	No potash added	Chlorotic leaf tips	0.108	125.6		
	Potash at 24 lbs. per acre	Slight leaf cupping	0.110	127.9		
	Potash at 120 lbs. per acre	Normal	0.086	100.0		
* 1940	No potash added	Chlorotic tips, dried				
	Potash at 30 lbs. per acre	margins	0.094	94.0		
		Leaf tip chlorosis				
	Potash at 240 lbs. per acre		0.093	93.0		
		Normal				
* 1945	No potash added		0.100	100.0		
	No potash added	Normal	0.098	100.0	0.053	0.034
		Frenched, few strapped leaves	0.188	191.8	0.112	0.051
* 1945	No fertilizer added	Normal	0.095	100.0	0.057	0.034
	No fertilizer added	Frenched, few strapped leaves	0.210	221.1	0.115	0.050

* Samples were taken from plants at same stage of maturity when possible; the third and fourth leaves from top being selected. The greater part of the frenched material consisted of leaves showing reticular chlorosis and little or no strapping. Both normal and frenched plants were in the same plots: In the "no potash" plot slightly less than half the plants showed symptoms of frenching, and in the "no fertilizer" plot about 65% of the plants.

The material obtained in 1946 (table II) falls under four headings. Frenching again caused a significant rise in free a-amino nitrogen amounting to 53.1%. The frenched and normal plants were frequently growing side by side. The severely strapped leaves could not be used because of

* See Errata,
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absence of leaf lamina. Mosaic disease, however, seemed to have little influence on the content of free a-amino nitrogen; though other strains of virus characterized by leaf strapping may give a different response. The controls were separated from the diseased plants by a 36-foot fallow plot.

The results with mineral deficiencies seemed definite and conclusive. A deficiency in potassium, magnesium, calcium, phosphorus, nitrogen, or boron brought about marked accumulations of free a-amino nitrogen in each instance. Potassium deficiency caused almost a 7-fold increase, magnesium almost a 4-fold increase and calcium a doubling in free a-amino nitrogen. In each of these cases the plants and the leaves analyzed showed extreme symptoms of deficiency. The moderate symptoms shown with phosphorus, and boron deficiency were accompanied with lesser increases in free a-amino nitrogen. It might be anticipated that a decrease in nitrogen supply would have the effect of minimizing any increase in free a-amino nitrogen, if it be assumed that accumulation is due to a block in protein breakdown as well.

TABLE II

THE FREE A-AMINO NITROGEN CONTENT IN DRY LEAF LAMINA OF MARYLAND MEDIUM BROADLEAF TOBACCO GROWN DURING 1946 AT UPPER MARLBORO, MARYLAND, AS AFFECTED BY FRENCHING AND MINERAL DEFICIENCIES

LOCATION OF PLOT OR DESIGNATION	TREATMENT	SYMPTOMS OF ABNORMALITY (SAMPLES TAKEN AUG. 13–22, 1946)	FREE a-AMINO NITROGEN	
			CONTENT	RELATIVE CONTENT
			%	%
Geo. Merricks Farm	Standard*	None	0.209	100.0
	"	Extreme frenching	0.320	153.1
Rotation Series	Standard	None	0.122	100.0
	"	Mosaic distinctly visible	0.127	104.1
Pure chemicals	Standard	None	0.098	100.0
	No B	Moderate boron deficiency	0.124	126.5
Fertilizer	Standard	None, but slight nitrogen deficiency	0.127	100.0
	No Ca	Extreme calcium deficiency	0.279	219.7
	No Mg	Extreme magnesium deficiency	0.486	382.6
	No N	Extreme nitrogen deficiency	0.167	131.5
	No P	Moderate phosphorus deficiency	0.188	148.0
	No K†	Extreme potassium deficiency	0.873	687.4

* These samples were prepared on August 13. Samples of dry soil collected on September 18 contained only a trace of amino nitrogen, whether the plant did or did not show symptoms of frenching.

† Total a-amino nitrogen was 0.949%.

Analyses of the material collected from the plants grown in 1947 (table III) gave results essentially identical with those of 1946. Marked increases in free a-amino nitrogen were found in each instance where a mineral deficiency caused the appearance of severe symptoms (magnesium, potassium). Moderate severity of deficiency symptoms were accompanied by a lesser accumulation of a-amino nitrogen (calcium, phosphorus). An insufficiency of nitrogen caused small decreases in a-amino nitrogen. Boron and sulphur

TABLE III

THE NON-PROTEIN FREE A-AMINO NITROGEN CONTENT IN DRY LEAF LAMINA OF MARYLAND MEDIUM BROADLEAF TOBACCO GROWN DURING 1947 AT UPPER MARLBORO, MARYLAND, AS AFFECTED BY FRENCHING AND MINERAL DEFICIENCIES

LOCATION OF PLOT OR DESIGNATION	TREATMENT	SYMPTOMS OF ABNORMALITY IN PLANTS AND ANALYTICAL SAMPLE	NITROGEN CONSTITUENTS IN MOISTURE-FREE LEAF LAMINA			
			FREE A-AMINO NITROGEN		TOTAL NON-PROTEIN A-AMINO NITROGEN	NON-PROTEIN COMBINED A-AMINO NITROGEN
			CONTENT	RELATIVE CONTENT		
			%	%	%	%
Fertilizer*	Standard	None	0.168	100.0	0.193	0.025
	No Ca	Moderate calcium deficiency in 75% of leaves sampled	0.311	185.1	0.356	0.045
	No Mg	Severe magnesium deficiency	0.561	333.9	0.617	0.056
	No K	Severe potassium deficiency	0.548	326.2	0.671	0.123
	No N	About 90% of leaves selected showed definite nitrogen deficiency	0.130	77.4	0.151	0.021
	No P	Small narrowed dark green leaves on small plants	0.244	145.2	0.298	0.054
	No B	Only 10% of leaves selected exhibited boron deficiency	0.163	97.0	0.188	0.025
	No Cl	None	0.211	125.6	0.275	0.064
	No S	Previous slight indication of sulphur deficiency	0.153	91.1	0.185	0.032
Merrick Farm*	Standard	None	0.432	100.0	0.492	0.060
	"	Moderate frenching	0.839	194.2	0.943	0.104
Deshield Farm†	Standard	Roots of normal plants	0.118	100.0	0.115	0
	"	Roots of severely frenched plants	0.130	110.2	0.158	0.028

* Middle leaves of plants were used for samples in the deficiency series and top leaves of the frenching plants from the Merrick farm.

† The roots collected at the Deshield farm for normal and frenched material were from plants showing frenched and normal suckers, respectively.

deficiencies were too slight to give any indication of an increase in amino acids. The influence of omission of chloride from the fertilizer mixture on a-amino nitrogen is of interest in view of its known effects (**3**) on drought resistance of tobacco and of leaf quality. Chlorine is not, so far as is known, an essential element in the nutrition of tobacco.

The quantities of a-amino nitrogen in plants showing symptoms of frenching and mineral deficiencies vary not only with the extent of the visible abnormalities in growth, but also with environmental conditions. Comparison of the percentages of free a-amino nitrogen in the controls of all the tables will reveal a variation between 0.053 and 0.432% with location and crop year.

The values for total non-protein, a-amino nitrogen parallel those for free amino nitrogen. They permit the computation by difference of non-protein combined a-amino nitrogen, which is relatively much smaller than the free a-amino nitrogen found. These values are presumed to include all non-protein a-amino nitrogen present in peptide, proteose, peptone, proline, and hydroxyproline. The nitrogen of the a-amino group of lysine, however, appears in the free a-amino nitrogen value.

The free a-amino nitrogen was approximately 76.7 to 100.0% of total non-protein a-amino nitrogen. The smallest value was given by plants where chloride was omitted from the fertilizer, the highest value with roots from normal plants. The other values can be readily computed. Their significance is not certain, since the variations were relatively small.

The last two rows deal with data obtained on the roots of frenched and normal plants. Only a slight difference in a-amino nitrogen (10.2%) was found. Greater weight should probably be assigned this difference than its magnitude would seem to demand, inasmuch as the normal plant was in process of forming frenched suckers and the frenched plant of becoming normal (suckers). It seems reasonable to assume that a comparison between better defined samples would show a definitely greater difference in free a-amino nitrogen.

Discussion

Further verification of the postulated free amino acid mechanism of symptom production in the tobacco plant is afforded to some extent by the analytical data on plants suffering from mineral deficiencies. In every case except perhaps boron, and nitrogen, the symptoms of mineral deficiency were paralleled by large abnormal increases in the free amino acids of the leaf tissues.

These exceptions may of course be due to the toxic accumulations of metabolites not containing a-amino nitrogen (13). Other explanations also exist however. One such is that the symptoms of boron deficiency were too mild and that too much normal tissue was included in the analytical samples. Plants with moderate minus boron symptoms (table II) gave an increase of 26.5% in free amino acid nitrogen even without rejection of more normal tissue from the analytical sample. A similar situation was encountered with minus potassium plants. Negative results were first obtained similarly with minus potassium plants since the analytical samples consisted of better than 90% normal leaf tissue.

An important contribution by WADLEIGH and SHIVE (**16**) presents evidence however that the cells of minus boron cotton plants are the seat of protein degenerations and increased acidities. An accumulation of sugar and ammonia was interpreted by them as due to a block in amino acid syntheses by the plants. This response would therefore seem to indicate too mild a boron deficiency as the most probable and important reason for the slight accumulation of excessive free amino acids with boron deficiency. The contrast in analytical results distinguishing calcium from boron deficiency actually obtained despite rather similar symptoms of deficiency appears striking. On the other hand boron deficiency symptoms originating through accumulation of the more toxic amino acids such as hydroxyproline (3 p.p.m.) would give no indication of any increase with the analytical methods employed.

The negative results with nitrogen deficiency are probably to be attributed to the prevention of amino acid synthesis. Symptoms of nitrogen deficiency may therefore possibly be due to loss of nitrogen from chlorophyll as well as accumulation of non-nitrogen metabolites in excess. No symptoms of sulphur deficiency were present in the plants at the time these were sampled for analysis.

Chlorosis accompanying a mineral deficiency however can no longer be casually assumed as indicative of an essential function in chlorophyll formation. The evidence would indicate rather that chloroses of this type are due to destruction of chlorophyll by excessive accumulations of amino acids and other metabolites. The characteristic pattern exhibited by the plant will, it is assumed, be found to be dependent on the particular metabolites that accumulate in excess, and the effects of anatomical structure in determining the regions in which toxic concentrations are permitted to become effective.

The marked fluctuations in a-amino nitrogen and protein nitrogen of plants subject to mineral deficiencies seem to be a general phenomenon. It would appear to indicate that the macronutrients and at least some of the micronutrients participate directly in the primary activity of the cell, namely the formation and breakdown of amino acids, proteins and intermediate products. Molybdenum also has recently fallen into this category since MULDER (**6**) found it to be as essential for nitrate reduction in green plants as for *Aspergillus niger* Van Tiegh.; and for denitrification as well as nitrogen fixation by soil bacteria. The points at which blocks are thus set up in the interlocking successive reactions of nitrogen metabolism of the cell are presumably as characteristic of the element in deficient supply as are the morphological symptoms. Thus RICHARDS and TEMPLEMAN (**10**) concluded that accumulation of free amino acids was due to breakdown of proteins in potash deficiency; and to inhibition of protein synthesis in phosphorus deficiency of barley. Nitrogen deficiency however led to " . . . little indication of departure from the usual protein cycle . . . . " A more adequate review of protein and carbohydrate responses to mineral deficiencies will shortly (**14**) appear.

The significance of variations in free a-amino nitrogen with alterations in environmental conditions is further emphasized by the results of a study by WOOD and PETRIE (**18**) with *Phalaris tuberosa* Link. These investigators reported that the interrelations between protein and amino acids are unaffected by changes in content of carbohydrates: sucrose, glucose, and fructose. Proteins they suggest, ". . . are formed from the whole of the amino acids in the cell."

Summary

Frenching was accompanied by a marked increase in isoleucine and other free amino acids in the leaf lamina of field plants of Maryland Medium Broadleaf tobacco. The assumption that the rise in free amino acids in frenching was the primary cause or mechanism of symptom production in the plant was also supported by the analytical data on field plants showing mineral deficiencies. Sharp increases in free amino acids accompanied calcium, magnesium, potassium and phosphorus deficiency, but not in nitrogen deficiency. Boron and particularly sulphur deficiencies were too slight to give definite chemical differences in tissues. It is probable that calcium, magnesium, potassium, phosphorus and perhaps boron function in amino acid and protein metabolism of the plant. Chloroses due to mineral deficiencies, excepting possibly magnesium and nitrogen, are attributed primarily to the direct toxic action of excessive accumulations of metabolites.

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