A NEW, NINHYDRINE-ISATINE POSITIVE AMINO ACID-LIKE COMPOUND IN THE LEAVES OF RICE PLANT

by

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The asparagine concentration of leaves increases parallel to the N amount present in the plants (11, 17). Thous the asparagine test may be the means of detection of N supply of plants. In the course of studying the asparagine test we have pointed out that as a rule the asparagine concentration of rice leaves changes parallel to the total concentration of free amino acids (13). This is also indicated by data of RATNER und UHINA (16). A greater asparagin concentration refers to a better N supply if the total N-content and dry-weight of the same variety is also greater (14). It is very difficult to establish deductions from the amount of free amino acids detected from the plants on their states and the nutrient substrate supply. Amino acid in great amount equally may indicate optimal N supply, vitality, or on the contrary, inadequate P-uptake, decrease of protein synthesis or anything else (1, 2, 3, 18, 20, 22).

It has already been established in pot experiments that the free amino acid content of rice shoots grown in alkalized by N-salts media is considerably higher than that of rice grown with normal nutrient solution — the NPK content of the nutrient medium being the same (14).

It appears that the cause of changes in the amino acid concentration has not been cleared up yet. In the course of our own experiments we aimed to answer the following questions:

1. How does the amino acid concentration in rice leaves change when all the leaves are completely developed?
2. Is there a dependence between total amino acid concentration and total N concentration in leaves of various levels?
3. Does the total amino acid and asparagine concentration change with the degree of N supply?

Materials and Methods

The experiment was carried out in Kopáncs Experimental Plantation. A Dunghan Shali variety was planted 12 March, 1963, and immediately flooded thereafter. The carrying of ammonium sulphate into the soil was done before planting as follows:

1. without fertilization,
2. 434 kg/ha (mediocre amount),
3. 695 kg/ha (high amount).

The area of separated parcels was 50 m^2. Beginning from flowering (8 August) samples were taken by 8 days, four times all together. Shoots were worked out by leaf-levels. They were fixed at 65° C and dried (air-dry weight). They were ground then fractured with perchloric acid salicil sulphuric acid; total N was measured with a PULFRICH photometer after NESSLER reaction.

Amino acids were detected by ascending paper chromatography. The air-dried plant parts were extracted with 70% ethanol and samples were carried on WHATMAN No 1 paper in four repetitions. The solvent was a butanol-glacial acetic acid-water mixture in a ratio of 2 : 1 : 1. Int two dimensional runs phenol-water of 4 : 1 ratio was applied as the second solvent. The chromatograms were sprayed with ninhydrine and isatin, on applying the methods of "universal standard mixture" and "rapid determination of total amino acid by eluation." In elaborating the new method we have started from KRETOVICS and KASZPAREK (8) and SZALAI's (19) works (13, 14). The essence of the method is that the composition of standard amino acids is similar to the amino acid composition of plant extracts. Therefore single amino acids of the standard run on the same level as the corresponding amino acids of the plant extracts (Figures 1 and 2). On determining the total amino acid content we have sprayed a standard mixture concentration series with ninhydrine on the same paper of the unknown extracts. Eluted spots being fixed by copper salt were examined by photometry. Extinction data of eluted standard spots yield the calibration curve.

Experimental Results

Table I shows measurement data. It appears that the greatest difference was obtained at a leaf length where in some cases the difference exceeds even 70—100 per cent. It also can be seen that increased N supply decreased the extent of tillering, namely the number of shoots by m^2 is less on these parcels.

Table 1

Rice shoot length and length of leaves, density of shoots during flowering on effect of different amounts of N.

Treatments	Length of leaves, cm				Length of shoots cm	density of shoots m^2
	lower 1.	middle 2.	middle 3.	upper 4.		
without manuring (control)	18,2 ±0,93	22,8 ±1,1	23,4 ±1,0	17,2 ±0,81	95,3 ±3,0	246 ±8,7
434 kg/ha $(NH_4)_2SO_4$	25,1 ±1,3	33,1 ±1,0	30,1 ±1,2	18,6 ±0,72	110,7 ±2,6	225 ±10,1
695 kg/ha $(NH_4)_2SO_4$	30,5 ±1,1	41,3 ±1,6	41,5 ±1,8	23,7 ±1,0	105,2 ±3,2	197 ±9,4
695 kg/ha, $(NH_4)_2SO_4$ diseased rice	30,6 ±1,3	45,6 ±1,7	43,2 ±1,9	28,4 ±1,3	105,4 ±3,7	190 ±12,3

On parcels manured with greater quantity of nitrogen the plants were partly lodged during flowering. Leaves became darkish green. The peaks dried-off, brownish spots appeared onthe leaves. Further these plants are called diseased rice.

It had already been published that the maximal number of living leaves of the variety studied is 5 in flowering however, as a rule, it is less, depending on the level of flooding and the nutrition. In the present case the shoots have 3-4 leaves. Figure 1 shows the amino acid content of seedlings with three leaves at three different amino acid concentrations of the universal standard mixture. It appears that the amino acid concentration in rice leaves of various insertions changes considerably upwards. There may be even more than 100 per cent difference between the total amino acid content of the uppermost and lowest leaves (Table IV). Regarding that in flowering 80% of the shoots were of four leaves, further we are referring to their data.

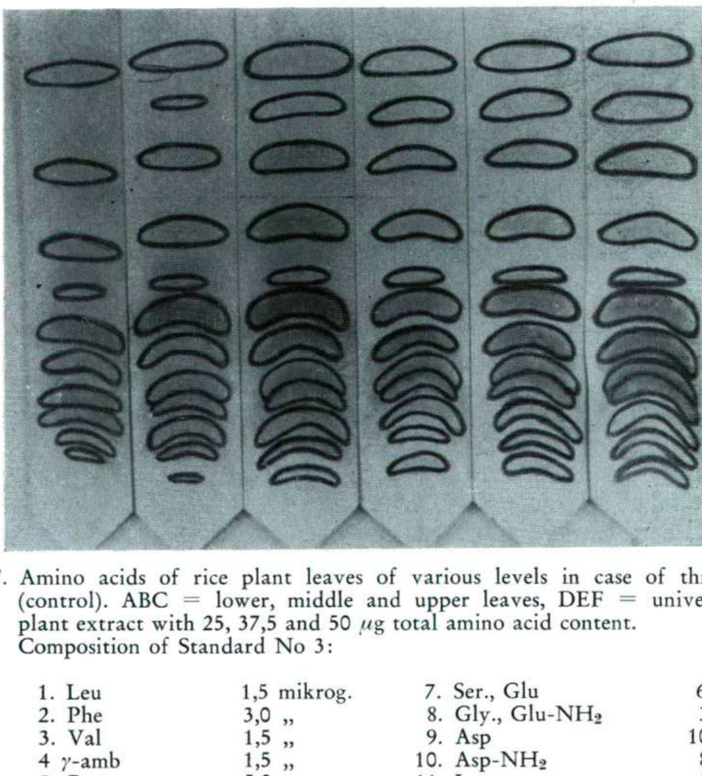

Figure 1. Amino acids of rice plant leaves of various levels in case of three living leaves (control). ABC = lower, middle and upper leaves, DEF = universal standard of plant extract with 25, 37,5 and 50 μg total amino acid content.
Composition of Standard No 3:

1. Leu	1,5 mikrog.	7. Ser., Glu	6,0 mikrog.
2. Phe	3,0 ,,	8. Gly., Glu-NH_2	3,5 ,,
3. Val	1,5 ,,	9. Asp	10,0 ,,
4 γ-amb	1,5 ,,	10. Asp-NH_2	8,0 ,,
5. Pro	5,0 ,,	11. Lys	1,0 ,,
6. Ala	5,0 ,,	12. Cys	4,0 ,,

The amino acid content in leaves of various levels of 4-leave rice without manuring also increases upwards to the third leaf. That of the fourth, in the uppermost leaf somewhat decreases (Table IV). A similar tendency is observed at rice supplied with 434 kg ammonium sulphate. However, on effect of 695 N the amino acid content of leaves is compensated. This compensation occurs in case of diseased rice (Figure 2).

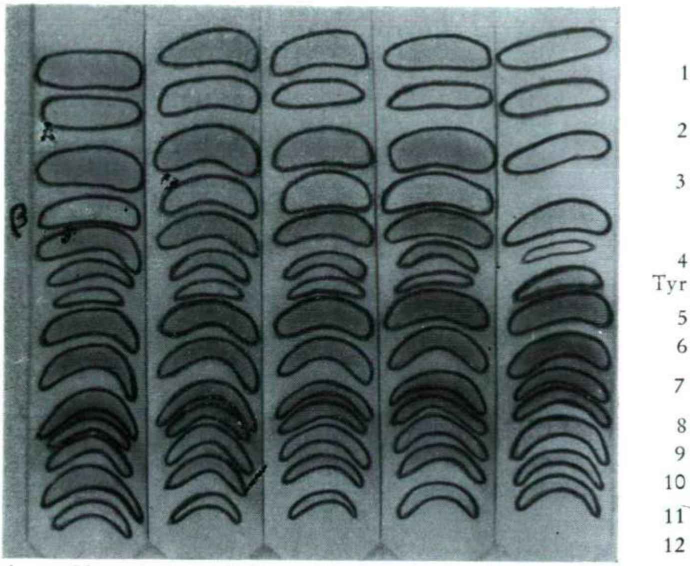

Figure 2. Amino acid content of lodged rice shoots manured with great amount of N by leaves of various levels. ABCD = lower, middle 1, middle 2 and upper leaves. E = Universal standard with 50 μg total amino acid content. Composition: as in Fig. 1. β = unknown amino-acid-like substance.

There is one important quantitative difference between the varieties: an amino-acid-like compound of unknown composition appearing at 0,64 Rf between γ-aminobutyric acid and valine. The spot denoted by us by β is the greatest at diseased rice (Figure 2), it is also considerable at healthy rice treated with 695 kg but here it decreases by levels. It could be observed only to a small extent at seedlings on parcels manured with 434 kg: in the two upper leaves. It could not be detected even in traces at rice without fertilizer (Figure 1). The ninhydrine reaction of the β-substance is not the customary reddish-purple, but bluish. After spraying with copper- or nickel-salt solution it is purple- blue and at spraying with isatin, bluish-green. It does not fluoresce after ultraviolet illumination. It does not give anilinephtalate and antron sugar reaction. In two dimensional chromatograms it is the fastest of all the amino acids of rice, as Figure 4 shows. Figures 3 and 4 also indicate that there is not other difference between diseased and healthy rice as the only β-spot. After a 5 n hydrochloric acid hydrolysis of the vaporized amino acid extract of the diseased rice, carried out for 72 hours at 105 C°, the unknown substance appeared after separation with the same intensity as a blue spot, as without hydrolysis. Thus β-compound is not peptide and is very stable. It does not take part in protein composition: being the free amino acids of the rice leaf homogenisatum removed and the hydrochloric acid hydrolysis of proteins carried out thereafter, it does not appear in the chromatograms. The analysis is further carried on.

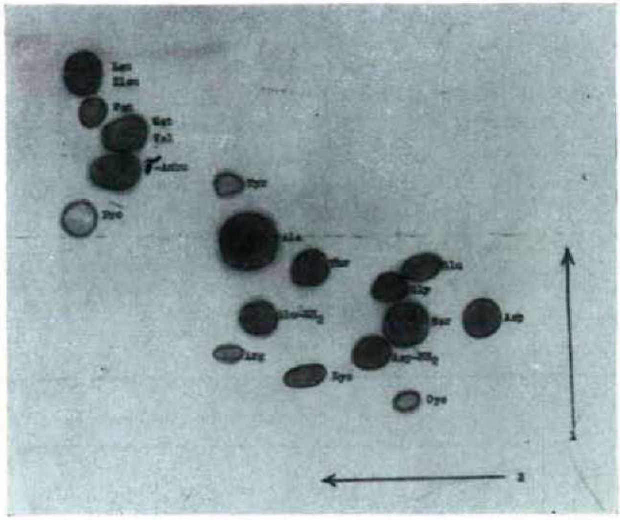

Figure 3. Amino acids of the middle leaves of rice without manuring. 1st direction: butanol-acetic acid-water. 2nd direction: phenol-water. ninhydrine, sprayed with copper salt solution.

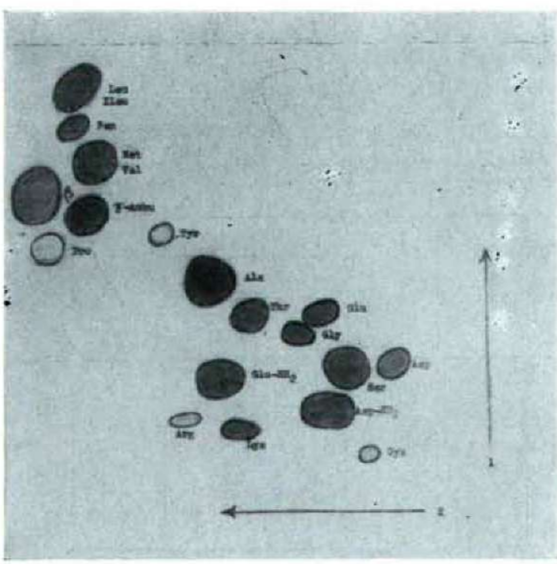

Figure 4. Amino acids of lodged rice shoots manured with great amount of N in their middle leaves. Outer-left spot is the β-substance.

As Table II shows, dry-weight increases parallel to the amount of N corresponding to the leaf-length (Table I) and it is the greatest in case of diseased rice. Total N-concentration increases within the shoots by levels. Being the total N concentrations of the single leaves of various level compared by varieties, it can be seen that there is an increase only at the 695 kg N manuring at the diseased rice. Thus it is very difficult to alter the "critical concentration" of leaves. Taking, however, into consideration the magnitude of dry-weights, too, it seems that varieties manured with N took up considerably more nitrogen from the soil (Table III).

Table 2

Dry-weight of rice leaves by leaves of various levels during flowering

Treatments	Dry, weight, mg.			
	1. lower	2. middle	3. middle	4. upper
without manuring (control)	73 ±2,4	74 ±2,3	80 ±2,7	64 ±2,1
434 kg/ha $(NH_4)_2SO_4$	91 ±3,8	110 ±4,2	100 ±4,3	85 ±3,5
695 kg/ha $(NH_4)_2SO_4$	110 ±4,3	150 ±5,7	150 ±5,5	120 ±4,1
695 kg/ha, $(NH_4)_2SO_4$ diseased rice	160 ±6,1	210 ±7,9	210 ±8,5	160 ±6,4

Table 3

Total N concentration of rice leaves in per cent of dry weight and the total N content in mg during flowering by leaves of various levels

Treatments	1. lower		2. middle		3. middle		4. upper	
	N con-centr. %	N con-tent mg	N con-centr. %	N con-tent mg	N con-centr. %	N con-tent mg	N con-centr. %	N con-tent mg
without manuring	0,72 ±0,028	0,53 ±0,017	1,02 ±0,04	0,75 ±0,04	1,10 ±0,05	0,88 ±0,03	1,40 ±0,05	0,89 ±0,03
434 kg/ha $(NH_4)_2SO_4$	0,71 ±0,03	0,64 ±0,028	0,92 ±0,04	1,01 ±0,05	1,10 ±0,05	1,10 ±0,04	1,40 ±0,07	1,19 ±0,05
695 kg/ha $(NH_4)_2SO_4$	0,72 ±0,04	0,79 ±0,03	1,20 ±0,04	1,80 ±0,07	1,40 ±0,06	2,10 ±0,08	1,70 ±0,06	2,04 ±0,09
695 kg/ha, diseased rice	0,92 ±0,042	1,47 ±0,07	1,30 ±0,05	2,73 ±0,09	1,50 ±0,07	3,15 ±0,14	1,40 ±0,06	2,24 ±0,09

Data of total amino acid content (Table IV) also indicate the increase of concentration by level of shoots manured with mediocre amount of nitrogen and of the control. But the concentration is compensated here too in case of great N amount. Asparagin concentration of the leaves generally also increases by levels and it reflects very well the degree of N supply.

Table 4

Total amino acid and asparagin concentration of rice leaves in per cent of dry weight by leaves of various levels during flowering

Treatments	1. lower		2. middle		3. middle		4. upper	
	total amino acids	asparagine	total amino acids	asparagine	total amino acids	asparagine	total amino acids	asparagine
without manuring	0,52 ±0,04	—	0,88 ±0,08	0,022 ±0,002	1,25 ±0,09	0,035 ±0,003	1,12 ±0,10	0,027 ±0,003
434 kg/ha $(NH_4)_2SO_4$	0,86 ±0,07	0,033 ±0,003	1,12 ±0,09	0,062 ±0,006	1,34 ±0,11	0,076 ±0,007	1,27 ±0,09	0,074 ±0,007
695 kg/ha $(NH_4)_2SO_4$	1,16 ±0,10	0,075 ±0,007	1,35 ±0,11	0,093 ±0,008	1,30 ±0,10	0,097 ±0,008	1,31 ±0,11	0,103 ±0,010
695 kg/ha, diseased rice	1,27 ±0,11	0,106 ±0,010	1,21 ±0,10	0,124 ±0,011	1,14 ±0,09	0,121 ±0,011	1,47 ±0,12	0,124 ±0,010

From all these data it is clear that the constant results of analyses were obtained with the middle leaves of rice seedlings, and in the following we give data of the second leaf from teh top.

Dry weight amounts obtained from flowering till ripening increases parallel to the amount of N supply (Figure 5). The total concentration of leaves does not give such a clear picture, although it somewhat increases proportional to the degree of N nutrition. It can be seen that the total N concentration of the leaves, independently of the treatment, markedly decreases during ripening. To a certain degree the extent of N supply can be seen from the total amino acid concentration of the leaves, too (Figure 6); it has a decreasing tendency during ripening but it has a sudden raise at the last, September sample. This fact may be ascribed to the emptying of the leaves before ripening, and the rushed translocation of the substances, respectively. The asparagine concentration of the leaves well shows the amount of N-supply (Figure 6).

The yield is significant because of the uneven diseased spots. Calculated for hectares it is 4,13 ton for the control; 4,56 ton for the variety manured with 434 kg; 3,28 ton for that manured with 695 kg. (Data and yield are given by. P. ADAMIK for which I express my thanks here, too.)

For the yield control a precisely evaluated analysis was carried out (Table V). It appeared that the panicle grain number, disregarding treatment, as a rule is low. It somewhat increases as well as the panicle length, when the N amount is increased. The thousand grain weight slightly decreases for the mediocre N amount, and with 25,8% for the great N amount as compared

Table 5

Yield analysis: panicle length and panicle grain number of rice: total N concentration of grain yield and thousand grain weight

Examination	without manuring	434 kg/ha $(NH_4)_2SO_4$	695 kg/ha $(NH_4)_2SO_4$
Length of panicle cm.	13,6 ±0,21	13,8 ±0,18	14,6 ±0,25
Grain number/panicle	42 ±1,8	47 ±2,1	54 ±2,6
Weight of 1000 grain	30,2 ±0,42	29,5 ±0,53	22,4 ±0,61
Total N concentr. of grain yield, %	0,56 ±0,022	0,60 ±0,019	0,65 ±0,025

with the control. Total N content of the grain yield did not give considerable difference. Data of yield analysis together with the density of shoots beyond any doubt testify the yield decreasing effect of the unisided N fertilization.

Figure 5

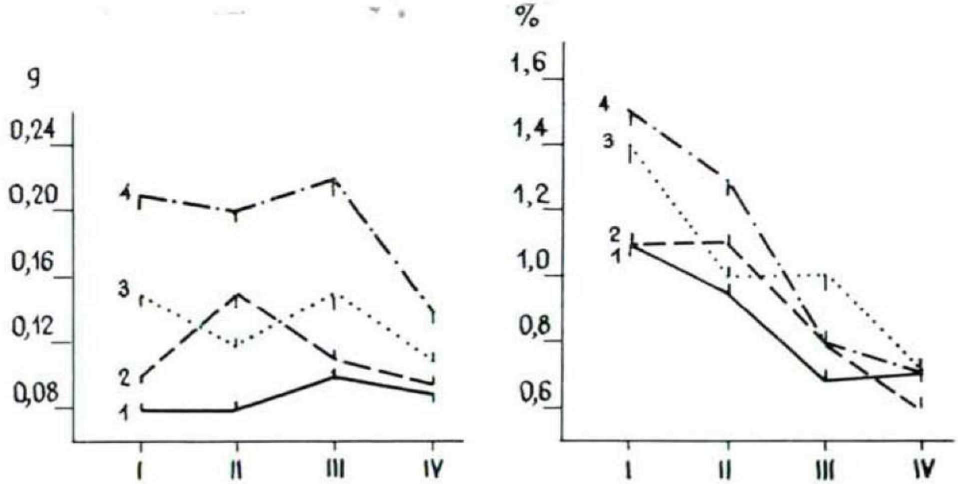

Figure 5. Dry-weight of middle leaves (on the left) and total N concentration as per cent of dry weight (on the right). I = flowering, II—IV = ripening. 1 — without manuring (controll); 2 — 434 kg/Ha N salt; 3— 695 kg/ha N salt, lodged rice. Length of liens means the magnitude of mean error.

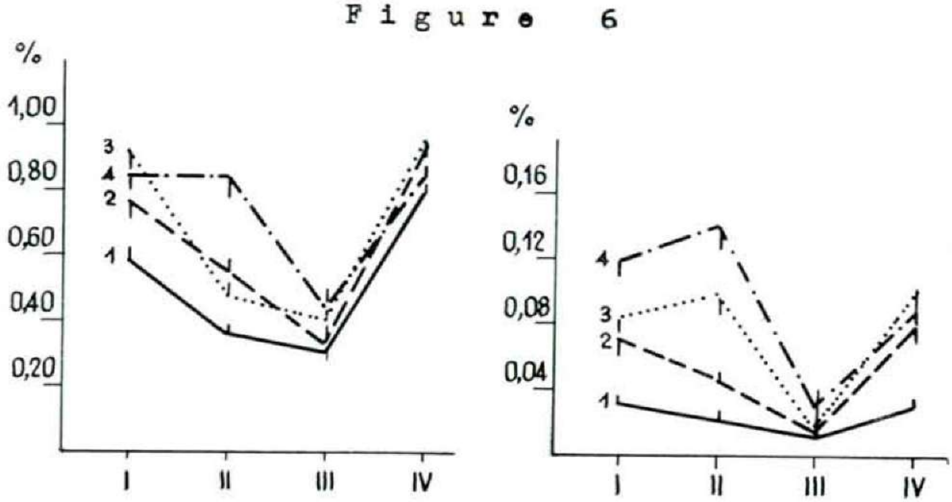

Figure 6. Total amino acid concentration of middle rice leaves (on the left) and asparagine concentration as per cent of dry weight (on the right). I = flowering, II—IV = ripening. 1—4 N salt magnitude as in Figure 5.

Discussion

Symptoms observed at N supply of great amount (Table I) agree with the P-deficiency symptoms described by HUGUET (7), but the relative P-deficiency of rice has already been described by us, too, (15) as well as by GRIST (5).

Increased amount of N did not cause considerable change in N concentration of the leaves (Table III), but the total N content of the leaves was markedly increased.

The amino acid content of the shoots by levels increases suddenly from bottom upwards in case of small N nutrition (Table IV). This fact can be ascribed to the ageing of the leaves, as pointed out by MOTHES (10) and PARTHIER (12). Then the decomposition of proteins and a rapid dislocation of amino acids occurs in the aged leaves. On effect of 695 kg N manure the difference between the amino acid concentration of the leaves of various levels disappears (Figure 2). Thus in case of increased N nutrition the leaves of various levels were saturated with free amino acids. This saturation may indicate the degree of N supply. This tendency can be observed at the asparagine concentration of the leaves of various levels, too, but to a less extent only. Thus the asparagine concentration of the single varieties by leaves of various levels increases according to the amount of N nutrition. (Table IV).

From our data it appears that a quantitative change of the amino acids is first of all caused by the N-manuring. Qualitatively a considerable difference is the positive ninhydrine and isatin spot at 0,64 Rf, which in the second dimension runs up to the highest Rf. This spot denoted by β may be one of the amino increases parallel to the magnitude of N supply, after a thoroughful study it can be an indicator of the magnitude of N nutrition.

In yield analysis the low thousand grain weight is a result of the bad fertilization as it had already been referred to by VÁMOS (21) and HARANJAN (6) in connection with N overnutrition.

Summary

In the course of study of free amino acids three characteristic features of the effect of great amount of N have been observed:

1. The amino acid concentration of leaves of various levels without fertilization and supplied with 434 kg ammonium sulphate per hectare rushedly increases upwards. At rice manured with 695 kg per hectare the concentration of the leaves is almost the same, especially at diseased rice. Thus on effect of great amount of N the leaves of various levels are saturated with free amino acids.

2. The asparagin concentration of the leaves increased parallel to the degree of N nutrition, especially on effect of increased amount of N.

3. New ninhydrine and isatin positive amino acid-like compound was pointed out at 0,64 Rf, between γ-aminobutyric acid and valin, which does not constitute protein. The amount of this substance denoted by β increased parallel acid derivates or precursors detected by MARÓTI (9) and FOWDEN (4). Since the amount of substance of unknown com position pointed out in the β-spot to the degree of N nutrition. Most of it was found in the diseased rice, in the control it could not be detected.

On effect of unisided N manuring the rice shoots show symptoms of P deficiency. The magnitude of N nutrition hardly could be detected in the N concentration of the leaves, but very well in dry weight and total N content.

The yield-decreasing effect of great amount of N reveals itself in the slight tillering and bad fertilization.

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