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ATTEMPTS OF SEARCHING AMINO ACID REQUIRING AUXPTROPHS AMONG CHLOROPHYLL MUTANTS OF PEARL MILLET AND QUANTIFYING THEIR FREE AMINO ACIDS

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Ten lethal chlorophyll mutants of Pearl millet (*Pennisetum americanum*) were grown on amino acid supplemented culture medium to isolate auxotrophs for amino acids, if any, in them. Only the necrotic yellows did show a little response with a little increase in chlorophyll content. None of them were found to be auxotrophs. Determination of free amino acids in these ten lethal mutants and in three viable mutants- light green 1. light green 2 and yellow virescent revealed enchnced quantities of most of the amino acids in all these mutants except in necrotic yellows which were deficient by about 36% over their control. Allelic mutants have been found to have differed in their amino acid patterns. Regression of total free amino acids or glycine on total chloropyll was significant in these mutants suggesting negetive relationship between total chlorophyll and total free amino acids or total chlorophyll and glycine. Accumulations of such large quantities of free amino acids were thought of as due to block in protein synthesis in these mutants.

Keywords: Amino acids; chlorophyll mutants; Pearl millet.

Introduction

In higher plants, chlorophyll deficient mutants result from either a block in chloropyll synthesis[1,2] or inability to synthesize carotenoids[3,4] or deficiency for certain metabolites like amino acids[5]. These latter ones behave as auxotrophs and are termed as nutritional mutants. A study of such mutants is useful to understand the relation between the phenotype and its biochemical nature. Several lethal clorophyl mutants were known in pearl millet, *Pennisetum americanum* (L.) Leeke[6,7] however, studies on nutritional requirements in such mutants is not known so far. In the present study attempt were made to study the reponse of ten sodium azide induced lethal chlorophyll mutants[8] to different amino acids exogenously supplied to the sterile culture medium.

Plastid development[2] and maturation of chloroplasts[9] involve protein synthesis; thus any lesion affecting protein synthesis is expected to result in free amino acid accumulation.Therefore it is aimed at determining the free amino acid quantities in the sodium azide induced chlorophyll mutants. The present paper describes the results of our attempts to isolate AA requiring autotrophs and free amino acid quantities among the ten lethal mutants (pale yellow 1 and 2, medium yellow 1 and 2,deep yellow 1 and 2, white 1,2 and 4 and necrotic yellows) and three viable mutants (yellow virescent, light green 1 and light green2) all obtained in sodium azide treatment[2] . Three control inbreds PDP. Vg272 TLD have also been included in this study.

Materials and Methods

Culture Experiments : Following the procedure used in *Arabidopsis*[10] and in maize[11], the minimal medium (MM) was prepared by dissolving the following ingredients (mgs/litre) in distilled water: NH_4NO_3 200, $MgSO_4$ 7 H_2O 100, Ca $H_4(PO_4)_2$, H_2O 100, KH_2PO_4 100, K_2HPO_4 50 and Fe $C_6H_5O_7$, $3H_2O$ 2.5; it was solidified with 0.8% agar and 2% dextrose was added to ensure more vigorous growth. For enriched medium (EM) 100 mgs/litre each of alanine, arginine, glutamine, phenylalanine, glycine, histidine, leucine, proline, isoleucine, methionine serine, threonine, tryptophan, tyrosine valine and *y* - amino butryric acid and 50 gms/ litre of aspartic acid and cystine were added to the medium. The agar melted MM and EM was poured into culture tubes (150 X 16 mm) with 5 ml per tube and were fitted with cotton plugs, finally tubes were sterilized by autocalving at 120°C and 15 lb/in^2 for 15 minutes.

Seeds, from plants heterozygous for each mutant, were surface sterilized with

mercuric chloride and were germinated in a sterilized glass Perti dish. At a stage when mutants could be indentifiable from greens (3-4 days)they were transferred to culture tubes in a vertical flow laminar chamber. The tubes were maintained under artificial illumination provided by fluoroscent lamps. Fresh weight of each type of these mutants seedlings in EM is taken and compared with fresh weight of corresponding mutants seedlings grown under field conditions. Leaf extracts were made in 80% acetone, readings were taken on Spectronic-20 (Bosch&Lamb) spectrophotometer at 645, 552 and 663 nm and quantites were determined[12]. Two replications were made for each mutant.

Quantification of free qmino acids: Leaf sample were weighed, owen dried at 50 ^{0}C and powdered using mortar and pestle. Free amino acids were extracted with 70% aqueous ethanol (about 20 ml/gram material); extract was filtered, washed with additional solvent and the filtrate was evaporated almost to dryness under reduced pressure,redissolved in 10ml of water and extracted thrice with chloroform (10 ml each time) in a separatory funnel to remove impurities. Purification of the sample was done by passing the aqueous extract through cation-exchange resin (Amberlite IR 120) following procedure of Lazarus [13]. The amino acids were eluted with 2N ammonium hydroxide, evaporated to dryness and redissolved in acetate buffer (pH 2.2) and centrifuged. Samples were run on an LKB 4101 automatic amino acid analyser. Two replications were made for each mutant and inbred. The amount of each amino acid present was determined by the method described in the technical manual provided along with the instrument.

Result and Disscussion

Amino acid requirement studies in the lethal mutant: Mutants grown on culture medium were associated with increased growth of the root system (as evident from increased length of the roots), but fresh weight of the shoot system did not differ significantly from those of the field grown mutant. However mutants grown on EM were viable for 3-4 days more than the field grown seedlings. In the case of deep yellow 1 and deep yellow 2, there was no such increased growth of the roots or prolonged survival. Control green seedlings grown on both minimal and and enriched medium has similar quantity of chlorophyll content which also did not differ significantly from samples obtained from field grown mutants suggesting little influence of amino acids on the greening in these control green seedlings. Of the ten lethal mutants tested for their response to amino acid supplementation, only the necrotic yellow exhibited a little response with about 36% increase in total chlorophll (Table 1).

Though the percentage increase in chlorophyll was considerably high (36%) in the necrotic yellows grown on EM than on MM (Table 1), this was only negligeable compared to the control green seeding. In barley the xantha-23 mutant response to leucine was as much as 75% which turned out to be an auxotroph[5]. This negligeable increase observed here lead us to conclude that even this mutant is also not a real auxotroph.

The mutants deep yellow 1 and 2 and white-4 of PDP were deficient in *y* -amino butyric acid (Table 2), but still have not responded to it when supplied in the medium. The absence of response to amino acid supplement, in spite of the deficiency of almost all amino acids (necrotic yellows) or one amino acid (deep yellow 1 and 2 and white 4) indicates that origin of these mutants may not be the result of simple metabolic blocks requiring amino acids.

Amino acid quantities: In addition to the 18 peaks present in the standard mixture, the mutants and inbreds of pearl millet studied here have two additional peaks-one in between phenylalanine and histidine and the other between NH_3 and arginine; they have been identified as y -amino butyric acid and tryptophan respectively (personal communication from Dr. Richard Andrews, Applications Chemist, L.K Biochrom,Ltd London). Quantities of free amino acids in all the mutants and inbreds were summarised in Table 2. Fighure 1 and 2 are amino grams of

on mutant and one inbred of the present study.

The mutants light green 1 and 2 were non allelic, but appear alike at plumule stage[8]. Correspondingly total free amino acid accumulation was similar in them (21% in Ig 2) compared to their respective controls. However, Ig I had about 163% more than PDP control.

The percent accumulation of total free amino acids appears to be more in the PDP mutants than in the mutants of the other two inbreds (TLDand Vg 272). This was due to the relatively lower content of the total amino acids in PDP then in the other two inbreds. But those mutants of TLD and Vg 272 have had as much as or even more accumulation compared to PDP control green. Thus the mutant pale yellow 1 which had about100% more total amino acids than its control TLD, was found to be having about 350% more than in PDP inbred (Table 2).

Quantities of free amino acids in the inbreds : The three inbreds PDP, TLD, and Vg 272 differed in quantity of both total and individual amino acids; total quantity in TLD was more or less similar to Vg 272 and was significantly greater than in PDP (Table 2) Thus PDP was having lesser amounts of all amino acids than TLD except proline; similarly it was having lesser quantities of all amino acids than Vg 272 except aspartic acid and proline. Amount of tryptophan remained similar in these three control inbreds.

Quantities of free amino acids in the mutants : Total quantity of free amino acids was about 36% less than in the control in the necrotic yellow (Table 2) which was due to the significantly low quantities of all amino acids except tryptophan which was higher than in control; aspartic acid and *y*-amino butyric acid were in similar quantities in the mutant and control.

In all other lethal mutants and in the three viable mutants (yellow virescent, light green 1 and light green 2), most amino acids were in higher quantities than in their respective control inbreds (Table 2). All the mutants had significantly enhanced amounts of threonine, serine, glycine, tryptophan, arginine, valine and isoleucine as common features. Except light green I and light green 2, significantly more quantity of ammonia in the mutants suggests the presence of large amounts of amides like glutamine and asparagine.

Significantly higher quantities of all the amino acids were seen in yellow virescent, pale yellow 1, deep yellow 1 and white 3, but alanine in yellow virescent was similar to its control (TLD) and *y* -amino butyric acid was less than in control (PDP) in deep yellow 1. Compared to their repective controls, tyrosine and phenylalanine differed only to a little extant in the mutants. Among the mutants, highest total accumulation was seen in white 3 (about 300% more than its control PDP) while least accumulation was seen in light green 1 (about 22%). Genetic tests made by us revealed that pale yellow I was allelic to

Table 1. Contents of chlotophyll (mg./g.f.wt.) in ten lethal mutants of pearl millet cultured on minimal medium (MM) and enriched medium (EM) (Mean from two replicates).

Mutants	Total chlorophyll		% increase in EM over MM
	MM	EM	
Pale Yellow 1	0.0782	0.0788	0.76
Pale Yellow 2	0.0764	0.0782	2.36
Medium Yellow 1	0.0678	0.0681	0.44
Medium Yellow 2	0.0752	0.0752	0
Deep Yellow 1	0.1489	0.1517	1.88
Deep Yellow 2	0.1476	0.1497	1.42
White 1	0.0926	0.0950	2.59
White 2	0.0951	0.0966	1.58
White 4	0.1011	0.1018	0.69
Necrotic Yellows	0.0599	0.0793	32.39

Table 2. Quantities of free amino acids (μ mol./g.f.wt.) in the first leaf of the inbreds PDP, vg 272 and TLD of pearl millet and in their mutents (Mean of the two replicates each)

Amino Acid	Inbreds			Mutents of TLD					Mutents of PDP					Mutents of vg 272	
	PDP	Vg 272	TLD	Light Green 1	Yellow Virescent	Pale Yellow 1	Medium Yellow 2	Necrotic Yellow	Light Green 2	Deep Yellow 1	Deep Yellow 2	White 3	White 4	White 1	White 2
Aspartic acid	0.69	0.46	0.85	0.95	1.30	1.33	0.98	0.97	0.91	2.17	1.11	1.12	1.57	1.90	1.61
Threonine	0.38	1.02	0.95	1.22	1.81	1.83	1.47	0.58	0.87	2.57	1.85	2.38	3.37	2.03	5.21
Serine	0.65	2.82	2.90	3.54	4.03	5.43	5.65	2.41	1.66	14.22	7.81	6.68	9.80	9.57	10.58
Glutamic acid	0.08	0.4	0.15	0.18	0.34	0.43	0.31	0.09	0.09	0.26	0.10	0.16	0.11	0.27	0.46
Proline	0.29	0.07	0.18	0.53	0.63	1.82	1.79	Trace	Trace	0.60	0.22	0.67		1.12	0.60
Glycine	0.19	0.63	0.47	0.55	0.80	0.89	0.73	0.35	0.35	0.56	0.38	0.61	0.65	0.83	0.66
Alanine	3.69	4.43	5.08	5.09	5.20	7.44	5.84	3.08	3.49	3.83	3.97	4.08	3.80	4.53	4.49
Cystine	Nil	0.13	0.05	0.09	0.23	0.39	0.28	Trace	Trace	0.25	0.28	0.18	0.24	0.48	0.26
Valine	0.37	1.70	1.52	1.99	2.70	3.15	2.23	0.86	0.59	2.67	2.26	2.96	2.64	2.99	2.68
Methionine	0.02	0.09	0.10	0.08	0.24	0.29	0.13	0.03	0.03	0.14	0.04	0.09	0.10	0.23	0.13
Iso-leucine	0.23	0.90	0.74	1.01	1.31	1.98	1.21	0.43	0.50	1.31	1.40	1.26	1.25	1.48	1.45
Leucine	0.49	1.06	1.21	1.32	1.93	2.52	2.37	0.57	0.57	1.53	1.22	1.12	1.22	1.26	1.16
Tyrosine	0.14	0.45	0.51	0.70	0.87	0.85	0.77	0.23	0.16	0.35	0.34	0.24	0.25	0.48	0.44
Phenylalanine	0.21	0.61	0.78	1.01	1.24	1.28	1.06	0.32	0.26	0.37	0.35	0.31	0.26	0.64	0.59
y-aminobutyric acid	0.64	1.04	0.86	1.06	1.02	1.11	0.86	0.98	0.36	0.13	0.15	1.35	0.17	1.08	1.38
Histidine	0.08	0.29	0.46	0.56	1.41	1.60	1.83	0.17	0.24	1.20	1.25	1.65	1.54	1.23	1.52
Lysine	0.39	0.87	0.92	1.09	1.57	2.22	1.73	0.32	0.33	1.09	0.75	0.99	0.61	0.85	0.91
Ammonia	0.13	0.43	0.58	0.68	1.13	1.86	1.57	0.17	0.20	1.29	0.93	0.58	0.48	1.45	1.28
Tryptophan	0.04	0.03	0.01	0.54	1.40	1.62	1.25	0.17	0.14	0.65	0.80	0.20	0.90	0.24	0.21
Arginine	0.24	0.77	0.96	1.33	1.87	2.03	1.65	0.49	0.38	1.92	1.93	1.24	0.98	1.21	1.48
Total	8.95	17.94	19.28	23.52	31.03	40.07	33.73	12.22	11.13	36.51	27.14	27.87	30.17	33.87	37.01
% increase over control				21.99	60.94	107.83	74.95	36.32*	24.36	211.40	237.93	307.93	203.24	88.80	106.30
% increase over PDP				162.69	246.70	347.71	26.87	36.54						278.44	313.52

* % decrease over control

Table 3. Quantities of total chlorophyll (mg./g.f.wt.), total amino acids (μ mol./g.f.wt.) in chlorophyll mutants and their controls.

Genotype	Total chlorophyll	Total free amino acids	Glycine
PDP	1.32	8.95	0.19
TLD	0.98	19.28	0.47
Vg 272	0.85	17.94	0.63
Light Green 1	0.61	23.52	0.55
Light Green 2	0.52	11.13	0.35
Yellow Virescent	0.32	31.03	0.80
Deep yellow 2	0.16	27.14	0.38
Deep Yellow 1	0.13	36.51	0.56
White 4	0.10	30.17	0.65
White 2	0.10	37.01	0.66
White 1	0.09	33.87	0.83
Pale Yellow 1	0.08	40.07	0.89
Pale Yellow 2	0.07	33.73	0.72
White 3	0	27.87	0.61

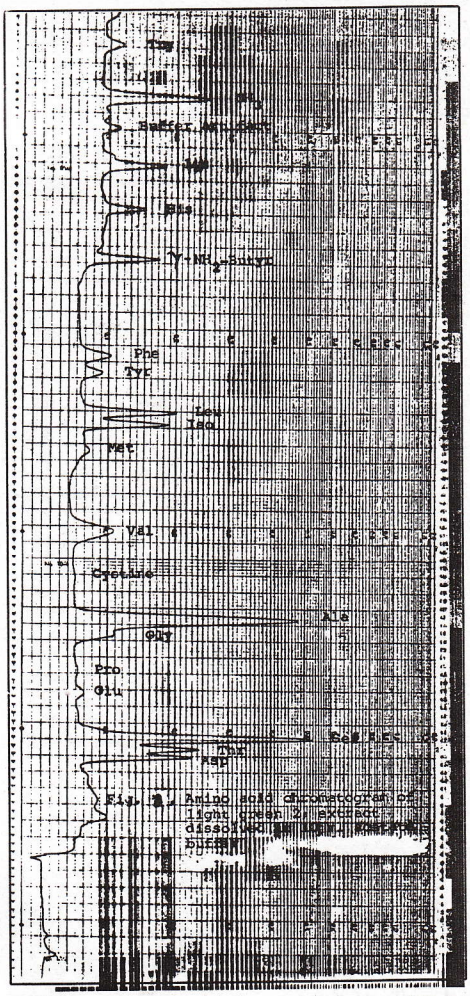

Fig.1. Amino acid chromatogram of light green 2, extract dissolved in 10 ml acetate buffer.

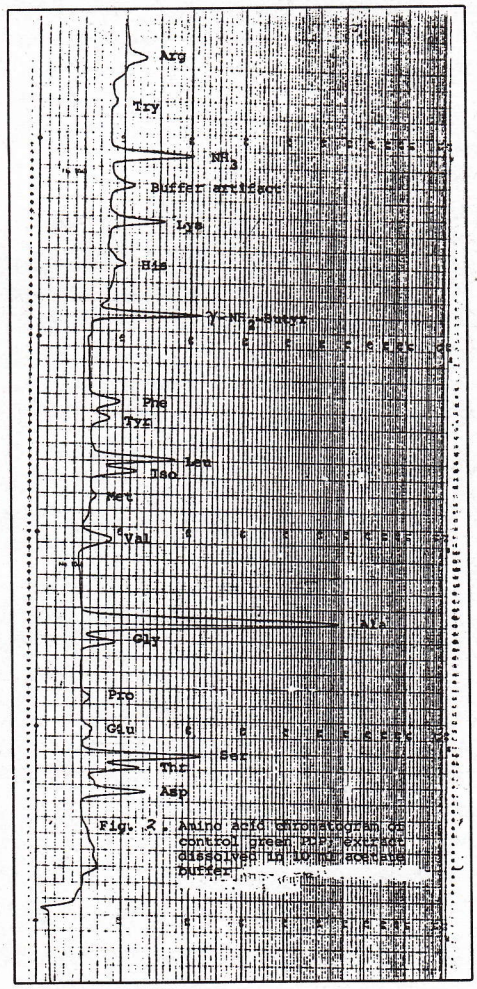

Fig.2. Amino acid chromatogram of control green PDP, extract dissolved in 10 ml acetate buffer.

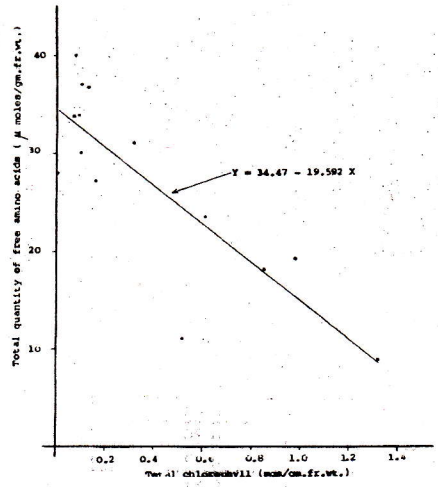

Fig.3. Regression of total free amino acids on total chlorophyll in chlorophyll mutants and thier control inbreds.

medium yellow 2 and deep yellow 1 was allelic to deep yellow 2, similarly white 1 was allelic to white 2[8].However a closer examination of table 2 revealed that these allelic mutants have differed in their patterns and total quantities.

Correlation between total chlorophyll and free amino acids : Quantities of total chlorophyll, total free amino acids contents of these mutants are summarised in Table 3. An examination of these values indicated that PDP with higher chlorophyll than the other two inbreds had a lower quantity. Furthermore accumlation was more in yellows and whites having comparatively less chlorphyll than the light green I and light green 2. Regression of total free amino acids on total chlorophyll was significant (t= 15.34,df=12, p=<0.0001) suggesting that the lower the chlorophyll content the higher was the accumulation of free amino acids (Fig.3). Glycine, an amino acid involved in porphyrin quantity (t=2.90, df=12,p=0.05-0.01) suggesting possible deficiency in porphyrin sysnthesis in these mutants.

These observations, that free amino acid quantity is related to pigment content parallel with those made in virescent mutant of barley[15] and pale green 13 of maize[16]. In the latter case the mutant pg 13 was deficient in two electrophoretic protein bands. In ground nut as well Tai *et al.* [17]observed that the chlorophyll was related to the contents of free amino acids and fatty acids of the seeds.

In the present studies the accumulated free amino acids in the mutants are thought of as due to a block in protein synthesis resulting in the accumulation such unused amino acids. Block in protein synthesis in pigment deficient mutants has been reported in *Pelargonium*[18]and barley[19]; in both cases ribosomes the site of protien synthesis were absent. In the virecent mutant of pea nut, Benedict and Metring[20]observed that chlorophyll formation was invariably associated with protien synthesis.

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