

Changes in proteins, amino and keto acids in different seedling parts of *Cyamopsis tetragonolobus* Linn. during growth in light and darkness

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Abstract. Comparative changes in protein, free amino and keto acids have been studied in different seedling parts of *Cyamopsis tetragonolobus* plants in light and dark. Endosperm recorded higher level of free amino acids in darkness than in light, while a low concentration of protein was exhibited both in light and dark. The breakdown of soluble protein was more in darkened cotyledon due to higher protease activity. The large increase in the free amino acids in the hypocotyl during seedling growth in the dark may be due to its restricted capacity to incorporate all the amino acids into proteins. Root samples from light recorded higher soluble protein as well as a higher free amino acid pool. α -Oxoglutaric acid (α -OGA) was recorded in low levels and at few growth stages in both light and dark. In light raised cotyledon samples, the dominating keto acids are phosphoenolpyruvate and pyruvic acid. Low levels of oxaloacetate in light, like α -OGA, indicate its rapid utilization during growth, but its accumulation in the dark may suggest sluggish protein synthesis thus sparing the utilization towards the synthesis of amino acids. Utilization of asparagine and glutamine was also affected in dark.

Keywords. Seedling parts ; protein ; amino acids ; keto acids ; protease activity ; *Cyamopsis tetragonolobus*.

1. Introduction

The correlative changes in amino and keto acids have been studied during seed germination and seedling growth (Fowden and Webb 1955 ; Webb and Fowden 1955 ; Krupka and Towers 1958a, b ; Mukherjee 1972 ; Mukherjee and Laloraya 1974, 1979, 1980). Recently, studies have been carried out in our laboratory (Gupta 1981 ; Afria and Mukherjee 1980, 1981) of the comparative changes in aforesaid metabolites along with organic acids and soluble protein in different seedling parts of various plants so that proper assessment could be made of their mobilization and/or breakdown at various growth stages. Leguminous plants can be divided into endospermic and non-endospermic ones depending upon whether the endosperm has been retained into maturity or not. Metabolic

changes during development of endospermic legumes have received less attention in comparison to the other group during seedling growth. For this reason, various biochemical changes with growth in *Cyamopsis tetragonolobus*, an endospermic legume, have been studied here. In this paper the comparative changes in soluble proteins, free amino acids, keto acids and protease activity have been described in endosperm, cotyledon, hypocotyl and root of this endospermic legume, during the early stages of seedling growth in light and dark.

2. Material and methods

Seeds of *Cyamopsis tetragonolobus* Linn. were surface sterilized with 0.1% mercuric chloride for 2 to 3 min followed by thorough washing. Acid treatment was given thereafter and washed thoroughly again with sterilized distilled water. Seeds were germinated on filter paper discs moistened with distilled water in Petri dishes and grown in darkness or light (2910 lux provided by fluorescent tubes) in a growth chamber maintained at $30 \pm 1^\circ$ C. Three replicates of 30 seeds each were taken for each experiment. Every care was taken to select morphologically uniform seeds and to ascertain least variability, experiments on growth in light and darkness (table 1) were repeated thrice. Protein, free amino and keto acids were determined quantitatively in endosperm, cotyledon, hypocotyl and root 48 hr after sowing (termed "initial") as well as 48, 72, 96 and 120 hr after 'initial' of both light and dark grown seedlings.

Soluble proteins from fresh plant material were measured according to the method of Lowry *et al* (1951) using Folin phenol reagent. The plant material was boiled in 80% ethanol for 2 min on a water bath. It was allowed to stand for 15 min at room temperature, ground in the same ethanol and centrifuged at 6000–7000 rpm for 5 min. Supernatant was discarded and the residue was again extracted with 80% ethanol. Supernatant was discarded again and the residue was extracted with 5% perchloric acid, followed by centrifugation at 6000–7000 rpm for 5 min. Supernatant was discarded and the residue was taken out in a test tube containing 1N NaOH and kept for 30 min. in warm water ($40\text{--}50^\circ$ C), 0.5 to 1.0 ml of this clear solution was taken and 10 ml of reagent C, which was prepared by adding reagent A (2% sodium carbonate in 0.1 N NaOH) and reagent B (0.5% copper sulphate in 1% sodium-potassium tartarate) in the ratio of 50 : 1 (v/v), was added to it and allowed to stand for 10 min at room temperature. Then added 1 ml of Folin's reagent (diluted twice) rapidly with immediate mixing and allowed to stand for 30 min. The OD was measured at 540 nm in a Bausch and Lomb Spectronic-20 colorimeter. The amount of protein was determined in terms of Bovine Serum Albumin.

The extraction procedure used for amino acids, their chromatographic separation and estimations were the same as recommended by Steward *et al* (1954). For keto acids the extraction procedure of Kaushik (1966) which is a slight modification of the method described by Towers and Steward (1954) has been followed. Free amino and keto acids were quantified as glycine and α -oxoglutaric acid equivalents, respectively.

Protease activity—The method of extraction of the enzyme was a slight modification of that described by Yomo and Varner (1973) and Ihnen (1976). 1% casein solution was prepared in 0.1 N NaOH. 100 mg of each seedling part (at least in 3 replicates) was homogenized in 10 ml of 100 mM phosphate buffer (pH 6.0) and centrifuged at 5000 rpm for 15 min. After filtration the pellet was homogenized with the 5 ml of buffer and the process repeated thrice for maximum recovery. All supernatants were combined so as to make the final volume to 25 ml. Each reaction set received 1 ml of the enzyme extract and 1 ml of casein solution and the pH was 10. The blank set received 1 ml each of enzyme extract, casein solution and 10% Trichloro acetic acid (TCAL) (cold). These sets were incubated at a temperature of $37 \pm 2^\circ \text{C}$ for 2.5 hr. 1 ml of 10% TCA (cold) was added to each reaction set after the incubation period was over and both the sets centrifuged. After discarding the residue 1 ml of filtrate was taken from each set and 2 ml of 0.5 N NaOH and 1 ml of 1 N Phenol Folin's reagent were added with immediate mixing. These sets were allowed to stand for 30 min and OD was taken at 540 nm in a ECI Junior Spectrophotometer. Protease activity was expressed in n mol of tyrosine equivalent $\text{hr}^{-1} \text{g}^{-1}$ tissue.

3. Results and discussion

Results have been summarized in tables 1-2 and figures 1-3.

3.1. Seedling growth in light and darkness

Table 1 shows that the growing axis did not differentiate 48 hr after soaking ('Initial' stage) but at 48 hr seedling stage roots and hypocotyls were noticed and the

Table 2. *C. tetragonolobus* showing protease activity (n mol) Tyrosine $\times 10^3 \text{ hr}^{-1} \text{g}^{-1}$ (fr. wt.) in different seedling parts in light and darkness.

Stages (hr)	Endosperm	Cotyledon	Hypocotyl	Root
Light				
Initial	2.772 \pm 0.036	2.592 \pm .249	*1.726 \pm .106	..
48	0.684 \pm 0.000	2.880 \pm .252	2.558 \pm .249	4.464 \pm .200
72	0.900 \pm 0.253	7.668 \pm .165	2.520 \pm .259	3.096 \pm .157
96	Trace	1.332 \pm .259	1.296 \pm .165	0.576 \pm .072
120	Trace	1.548 \pm .190	1.440 \pm .252	2.916 \pm .225
Dark				
Initial	34.128 \pm .374	59.148 \pm .655	*16.488 \pm 1.590	..
48	11.592 \pm .538	11.988 \pm .124	35.100 \pm 0.561	40.068 \pm .533
72	8.100 \pm .272	8.532 \pm .409	2.772 \pm 0.036	4.068 \pm .252
96	0.792 \pm .060	1.224 \pm .252	0.648 \pm 0.000	3.060 \pm .095
120	0.432 \pm .000	1.728 \pm .286	1.836 \pm 0.000	1.404 \pm .124

* Data represents growing axis as hypocotyl and root did not differentiate.

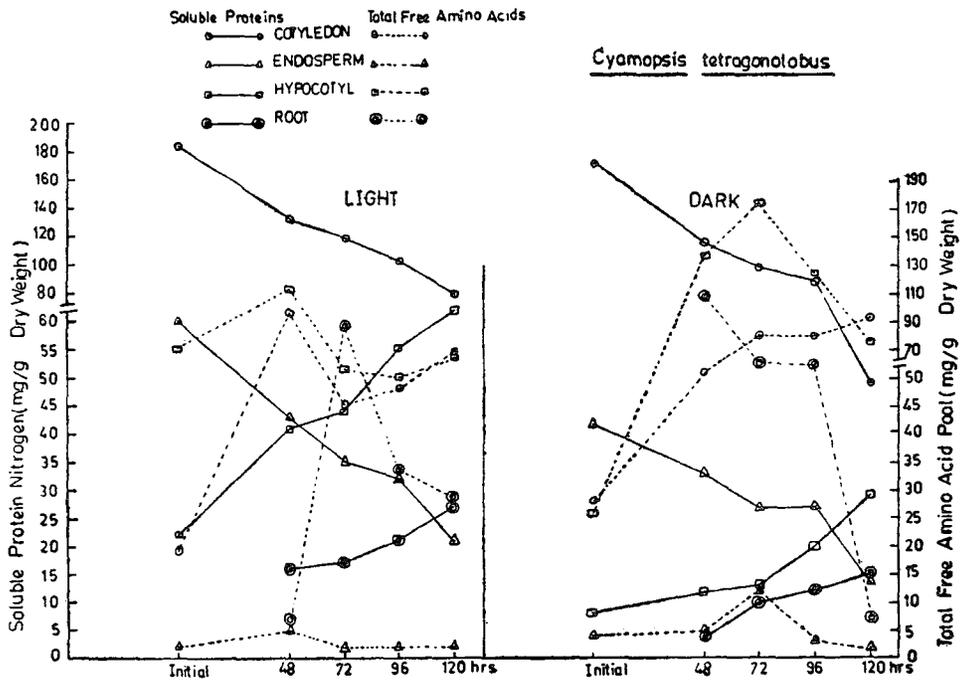


Figure 1. *Cyamopsis tetragonolobus* : showing levels of soluble protein—N and total free amino acid pool in light and darkness in different seedling parts at various growth stages.

length of hypocotyls was greater in darkness than in light. At 120 hr stage the hypocotyls length of dark grown seedlings were 181.60% more than those raised in light. Root growth also exhibited the same pattern as recorded for hypocotyls but the increase ranged between 15 to 25% in dark as compared with light.

As regards the fresh weight changes, the endosperm was of greater weight in darkness than in light at 'initial' stage. But with further seedling growth, at 120 hr stage, the value in dark was lower than in light. Changes in percent dry-mass showed a different pattern in that the endosperm from seedlings raised in dark had always a higher value than those in light irrespective of the seedling growth stages (table 1). Cotyledons at 120 hr stage also had more fresh weight in light than in dark. Moreover, percent dry mass although initially more or less of the same value in light and dark decreased much less with further growth in the dark.

Hypocotyls, after differentiation, show more than three-fold increase in fresh weight in dark compared with those in light. However, the dry weight values were slightly lower in the former. Roots exhibited a small decrease in their fresh weight both in light and dark.

Growth data presented here illustrate the two common phenomena of photomorphogenesis and etiolation in light and dark. Dark-grown seedlings having stimulatory effects on hypocotyl lengthening recorded a linear increase and direct relationship with the fresh weight but percent dry mass exhibited inverse relationship indicating the failure of translocation of the products of reserve hydrolysis to keep pace with the extension growth.

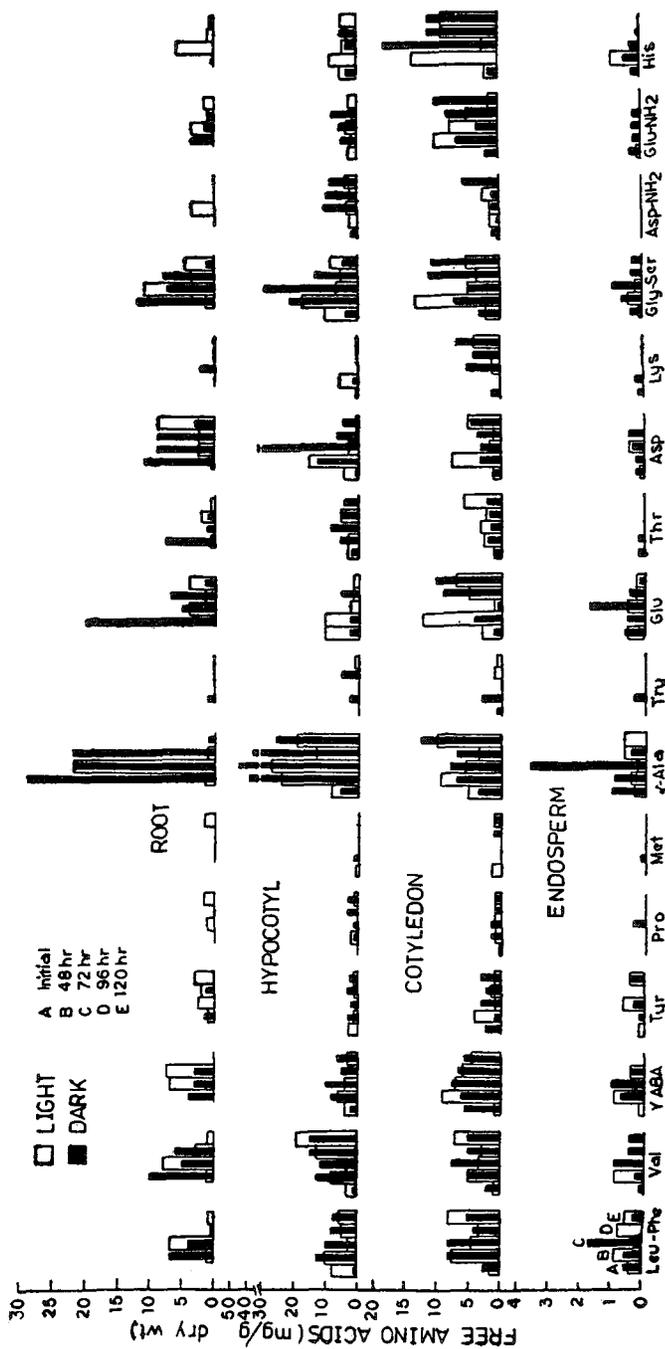


Figure 2. *C. tetragonolobus* : Free amino acid changes in different seedling parts at various growth stages.

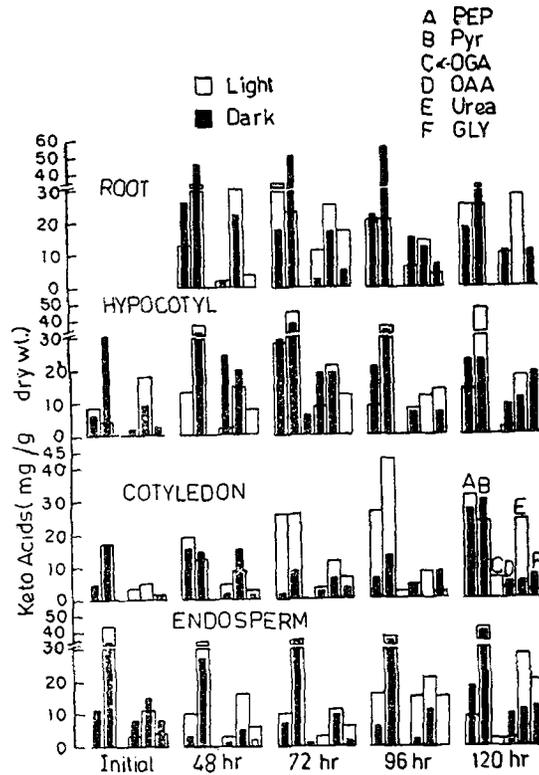


Figure 3. *C. tetragonolobus*: Keto acid changes in different seedling parts at various growth stages. Abbreviations: PEP, phosphoenolpyruvate; PYR, pyruvic acid; GLY, glyoxylic acid; a-OGA, a-oxoglutaric acid; OAA, oxaloacetic acid.

3.2. Biochemical studies

The studies with this endospermic legume revealed that the endosperm had low soluble protein values initially which further decreased with seedling growth in both light and dark (figure 1). Endosperm samples from dark treatments recorded a higher number and amount of free amino acids, when compared to corresponding light samples. In light, levels of free amino acids remained low during early stage but dark-raised endosperm samples recorded increasing values up to the 72 hr stage, whereafter they declined (figure 1). Glutamic acid, α -alanine, leucine-phenylalanine, serine-glycine, glutamine and histidine dominated quantitatively in dark-raised endosperm samples (figure 2).

The breakdown of proteins could also be detected in cotyledons of both light and dark grown seedlings. However, the depletion was more in dark since light causes a retention of proteins as mentioned earlier (Rai and Laloraya 1967; Mukherjee and Laloraya 1979). Along with the protein depletion although an enhancement in the free amino acid pool was expected in endosperm and cotyledons, their marked difference and a large increase in the latter suggests different rates and pattern of accumulation in light and dark and a rapid translocation of these

metabolites (figures 1-2). The hydrolysis of endosperm reserves by enzymes released from the aleurone layer and their absorption by cotyledons followed by the translocation to the growing axis has also been noted by Bewley and Black (1978). Moreover, unequal rate of protein breakdown and free amino acid formation in relation to light and darkness will also influence the transport of amino acids to the growing axes. Further, many-fold increase in the free amino acids in the hypocotyl during seedling growth in dark (figures 1-2) may be due to restricted capacity of dark grown seedlings to convert all the amino acids into proteins as also noticed by Srivastava and Kooner (1972) in *Phaseolus aureus* L. Oota *et al* (1953) while studying the changes in the content of various primary metabolites in germinating *Vigna sesquipedalis* beans showed a decline of these metabolites in the cotyledons while the hypocotyls and roots recorded an increase during early germination for six days. It is also proposed that the growing axes of light grown seedlings are capable of amino acid biosynthesis by amination of carbon skeleton produced in photosynthesis and this process accounts for increase in amino acid levels (Bewley and Black 1978). There is a differential concentration of amino acids in the hypocotyls under the two situations (figures 1-2).

Root samples in the light showed higher protein content in comparison to dark raised samples at all stages and a very marked increase was noticed in the total free amino acid pool at 48 hr stage. Further growth gave a sharp increase in the free amino acid pool in light whereas in dark the decline was more marked and could be observed from the beginning (figure 2).

Proline and methionine were not widely distributed in different seedling parts of *C. tetragonolobus*. Proline was recorded in cotyledons in both light and dark while endosperm and root contained the same at only few growth stages. Proline may be converted to glutamic acid thus increasing the pool size of this amino acid as reported by Bewley and Black (1978). Cysteic acid was not traced in endosperm and root at any growth stage of light and dark while cotyledon and hypocotyl recorded the same in very low concentrations at few growth stages. Methionine was also traced at few stages. γ -Aminobutyric acid was found in higher amounts in cotyledons of *C. tetragonolobus* in comparison to other seedling parts although it was of widespread occurrence in different organs. Its higher amount had been reported earlier also (Altschul 1958).

Changes in protease activity in various seedling parts in the light and darkness are shown in table 2. Dark-grown seedlings showed a significantly higher activity of this enzyme than those in light in all parts during growth. Initial stages of all seedling parts were unique in having maximum protease activity. The maximum decline in the activity was noticed in the endosperm and cotyledon which correlated with their protein depletion.

α -Oxoglutaric acid, the predominant keto acid, was recorded only at few growth stages mostly in low levels in both light and dark seedling parts of *C. tetragonolobus*. In cotyledons of light grown seedlings, the dominant keto acids were phosphoenolpyruvate and pyruvic acid (figure 3), the levels of which were maintained during seedling growth. Dark raised cotyledon samples had low values which declined further (figure 3). A characteristic feature of the keto acids was a rapid increase in their concentrations followed by later decline. Hypocotyls of light-raised seed-

lings maintained higher levels of phosphoenolpyruvate, pyruvic acid, oxaloacetate and urea up to 72 hr stage followed by a decline and then a small increase, while in dark although above-mentioned keto acids could be detected in higher concentrations, levels of hydrazones recorded a gradual decline after 72 hr stage (figure 3). Higher levels of keto acids could also be found in roots. Low levels, in most of the samples of oxaloacetate, the keto analogue and precursor of aspartic acid like α -oxoglutaric acid, can be explained by their rapid utilization during seedling growth. The tendency for accumulation of oxaloacetate especially at 120 hr stage in endosperm, cotyledon and hypocotyl of dark-raised samples may suggest that this keto acid was rapidly utilized in the light-induced growth of seedlings. It has been suggested by Webb and Fowden (1955) that accumulation of keto acids is related to sluggish rate of protein synthesis thus sparing the utilization of keto acids for the synthesis of amino acids.

Higher amounts of keto acid hydrazones of urea in light and dark in *C. tetragonolobus* account for its active role in nitrogen metabolism during seedling growth (figure 3). Asparagine and glutamine, the two common amides, which store excess ammonia to get rid of the toxic compound, recorded higher amounts from hypocotyl of dark grown seedlings in comparison to light while root samples from dark contained no detectable glutamine and asparagine content declined with seedling growth (figure 2). Cotyledons of 120 hr seedling stage were unique in exhibiting accumulation of asparagine and glutamine in dark in comparison to those in light thus sparing the utilization of these compounds in protein synthesis which is affected by dark.

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