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Stimulation of nitrate reductase activity by delta amino levulinic acid in excised maize leaves[1]

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Summary. Supply of 0.2 mM delta amino levulinic acid to maize leaf segments in light increased nitrate reductase activity, total organic nitrogen and to some extent chlorophyll also, but it had no effect on glutamate dehydrogenase or peroxidase activities, in the absence or presence of nitrate or ammonium as nitrogen source.

Amino levulinic acid (ALA) is believed to be the rate-limiting precursor for the synthesis of tetrapyrroles such as chlorophylls, heme and bile pigments[3,4]. It stimulates the synthesis of chlorophyll in dark-cultured *Euglena gracilis*[5] and of protochlorophyllides in etiolated wheat[6] and barley[7] leaves. Radioactive ALA is incorporated into peroxidase tetrapyrroles in light, in cultured peanut cells[8]. However, prolonged treatment of plant material with ALA results in a decreased phytochrome content[9]. As a precursor for chlorophyll synthesis, ALA can affect biogenesis and metabolism of chloroplasts, which in turn may affect other plant processes also. However, the effects of ALA on processes other than chloroplast metabolism are also known. For example, it causes swelling of mitochondria from barley leaves[10] and stimulates oxygen uptake in etiolated wheat leaves in the dark[10]. To examine whether the effect of ALA extended to other plant processes as well, the assimilation of nitrate and ammonium and relevant enzyme activities in excised maize leaves, were studied.

Experimental procedure and results. *Zea mays* L. (cv. Ganga safed-2) seeds were purchased from National Seed Corporation, New Delhi. Seeds were surface sterilized, washed thoroughly and grown in continuous light (supplied by 100 W incandescent bulbs and 40 W fluorescent tubes) of about 65 W m^{-2} density for 10 days at 25 ± 2 °C, with modified $^1/_2$ strength Hoagland's solution without nitrogen. Primary leaf segments from uniformly grown seedlings were incubated in $^1/_4$ strength Hoagland's solution, containing either no nitrogen or 10 mM KNO_3 or NH_4Cl as nitrogen source. Delta amino levulinic acid (purchased from Sigma Chemical company, St. Louis, Mo.) was added as indicated at a concentration of 0.2 mM and the incubation was carried on for 24 h at 25 °C, either in light or in dark. The pH of the nutrient solution and of incubation medium was 6.0. Total chlorophyll, peroxidase, in vivo nitrate reductase activity and NADH specific aminating glutamate dehydrogenase activity were measured by the methods of Strain and Svec[11], Maehly[12], Srivastava[13], and Singh and Srivastava[14] respectively. Nitrogen was measured by a modified micro-kjeldahl method[15], of 80% ethanol soluble and insoluble fractions separately. Protein was calculated by multiplying the insoluble nitrogen value by a factor of 6.25[16]. Values are expressed as their mean ± SE for 3 experiments. The paired t-test (between the replicates of −ALA and +ALA) was applied to evaluate the significance of effect of ALA on different parameters.

In the light, the exogenous supply of ALA to excised maize leaves increased protein content, total nitrogen and total chlorophyll slightly (table 1). The increase in protein in the presence of nitrogenous salts was however, insignificant. In the dark also, some increase in protein and organic nitrogen contents was observed in control or in KNO_3. A slight increase (14–29%) was observed in total chlorophyll also, during ALA supply. In light, supply of ALA increased total and specific activities of nitrate reductase significantly and this stimulation was more pronounced in control or KNO_3 than in NH_4Cl leaves (table 2). However, it had either no effect, or inhibited the activity of NADH-glutamate dehydrogenase slightly. The activity of peroxidase increased slightly in the control but was unaffected in the presence of KNO_3 or NH_4Cl. In the dark, supply of ALA did not increase either total or specific activity of nitrate reductase significantly. Total activity of nitrate reductase increased significantly with the supply of glycine, glutamate, glutamine and alpha ketoglutarate as well (table 3). The effect of glutamate was most pronounced and the effects varied slightly with the variation in concentration of glutamate or alpha ketoglutarate.

Discussion. The stimulating effect of ALA on nitrate reductase activity and nitrogen assimilation is reported for the 1st time. The assimilated nitrogen is partitioned more in the non-proteinaceous fraction in the presence of ALA, as the increase in protein was often insignificant. The stimulation was more apparent when the source of inorganic nitrogen was nitrate than when it was ammonium. Apparently, the increase in inorganic nitrogen assimilation is through nitrate reductase activity, as it is believed to be the rate limiting enzyme in nitrate assimilation pathway[17]. Further, the increase in nitrate reductase activity appears to be specific, as another important enzyme of the pathway, the NADH glutamate dehydrogenase, was little affected. Effect on peroxidase, an enzyme unrelated to nitrogen metabolism pathway but containing tetrapyrroles, was also generally insignificant.

Nitrate reductase is a sensitive enzyme and its activity is regulated by several nutritional and environmental factors[18]. Amino levulinic acid may increase its activity either by some direct activation process or through some indirect products. As a precursor of tetrapyrroles, it may increase nitrate reductase level by inducing the cytochrome b component of the enzyme complex. In our experiments, ALA did not increase nitrate reductase activity in the dark, when

Table 1. Effect of delta amino levulinic acid on some nitrogenous components of maize leaf segments floated on nutrient solution containing KNO_3 or NH_4Cl as nitrogen source, in light or in dark

Parameter	mg g^{-1} fresh wt Control −ALA	+ALA	KNO_3 −ALA	+ALA	NH_4Cl −ALA	+ALA
	In light					
Protein	10.6 ± 0.33	11.2 ± 0.32 (5)	13.9 ± 0.45	17.6 ± 2.7 (27) n.	13.4 ± 2.4	14.2 ± 0.63 (7) ns
Total nitrogen	2.07 ± 0.06	2.45 ± 0.13 (18)	3.11 ± 0.32	4.36 ± 0.32 (40)	3.05 ± 0.01	3.72 ± 0.02 (22)
Total chlorophyll	1.06 ± 0.06	1.23 ± 0.08 (16)	1.30 ± 0.05	1.34 ± 0.05 (3)	1.28 ± 0.03	1.42 ± 0.02 (11)
	In dark					
Protein	5.58 ± 0.14	7.85 ± 1.07 (41) ns	8.23 ± 0.95	9.87 ± 0.35 (20) ns	9.80 ± 0.04	9.87 ± 0.35 (1) ns
Total nitrogen	1.11 ± 0.07	1.42 ± 0.01 (27) ns	1.83 ± 0.19	2.18 ± 0.09 (19)	2.23 ± 0.05	2.32 ± 0.12 (4)
Total chlorophyll	0.69 ± 0.07	0.79 ± 0.05 (14) ns	0.89 ± 0.06	1.13 ± 0.08 (27)	0.87 ± 0.06	1.13 ± 0.09 (29)

Leaf segments were floated on ¼ strength Hoagland's solution containing the desired nitrogenous salts in the presence or absence of ALA either in light or in dark for 24 h at 25°C. Percent increase in the presence of ALA is given in parentheses. ns, effect of ALA insignificant at $p = 0.05$.

Table 2. Effect of delta amino levulinic acid on enzyme activities in maize leaf segments floated on nutrient solution containing either KNO_3 or NH_4Cl as nitrogenous source, in light or in dark

Enzyme activity	Nitrogenous salt Control −ALA	+ALA	KNO_3 −ALA	+ALA	NH_4Cl −ALA	+ALA
	In light					
Nitrate reductase, nmoles NO_2, h^{-1}						
a) g^{-1} fresh wt	952 ± 80	2110 ± 40 (121)	2040 ± 80	5160 ± 100 (153)	2770 ± 80	4190 ± 190 (151)
b) mg^{-1} protein	89 ± 8	189 ± 3.5 (212)	147 ± 11	313 ± 44 (213)	208 ± 2	298 ± 24 (142)
Glutamate dehydrogenase, nmoles NADH min^{-1}						
a) g^{-1} fresh wt	733 ± 20	729 ± 81 (−1) ns	819 ± 34	756 ± 42 (−8) ns	756 ± 26	704 ± 24 (−7) ns
b) mg^{-1} protein	69 ± 0.9	65 ± 3.3 (−6) ns	59 ± 2.3	47 ± 1.8 (−21) ns	57 ± 2.5	50 ± 2.1 (−13) ns
Peroxidase, OD min^{-1}						
a) g^{-1} fresh wt	16 ± 0.7	20 ± 0.7 (19)	22 ± 0.3	25 ± 1.3 (16) ns	23 ± 1.1	24 ± 1.3 (4) ns
b) mg^{-1} protein	1.52 ± 0.05	1.72 ± 0.04 (13)	1.57 ± 0.06	1.44 ± 0.2 (−8)	1.70 ± 0.2	1.65 ± 0.01 (−3)
	In dark					
Nitrate reductase, nmoles NO_2, h^{-1}						
a) g^{-1} fresh wt	623 ± 40	652 ± 45 (5)	1169 ± 50	883 ± 30 (−24)	776 ± 80	756 ± 70 (−3) ns
b) mg^{-1} protein	113 ± 7.4	86 ± 9 (−24) ns	146 ± 15	89 ± 4 (−40) ns	79 ± 7	77 ± 5 (−3) ns

Details as in table 1.

its assimilation into end products is limited[19]. This may indicate that assimilation of ALA into a specific component of the nitrate reductase molecule is involved, either in normal or ALA-induced synthesis of the nitrate reductase molecule. Increase in organic nitrogen of leaves in the dark, without an increase in nitrate reductase activity, may be on account of accumulated ALA itself.

Glycine, glutamate, glutamine and alpha ketoglutarate are possible precursors of ALA synthesis in green plants[3]. It is possible that the biosynthetic route of ALA formation is reversible and these metabolites are formed during ALA supply. However, these metabolites are more active compounds in cellular metabolism than ALA itself, and have diverse metabolic fates. This realization prompted us to supply these compounds at a concentration higher than ALA. The effect of individual amino acids on nitrate reductase activity varies from plant species to plant species[18]. Glutamate and glutamine have no effect on enzyme activity in cultured soybean cells[20], while glycine and gluta-

Table 3. Effect of possible catabolic products of amino levulinic acid on nitrate reductase activity in maize leaf segments floated on nutrient solution containing either KNO_3 or NH_4Cl as nitrogen source, in light

Catabolite	Enzyme activity, nmoles NO_2 h^{-1} g^{-1} fresh wt Control	KNO_3	NH_4Cl
None	952 ± 80 (100)	2040 ± 80 (100)	2770 ± 80 (100)
Glycine 5 mM	1160 ± 140 (121)	2720 ± 190 (133)	3920 ± 270 (141)
Glutamine 5 mM	2090 ± 160 (219)	4790 ± 70 (234)	4160 ± 230 (166)
Glutamic acid 1 mM	6069 ± 230 (637)	7233 ± 440 (354)	6252 ± 540 (225)
5 mM	5088 ± 310 (533)	8949 ± 120 (438)	6463 ± 230 (232)
Alpha keto-glutarate 1 mM	3113 ± 210 (326)	4120 ± 220 (202)	7807 ± 150 (281)
5 mM	4501 ± 280 (472)	9125 ± 550 (447)	4481 ± 100 (162)

The excised leaf segments were floated on the desired solution for 24 h in light at 25°C. Values relative (× 100) to control are given in parentheses. The effect of each catabolite was significant at $p = 0.05$.

mine inhibit the same in cotton roots[21]. The variations possibly reflect the differences in either uptake and metabolism of individual amino acids or in the regulatory nature of the enzyme itself, in different species. Stimulation of in vivo nitrate reductase activity by alpha ketoglutarate is possibly due to an increased reductant (NADH) level through the stimulation of the tricarboxylic acid cycle.

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Definitive evidence for lack of phytosterol dealkylation in honey bees

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Summary. Neither developing honey bee larvae nor queens were able to convert dietary ^{14}C-desmosterol to cholesterol, nor was larval development of sterol metabolism affected when the inhibitor, 25-azacoprostane, was included in the worker bee diet. These results provide strong evidence that this phytophagous insect is unable to dealkylate phytosterols at the C-24 position in order to produce cholesterol.

Results from previous studies[1-3] on sterol utilization in the honey bee, *Apis mellifera* L., indicated that this phytophagous insect is incapable of dealkylating C_{28} and C_{29} phytosterols to produce cholesterol. In recent years, several species of phytophagous insects[4-6] have been found to be unable to make this conversion, although it had previously been held that the dealkylation and conversion of C-24 alkylated phytosterols to cholesterol was a trait common to phytophagous as well as many omnivorous insects[7]. The constant occurrence of low levels of cholesterol (from trace to 2.2% of total sterols) and desmosterol (from trace to 2.5% of total sterols), found in previous honey bee studies, regardless of the dietary sterol[1-3], suggested that a more definitive study should be carried out to determine whether conversion of the C_{28} and C_{29} phytosterols might occur at a very low level to produce these small quantities of desmosterol and cholesterol. Two experiments would lend themselves well to providing this information. First, feeding ^{14}C-desmosterol as the sole added dietary sterol in an artificial diet would result in easily measurable amounts of ^{14}C-cholesterol if the biochemical pathway for conversion of 28- or 29-carbon phytosterols to cholesterol is present in the insect[8,9]. Second, the addition of a potent inhibitor of the Δ^{24}-sterol reductase enzyme to the diet in combination with a 24-alkylated sterol would cause an accumulation of desmosterol in the insect sterols and perhaps adversely affect larval development if this pathway exists[10-12].

Test colonies were set up with 400 g of newly emerged 'Italian' bees (about 4000 bees) plus a mated laying queen and maintained in small hives in flight cages as previously described[13]. The chemically defined artificial diet was also prepared as in prior studies[13]. The [26(27)-^{14}C] desmosterol was purchased from Amersham Corporation, Arlington Heights, IL, USA[14] and purified by argentation column chromatography of the acetate and recrystallization. The sterol was then examined for radiochemical purity by counting areas of adsorbent scraped from thin-layer chromatography (TLC) plates in a Packard Tricarb-Liquid Scintillation Spectrometer and by trapping and counting fractions from the gas-liquid chromatography (GLC) effluent. For one test diet, ^{14}C-desmosterol (> 97% pure, sp. act. 209 cpm/µg) was coated at 0.1% (dry weight) on the dry dietary components with dichloromethane. A second test diet was coated with 0.1% (dry weight) of 24-methylenecholesterol in combination with 0.01% (dry weight) of 25-azacoprostane (prepared as published[11]). 24-Methylenecholesterol is converted to cholesterol through the intermediate desmosterol by insects having the capacity to convert 24-methyl sterols to cholesterol[15]. Two units were set up on each diet; 2 samples of prepupae (130 and 89, respectively) from the ^{14}C-desmosterol-fed colony, and 2 samples (127 and 129 prepupae, respectively) from the 24-methylenecholesterol plus 25-azacoprostane-fed colony were weighed and stored frozen until work-up for sterol analysis. The 2