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REGULATION OF ARGININE DECARBOXYLASE AND PUTRESCINE LEVELS IN *CUCUMIS SATIVUS* COTYLEDONS

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; cotyledons; cytokinins; gibberellins; arginine decarboxylase; putrescine; potassium; abscisic acid.

Abstract—Arginine decarboxylase (arginine carboxy-lyase EC 4.1.1.19) of *Cucumis sativus* cotyledons, has a pH optimum of 8.3 and a temperature optimum of 40°. Among the various plant hormones administered to excised cotyledons in culture, benzyladenine and its riboside were most effective in increasing the arginine decarboxylase activity and putrescine content. The enzyme activity and putrescine content were significantly increased on acid feeding of the cotyledons and decreased by KCl treatment. The KCl effect could be only partially reversed by benzyladenine. Abscisic acid inhibited cotyledon growth and also reduced arginine decarboxylase and putrescine levels. This effect was overcome by cytokinins. The half life of the enzyme using cycloheximide was 3.7 hr. Dibutyryl cyclic AMP and 5′-AMP also marginally stimulated the enzyme and putrescine levels. Mixing experiments indicate that there is neither a non-dialysable activator nor inhibitor of the enzyme.

INTRODUCTION

It is now well documented that in a number of biological systems, the increased synthesis and accumulation of polyamines preceded by enhanced activity of the enzymes mediating their formation are intimately associated with growth and development [1, 2]. In animals, augmented levels of the key enzymes of polyamine biogenesis, viz ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase—and elevated spermidine and spermine contents are encountered in regenerating rat liver [3], in developing rat brain [4], in tropic hormone-stimulated target tissues [5], on refeeding following starvation stress [6] and in natural and induced neoplasias [2, 7]. In many of these systems, a close parallelism exists between the enzyme and polyamine levels on the one hand and macromolecular metabolism on the other, implying a crucial role for the amines in developmental processes. In fact, a direct growth-promoting action of polyamines has been demonstrated in various microorganisms, animals and plant cells in culture [8, 9].

While the occurrence and distribution of di- and polyamines have been established in widely different taxonomic groups of plants [10], studies on the enzymes concerned with their biogenesis and the hormonal and other regulatory factors involved therein are meagre. Arginine decarboxylase, the first enzyme of the biosynthetic pathway (analogous to ornithine decarboxylase in animals) has been purified to homogeneity and characterized from seedlings of *Lathyrus sativus* [11]. *S*-Adenosyl-L-methionine decarboxylase activity has been detected in a few plants [10], pointing to the operation of a biogenetic sequence in plants similar to that in animals [9]. The growth promoting effect of polyamines shown in *in vitro* explants of Jerusalem Artichoke tubers [12], and the relationship established between K^+-status and putrescine levels in a number of plants [13] suggest some functional role for these amines in plants. However it has not been established whether arginine decarboxylase in plants is modulated by growth regulatory factors in a fashion similar to ornithine decarboxylase in mammalian systems. In the present study, we have chosen excised cucumber cotyledons growing in organ culture as a model system and investigated changes in the activities of arginine decarboxylase and putrescine levels in response to several phytohormones, inhibitors and stress conditions.

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RESULTS

Temperature and pH optima of arginine decarboxylase and putrescine levels

The enzyme exhibited a single pH optimum at 8.3 in 100 mM Tris–HCl buffer and had less than 30% of this activity below pH 6.5 and above pH 10. The optimal temperature for the enzyme at pH 8.3 was 40°. More than 50% of the activity was still retained at 50°.

Since putrescine levels exceeded even the total amount of all the other amines together (data not given), we have evaluated the fluctuation in the diamine levels as an additional index of arginine decarboxylase activity. The control levels of putrescine per pair of cotyledons after 3 days culture was 98 ± 13.4 nmol. In all experiments the absolute contents of the diamine have been given. When the diamine levels were expressed per mg protein a similar pattern of results was obtained due to the fact that during the culture period no significant change in the net protein content of cotyledons occurs. However, a marked increase in RNA and DNA content has been noted under these conditions (unpublished observations).

In studies employing various buffers as the culture medium in the pH range of 4–8 to determine optimal conditions for growth, responses of arginine decarboxylase and putrescine levels to various stimuli, 2 mM sodium phosphate buffer (pH 6.5) proved most satisfactory.

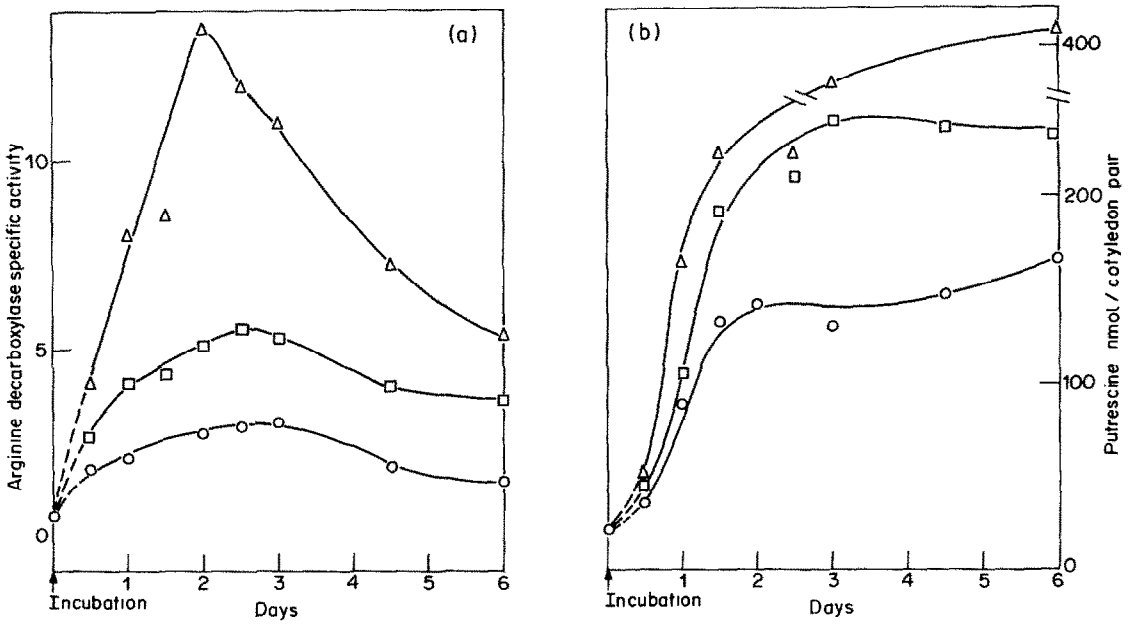

Fig. 1. Changes in *Cucumis sativus* arginine decarboxylase (a), and putrescine levels (b) with duration of culture. Controls (○——○), 22 μM benzyladenine (△——△), 46.5 μM kinetin (□——□).

Arginine decarboxylase activity and putrescine levels in response to varying hormone concentrations.

The sp. act. of arginine decarboxylase increased with the time of incubation under continuous light conditions reaching a maximum around 2.5 days and declining thereafter (Fig. 1a). Treating the cotyledons with benzyladenine and kinetin elicited a measurable increase in the enzyme activity and putrescine content as early as 12 hr after incubation. The sp. act. of the enzyme was progressively elevated in the cytokinin-treated tissue and around 2.5 days exhibited the maximum level over control values (*ca* 4- and 2-fold with benzyladenine and kinetin respectively). Despite subsequent decrease in the enzyme activity it always remained higher than that in the untreated cotyledons. The pattern of putrescine accumulation was slightly different in that the plateau levels reached at *ca* day 3 were maintained thereafter during the 6 day incubation period (Fig. 1b). Around day 3 following treatment

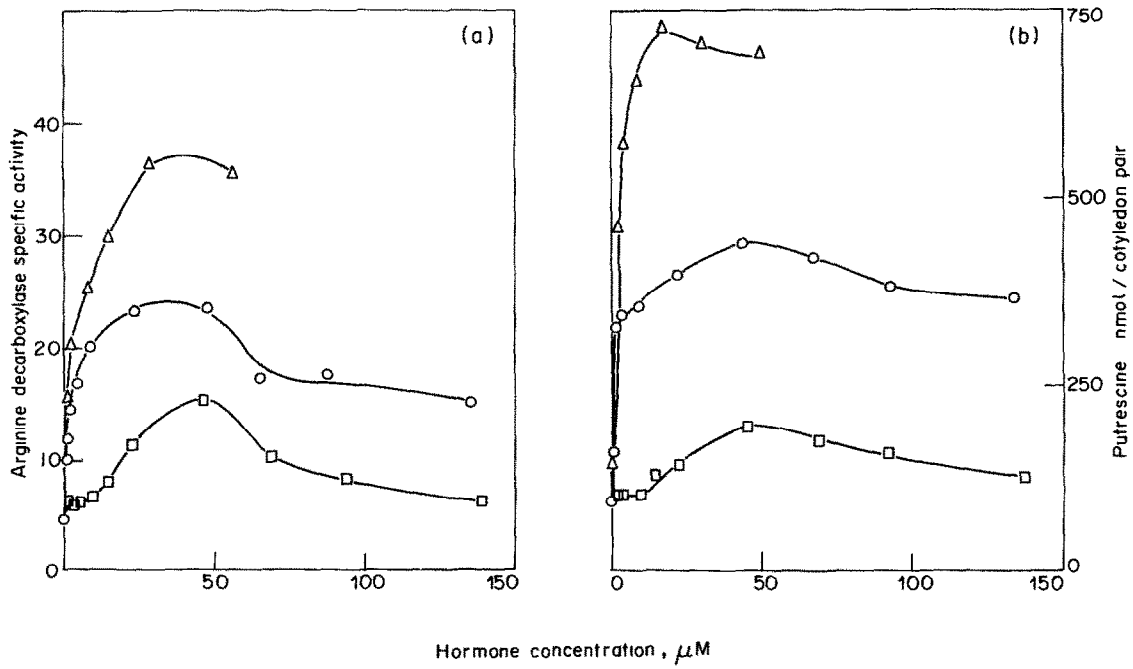

Fig. 2. The effect of varying hormone concentration on *Cucumis sativus* arginine decarboxylase (a) and putrescine levels (b). Benzyladenine (○——○). Benzyladenosine (△——△) and Kinetin (□——□)

with benzyladenine, a 2.5-fold increase in the amine content was registered while with kinetin the enhancement was of the order of 2-fold.

The response to increasing concentrations of the various cytokinins administered for a period of 3 days was dose-dependent with respect to the two parameters. There was a parallel progressive enhancement of the decarboxylase activity (Fig. 2a) and putrescine content (Fig. 2b) with initial increase in the cytokinin concentrations. Much higher concentrations exhibited a distinctly reduced response. At their optimum concentrations the sp. act. of the enzyme was 4.5, 6- and 3-fold greater than controls, with benzyladenine, benzyladenosine and kinetin respectively. The corresponding putrescine levels were approximately 4-, 4.4- and 2-fold respectively than in untreated cotyledons.

Gibberellic acid also stimulated the enzyme activity over a range of concentrations tested, and at 1 mM a 3-fold increase was registered (Table 1). Putrescine levels were augmented 2-fold under these conditions.

Effect of KCl and HCl feeding

During KCl-treatment and acid stress a dramatic influence on the growth on the one hand and on arginine decarboxylase and putrescine levels on the other were evident. KCl treatment (5 to 100 mM) markedly increased the fr. wt of the cotyledons without a corresponding change in dry wt. Higher concentrations of both KCl (> 100 mM) and acid (> 10 mM) were deleterious to the growth of cotyledons and precipitated necrosis and death. Even lower HCl concentrations inhibited growth as well as greening of the cotyledons in the presence of light.

A decrease in arginine decarboxylase and in putrescine content ($\times \frac{1}{3}$) were discernible on KCl treatment at 50 mM level (Fig. 3). At 75–100 mM salt concentrations, the putrescine levels were barely detectable. On the other hand acid treatment, despite being growth-inhibitory, nearly doubled the enzyme activity and putrescine levels (Table 1).

Table 1. Effect of gibberellic acid, nucleotides and H^+ on arginine decarboxylase and putrescine content of *Cucumis sativus* cotyledons

Effector	Arginine decarboxylase sp. act. pkat/mg protein	Putrescine content nmol/cotyledon pair
None	5.28 ± 0.69*	98 ± 13.4*
GA_3 10 µM	8.03 ± 0.40	186 ± 14
100 µM	10.43 ± 1.20	246 ± 23
1 mM	17.63 ± 2.53	267 ± 25
db-Cyclic AMP		
2 µM	9.50 ± 0.87	158 ± 6
20 µM	13.83 ± 1.35	143 ± 4
5′-AMP		
10 µM	7.38 ± 0.2**	144 ± 6
100 µM	9.33 ± 0.36	154 ± 5
HCl 5 mM	9.31 ± 0.4	198 ± 13
10 mM	11.28 ± 0.78	214 ± 12

*SD (n = 3). **Significant at $P < 0.01$. GA_3 = Gibberellic acid; db-cyclic AMP = dibutyryl cyclic adenosine monophosphate.

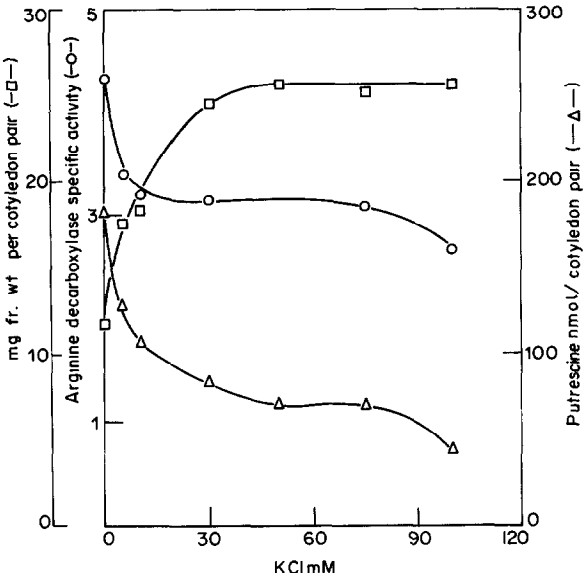

Fig. 3. The effect of KCl on arginine decarboxylase (○———○), putrescine levels (△———△) and fresh weight (□———□) of *Cucumis sativus* cotyledons.

Reversal of KCl inhibition by phytohormones

When 30 mM KCl was included in the culture medium along with varying concentrations of either benzyladenine, kinetin or gibberellic acid, the fr. wt increase was further enhanced. The differentiation of the cotyledons into succulent leaf-like structures showing prominent veins was even more marked. Among the above 3 hormones, benzyladenine brought about a complete reversal of the KCl-inhibited enzyme activity and reduced putrescine content to near normal levels (Table 2). Kinetin and gibberellic acid augmented arginine decarboxylase activity to control values. However, the corresponding putrescine levels were more or less unchanged. The reason for this is not clearly understood at present and may be due to the fact that enzyme levels are more sensitive to perturbations than are putrescine levels. It is also likely that K^+ is further affecting the metabolism of putrescine. It is pertinent to recall that the above hormones

Table 2. Reversal of KCl inhibition of arginine decarboxylase and putrescine levels of *Cucumis sativus* cotyledons by phytohormones

Treatment	Arginine decarboxylase sp. act. pkat/mg protein	Putrescine content nmol/cotyledon pair
None	5.28 ± 0.69*	98 ± 13.4*
KCl	2.68 ± 0.26	35 ± 4
KCl + BA 4.4 µM	4.60 ± 0.52	40 ± 7
KCl + BA 22.2 µM	4.73 ± 0.53	110 ± 9
KCl + Kinetin 23 µM	3.81 ± 0.51	45 ± 6
KCl + Kinetin 46 µM	4.26 ± 0.78	43 ± 4
KCl + GA_3 50 µM	4.06 ± 0.55	43 ± 5
KCl + GA_3 100 µM	4.00 ± 0.58	40 ± 4

*SD (n = 3). 30 mM KCl was used. BA = Benzyladenine; GA_3 = Gibberellic acid.

per se have a significantly positive effect on these parameters. However, on combined treatment the overriding inhibitory influence of K^+ on the amine metabolism is noteworthy.

Abscisic acid inhibition and reversal by phytohormones

Abscisic acid interfered with growth of the excised cotyledons even at 1 μM [16] as evidenced by a clear decrease in fr. wt and greening. A dose-dependent decline in arginine decarboxylase activity and putrescine levels on abscisic acid administration was clearly discernible and at 100 μM, about 65% and 50%, reduction respectively in the two parameters was noticeable (Table 3). The inhibitions in growth, enzyme activity and marginally reduced putrescine levels due to 50 μM absicic acid were overcome completely by as low as 5 μM benzyladenine or kinetin or 10 μM gibberellic acid (data not given). As could be expected, higher hormone concentrations, besides annulling abscisic acid inhibition, enhanced arginine decarboxylase and putrescine levels above control values, unlike the persisting inhibitory influence of KCl especially with respect to putrescine accumulation.

Effect of cycloheximide on hormone induced arginine decarboxylase and putrescine levels

To test whether the augmented total and sp. act. of arginine decarboxylase were due to a *de novo* synthesis of the enzyme protein, cycloheximide was included in the culture medium along with the phytohormones. This inhibitor of protein synthesis at concentrations above 20 μg/ml effectively checked the increase in enzyme activity and also of putrescine levels (Table 4). This was also true when the two cytokinins were present. Morphologically there was no greening of cotyledons on treatment with the inhibitor even in the presence of cytokinins.

Decay of arginine decarboxylase activity on treatment with cycloheximide, putrescine and KCl

The cotyledons previously exposed for two days to benzyladenine in order to stimulate basal arginine decarboxylase activity, were washed thoroughly and subsequently incubated for various periods of time with cycloheximide (25 and 50 μg/ml), putrescine (20 mM) and KCl (50 mM). The enzyme activity decayed with a $t_{\frac{1}{2}}$ of 3.7 hr, 3.0 hr and 10.7 hr respectively (Fig. 4). Both concentrations of cycloheximide gave the same $t_{\frac{1}{2}}$ for the enzyme. The enzyme recovered activity consistently after 24–36 hr of treatment with the inhibitors. It is interesting to note that, unlike the previous experiment where chlorophyll development was totally blocked, administration of cycloheximide to the above green cotyledons had no apparent morphological effect, like loss of chlorophyll, during the 36 hr incubation.

Influence of nucleotides

In view of the recent reports that cyclic AMP may be a mediator of action of some phytohormones [17], the dibutyryl derivative of the cyclic nucleotide was tested in this system. Arginine decarboxylase sp. act. was increased 2-fold with 20 μM dibutyryl adenosine 3′-5′ monophosphate and 70% increase with 100 μM 5′-AMP. The

Table 3. Inhibition by abscisic acid of arginine decarboxylase and putrescine levels of *Cucumis sativus* cotyledons and the reversal by hormones

Treatment	Arginine decarboxylase sp. act. pkat/mg protein	Putrescine content nmol/cotyledon pair
None	5.28 ± 0.69*	98 ± 13.4*
ABA 10 μM	3.18 ± 0.18***	88 ± 8†
50 μM	3.65 ± 0.45**	82 ± 8†*
100 μM	2.28 ± 0.18***	70 ± 8**
ABA 50 μM		
+ BA 22 μM	14.93 ± 2.23	209 ± 18
+ BA 44 μM	12.45 ± 1.15	240 ± 11
+ Kinetin 23 μM	9.05 ± 0.48	168 ± 12
+ Kinetin 46 μM	8.08 ± 0.4	209 ± 19
+ GA_3 10 μM	7.91 ± 0.41	139 ± 12
+ GA_3 50 μM	9.05 ± 0.4	138 ± 8

*SD (n = 3). **Significant at P = 0.02. ***Significant at P < 0.002. †not significant. †*significant at P = 0.1. ABA = Abscisic acid; BA = Benzyladenine; GA_3 = Gibberellic acid

Table 4. Inhibition by cycloheximide of stimulated arginine decarboxylase and putrescine levels of *Cucumis sativus* cotyledons by hormones

Treatment	Arginine decarboxylase sp. act. pkat/mg protein	Putrescine content nmol/cotyledon pair
None	4.8 ± 0.18*	83 ± 6*
CHI	1.0 ± 0.08	31 ± 5
BA	15.16 ± 2.16	161 ± 17
BA + CHI	1 33 ± 0.2	36 ± 4
Kinetin	8.5 ± 0.49	120 ± 9**
Kinetin + CHI	0.96 ± 0.06	30 ± 3

*SD (n = 3). **Significant at P < 0.01. Incubations in the above experiment were for 24 hr only. BA = Benzyladenine 22 μM; CHI = cycloheximide 25 μg/ml; Kinetin = 46 μM.

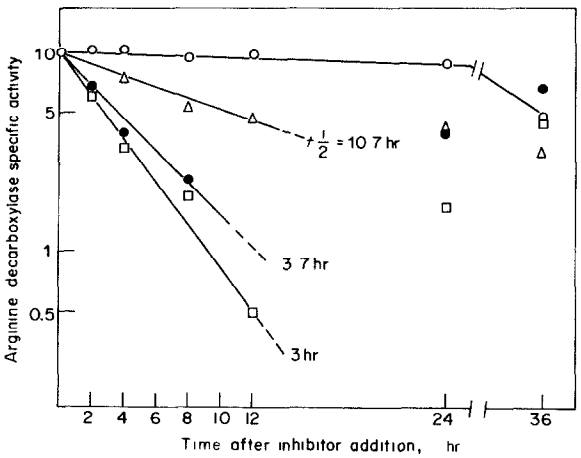

Fig 4. Half-life of arginine decarboxylase of *Cucumis sativus* cotyledons. Control (O——O), 50 μg/ml cycloheximide (●——●), 20 mM Putrescine (□——□) and 50 mM KCl (△——△). The $+_{\frac{1}{2}}$ data have been computed by regression analysis. Note log scale for enzyme activity.

Table 5. Effect of mixing crude extracts of untreated cotyledons with hormone, KCl and putrescine treated cotyledon extracts on the specific activity of arginine decarboxylase

Sl. No.	Treatment	Arginine decarboxylase sp. act. pkat/mg protein
1	None	5.95
2	BA 22 μM	16.63
3	Kinetin 46 μM	9.41
4	KCl 50 mM	3.00
5	Putrescine 20 mM	2.86
6	Abscisic acid 50 μM	3.43
7	CHI 25 μg/ml	1.48
	1 + 2	12.00 (11.29)
	1 + 3	6.73 (7.68)
	1 + 4	3.60 (4.46)
	1 + 5	5.01 (4.4)
	1 + 6	4.83 (4.68)
	1 + 7	3.33 (3.70)

Equal vols of control and treated extracts were mixed for 30 min at room temp. prior to enzyme assay. Incubations with hormones were for 3 days while those with KCl, putrescine and cycloheximide (CHI) were for 24 hr. Values in parenthesis are the expected sp. act. BA = benzyladenine.

corresponding increase in putrescine contents was marginal, being of the order of 35% and 27% respectively (Table 1).

Effect of mixing crude extracts of control cotyledons with treated cotyledons on the activity of arginine decarboxylase

On mixing equal volumes of the control crude extracts with those of variously treated cotyledons for 30 min at room temperature, the resultant sp. act. of arginine decarboxylase were merely the mathematical means (within experimental errors) of the individual values (Table 5). No greater inhibition was observed on mixing control crude extracts with those of cotyledons initially treated with benzyladenine and subsequently with cycloheximide, putrescine or KCl (as in the half-life experiment).

DISCUSSION

Prompted by the observation that the cytokinins promote growth and expansion of excised cotyledons of some plants including *Cucumis sativus* [16, 18, 19], we have exploited the cucumber cotyledons in organ culture as a model system to study the regulation of arginine decarboxylase and putrescine levels by phytohormones and other environmental factors. In the present study it has been consistently noted that the synthetic cytokinins viz benzyladenine and its riboside elicit marked responses in terms of expansion and greening of the cotyledons culminating in differentiation into leaf-like structures. It is noteworthy that this phenomenon is simulated by K^+ and inhibited by abscicic acid. Anatomical studies suggest that the differentiation brought about by cytokinin or KCl results in a dorsiventral leaf-like structure (unpublished observations).

The routine use of L-arginine-[U-^{14}C] as the substrate raises the possibility that $^{14}CO_2$ released during the enzyme assay may at least in part be contributed by the presence in the crude extracts of arginase + urease activities with the resultant release of $^{14}CO_2$ from the guanidino group rather than the carboxyl function alone as already found in *Cucurbita* seedlings [20]. However, the following observations completely rule out the above premise: (1) Omission of pyridoxal phosphate and/or dithiothreitol, the obligatory co-factors for arginine decarboxylase as the *L. sativus* enzyme [11] in the assay, did not result in measurable $^{14}CO_2$ release even in the presence of Mn^{2+} (1–10 mM), a cofactor for arginase activity [20]; (2) Substitution of DL-arginine-[1-^{14}C] (prepared from DL-ornithine-[1-^{14}C] by guanidination with *O*-methylisourea [21]) for L-arginine-[U-^{14}C] resulted in $^{14}CO_2$ release in exactly equivalent amounts when the assay was carried out either with control, K^+- or cytokinin-treated cotyledons. The latter observation also eliminates the possibility of any significant contribution of $^{14}CO_2$ during subsequent metabolism of the product agmatine by agmatine iminohydrolase and *N*-carbamylputrescine amidohydrolase activities [9] under the above assay conditions.

The presence of arginine decarboxylase and its modulation in concert with putrescine in response to various stimuli, provides presumptive evidence for the operation of the arginine → agmatine → putrescine pathway [9] in this plant system. The relatively high pH (8.3) and temperature (40°) optima exhibited by *Cucumis sativus* enzyme is in line with the properties of other plant arginine decarboxylases [11, 22]. The changes in the sp. act. of the enzyme always run parallel to those in total activity indicating that the modulation in the enzyme activity represents a true picture of the enzyme content, uninfluenced by the possible fluctuations in the total reserve protein contents of the cotyledons during incubation. It could also be shown that the changes in arginine decarboxylase activity due to hormonal or environmental influences are not accompanied by the appearance or disappearance of any non-dialysable enzyme inhibitor or activator, as revealed by mixing experiments. This observation was pertinent in view of the finding that an ornithine decarboxylase-specific protein inhibitor is induced by putrescine [23].

At the pH optimum of culture (pH 6.5), the enzyme attained maximum specific, as well as total activity between 60–72 hr and decreased thereafter and this pattern was qualitatively uninfluenced by the presence of hormones. Concomitantly, putrescine levels and fr. wt also increased up to 72 hr and then remained constant. The time-course of enzyme increase did not show dramatic changes even after 12 hr of cytokinin treatment, unlike fluctuations in ornithine decarboxylase on administration of the animal hormones [5]. This is understandable since the half-life of the plant enzyme was relatively longer (3–4 hr) when compared to that of the mammalian ornithine decarboxylase viz 11 min [24]. It is well established that at a given time the increase in enzyme activity is inversely related to the half-life of the enzyme when there is a general increase in protein synthesis [25]. The higher value for $t_{\frac{1}{2}}$ obtained with KCl may be due to the fact that protein synthesis is unaffected. Its action may be at a site other than inhibition of enzyme synthesis. The half-life of the enzyme obtained on addition of a protein synthetic inhibitor or excess of product was nearly the same and the slightly higher value with cycloheximide may be due to its inhibition of protein degradation as well. Such a comparable half-life for an enzyme obtained by employing cycloheximide and the end product hemin, in allylisopropylacetamide-induced rat-liver mitochondrial δ-aminolevulinate synthetase has been reported [26]. In

this system the decay of the enzyme activity was not as rapid with actinomycin D due to the fairly long half-life of the messenger RNA.

While the exaggerated amine elaboration accompanying growth and development in animal systems has been amply documented [2, 7], the existence of an analogous situation in plants is suggested by (i) augmented amine content in indole acetic acid-stimualted *Helianthus tuberosus* explants in culture [12], and (ii) indole acetic acid- and gibberellic acid-treated *Lathyrus sativus* embryos wherein arginine decarboxylase was significantly increased [27]. The present study with cucumber cotyledons substantiates this further and extends the same to cytokinins and to other environmental factors. In addition to cytokinins, dibutyryl cyclic AMP also enhanced arginine decarboxylase and putrescine levels in this system. The ability of the cyclic nucleotide *per se* to induce the parallel enzyme ornithine decarboxylase in animal systems wherein it mediates the action of a number of hormones is now well established [28–30]. Several reports provide evidence that cyclic AMP could also be the second messenger for auxins, gibberellins and cytokinins [17]. However since 5'-AMP could mimic cylcic AMP to some extent in the present study it is likely that both, being adenine derivatives, are functioning rather as cytokinin analogues, a view also advocated in the case of induction of betacyanin synthesis by cyclic AMP and cytokinins [31]

The data regarding the two stress conditions viz, K^+- and H^+-feeding, serve to emphasize that the obligatory relationship between accelerated growth and enhanced amine metabolism is not always valid. This is borne out by the observation that K^+ supply, while markedly stimulating growth of the excised cotyledons (as evidenced by enhanced RNA and DNA synthesis) drastically suppressed the corresponding levels of both the enzyme and putrescine levels. In line with earlier observations on barley seedlings [32] acid feeding resulted in a significant increase in both arginine decarboxylase and putrescine levels, but was unaccompanied by corresponding growth stimulation. This phenomenon was very striking when K^+ and hormones were supplied together. In this case while the two effectors functioned synergistically in terms of growth, the enzyme and putrescine levels remained unaltered and were comparable to the untreated controls at best. It is obvious that K^+ has an overriding influence in determining the levels of these two parameters in the cotyledons and that the cytokinins are only partially effective in annulling the inhibitory response. In contrast to this specific inhibitory effect of K^+ on amine metabolism, abscisic acid brought about generalized inhibition of not only arginine decarboxylase and the amine content, but also growth and tissue expansion. Abscisic acid inhibition was completely reversed by the phytohormones in line with the well established biological relationship among them [33].

EXPERIMENTAL

Materials. Seeds of *C. sativus* L. cv Guntur, were provided by Dr. M. Udayakumar of the University of Agricultural Sciences, Hebbal, Bangalore Putrescine 2HCl, benzyladenine, benzyladenosine, kinetin, gibberellic acid A_3, abscisic acid, 5'-AMP and dibutyryl cyclic AMP were products of Sigma. L-Arginine-[U-^{14}C], sp. act. 66 mCi/mmol, was purchased from Bhabha Atomic Research Centre, Trombay, Bombay, India. The sources of other chemicals employed have been referred to earlier [11].

Germination and growth conditions. Washed cucumber seeds were germinated in the dark on moistened Whatman No.1 filter paper discs placed on a wet cotton layer in Petri dishes in an incubator at 28° for 2 days. About 20–50 pairs of cotyledons excised from the embryo axes were transferred to each sterilized Petri dishes containing Whatman No.1 filter disc moistened with 4 ml of test solns freshly prepared in 2 mM NaPi (pH 6.5) The Petri dishes were incubated for various indicated periods in a sterile chamber equipped with two 40 W fluorescent tubes at *ca* 40 cm ht. The cotyledons were washed with H_2O and blotted before using for either enzyme assays or putrescine estimation

Arginine decarboxylase assay. Cell-free extracts of the cotyledons were prepared by grinding them with 2 ml of 50 mM Na_2HPO_4 containing 5 mM β-mercaptoethanol and 20 μM pyridoxal-5' phosphate in a chilled pestle and mortar Unless otherwise stated, all operations were carried out at 4°. The homogenates were centriguged at 25 000 *g* for 20 min and supernatants dialyzed against 3 changes of 200 vols of 10 mM NaPi buffer (pH 7.5) containing 2 mM β-mercaptoethanol and 20 μM pyridoxal-5' phosphate. Following dialysis, the extracts were clarified and the supernatants used for enzyme assay. Arginine decarboxylase activity in the cell-free extracts was determined as described elsewhere [11]. The assay mixture consisted of 100 μmol Tris–HCl buffer (pH 8.3), protein 0.5–2.0 mg, 2 μmol arginine (sp. act. 5.5×10^4 cpm/μmol), 100 nmol pyridoxal-5' phosphate and 2.5 μmol dithiothreitol in a total vol. of 1 ml and was incubated in a Dubnoff metabolic shaker at 40° for 2 hr. One pkat of decarboxylase activity is defined as the amount of enzyme catalyzing the release of 1 pmol of CO_2/sec. The sp. act. is represented as pkat/mg protein Protein was estimated by the procedure of ref. [14]

Putrescine estimation. Crude amine fraction was prepared from acid-soluble supernatant by BuOH extraction under alkaline conditions, purified on a Dowex 50 × 8 (50–100 mesh) column to remove traces of amino acids and quantitated with ninhydrin following resolution from other amines by circular PC with OH–HCl–KCl buffer (pH < 2, 4:1) as described in ref. [15].

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