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AMINE LEVELS IN *LATHYRUS SATIVUS* SEEDLINGS DURING DEVELOPMENT

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Key Word Index—*Lathyrus sativus*; Leguminosae; chick pea; growth and development; distribution in the embryo; DNA; RNA; protein; agmatine; putrescine; spermidine; spermine; homoagmatine; cadaverine.

Abstract—In growing *Lathyrus sativus* seedlings, the levels of DNA, RNA and protein markedly decreased in the cotyledons and progressively increased in the embryo-axis. In cotyledons, spermidine and spermine contents were substantially reduced while those of agmatine and putrescine were sharply increased. By contrast the embryo-axis progressively accumulated relatively larger amounts of agmatine, homoagmatine, putrescine, cadaverine, spermidine and spermine in parallel with similar changes in its DNA, RNA and protein content. While the cotyledons contained *ca* 50% of the total agmatine and putrescine present in the plant embryo by day 10, the embryo-axis, though representing less than 20% of the dry wt, contained 90 and 75% of total cadaverine and homoagmatine respectively of the seedlings. Spermidine and spermine levels of this tissue were also comparatively higher, being of the order of 80 and 50% respectively of the total. The root and shoot portions of the embryo-axis also exhibited a similar relationship between changes in DNA, RNA and protein and all the above amines during development. However, the polyamine content of the shoots was relatively higher than those of the roots during the growth period.

INTRODUCTION

In recent years, increasing attention has been given to the possibility that the polyamines spermidine and spermine play a significant role in the structure, function and biosynthesis of macromolecules like DNA, RNA and protein in a variety of biological systems, especially during growth and development [1–4]. In rapidly proliferating animal systems like chick embryo [5] and regenerating rat liver [6], increased accumulation and enhanced activity of anabolic enzymes of polyamines precede or parallel the changes in RNA levels. The observation that spermine or spermidine could accelerate RNA synthesis in chick embryo [7] and rat prostate [8] supports the suggestion that the polyamines are involved in nucleic acid metabolism. The available, though limited, evidence points to an analogous relationship between these amines and macromolecular metabolism in plant growth and development. Thus, in higher plants, polyamines promote protein synthesis *in vitro* and *in vivo* besides serving as a growth factor for certain plant cells in culture [9–11]. Further, Bagni [12] has shown that during the growth of *Phaseolus vulgaris* seedlings, spermidine and spermine decrease in cotyledons and simultaneously increase in shoots in association with similar alterations in the levels of RNA and protein.

We have earlier reported [13,14] that during development of *L. sativus* seedlings, substantial amounts of the guanidino amines agmatine and homoagmatine, the diamines putrescine and cadaverine, and the polyamines spermidine and spermine progressively accumulate. More recently, we have demonstrated [15] that agmatine and putrescine, on the one hand, and homoagmatine and cadaverine on the other, are connected by precursor-product relationships in this plant system. Of particular interest was the parallel accumulation of large amounts of cadaverine and putrescine during growth of the seedling and this raised the question of physiological significance of cadaverine in this plant. In this paper, we have investigated the distribution and the changes in the levels of all the amines in different parts of the plant embryo during development with a view to correlate the same

with the concomitant changes in the contents of DNA, RNA and protein.

RESULTS AND DISCUSSION

The data presented were obtained with etiolated *L. sativus* seedlings growing in the absence of exogenous nutrients and thus represent changes solely at the expense of nutrient reserves of the seed stored during embryogenesis. The changes in DNA, RNA and protein contents in the whole seedlings and their distribution patterns in isolated parts of plant embryo at various stages of development during an arbitrary period of 10 days are shown in Fig. 1. In whole seedlings (1a) the protein content progressively diminished up to day 7 and attained a steady state thereafter, corresponding to *ca* 55% of the initial value. The levels of DNA and RNA also markedly declined, but the steep fall was followed by a significant increase, particularly of RNA, during later stages. By day 10, the net reduction in DNA and RNA contents corresponded to *ca* 40 and 6% respectively of the initial values. During this period there was a net decrease in dry wt of the seedlings by *ca* 20% as expected, due to the metabolic activity of the embryo.

Figure 1b shows that the changes taking place in the cotyledons account for the diminution in the macromolecular contents in the whole seedling. This is to be expected from the fact that this tissue accounts for more than 75% of the embryo in terms of fr. wt, dry wt and protein nitrogen even at the end of the 10-day period. Thus the steep fall in the protein content of cotyledons throughout the growth period (by *ca* 62% by day 10), presumably due to intense proteolytic degradation of globulins stored in this tissue [16], is in agreement with the observation of Bagni with *P. vulgaris* [12]. Moreover, a sharp decrease (by 55%) of DNA content up to day 3 reaching a steady state thereafter, could probably be attributed to the lack of cell-division in cotyledons [17]. Again, RNA changes in this portion of the embryo reflected the corresponding pattern observed with the whole seedlings (Fig. 1a). Thus, it is clear that RNA content was reduced up to day 5 (by *ca* 55%) and then showed an upward trend, indicative of synthesis of RNA at later stages of development. However, even on day 10 there was a net decrease of RNA (by 30%) compared to that at the beginning of the experiment, in agreement with earlier observations with several other higher plant systems [12,17–19]. The only discrepancy between the present data and those of Bagni with light-grown *P. vulgaris* is that a continuous decrease in RNA content of cotyledons of *P. vulgaris* with enhancement of growth has been noted [12]. On the contrary, there is now substantial evidence to show that cotyledons or endosperm are capable of RNA synthesis [20, 21]. In fact, a more recent study with *P. vulgaris* germinated in a nutrient medium revealed

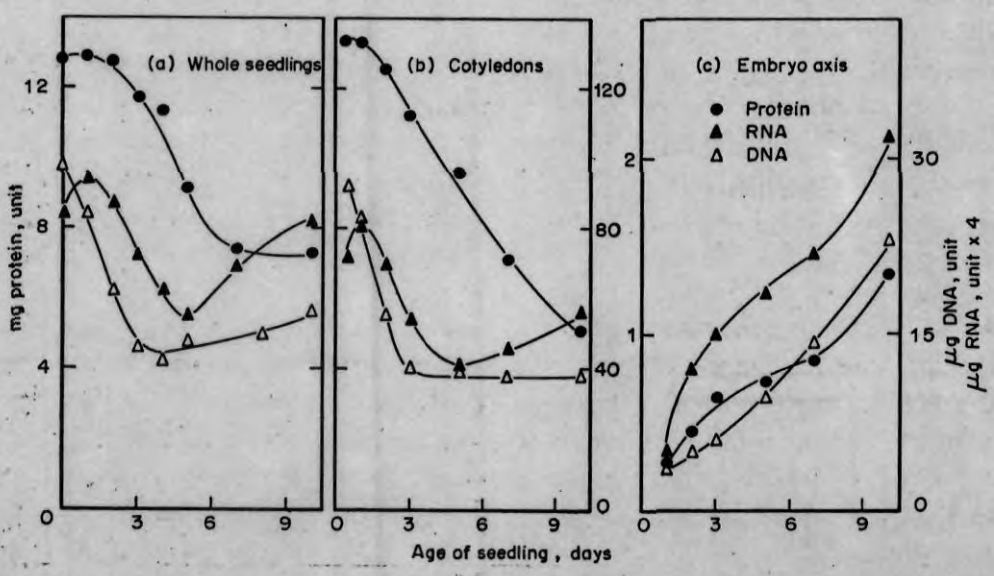

Fig. 1. Quantitative changes in protein, RNA and DNA contents of *Lathyrus sativus* seedlings with growth. Unit represents a whole seedling in (a), a pair of cotyledons in (b) and an embryo-axis in (c). Data represent mean value of 6 independent determinations and s.e. was less than 6% of the values indicated.

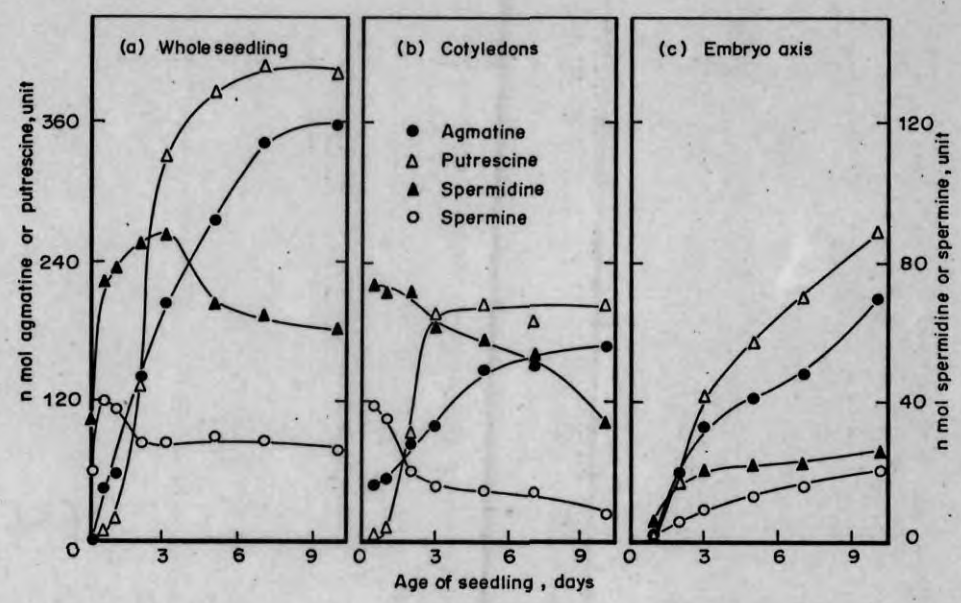

Fig. 2. Changes in the concentrations of agmatine, putrescine, spermidine and spermine in *L. sativus* seedlings with growth. For details see the legend to Fig. 1.

that during growth, a net accumulation of RNA occurred in the cotyledons [21]. Conditions of germination and seedling growth such as the presence of light, nitrate and sugar in the growth medium could influence macromolecular metabolism in cotyledons, as shown by studies with radish [20]. Another conceivable explanation for the apparent difference in RNA changes between cotyledons of *L. sativus* and those of *P. vulgaris*, apart from the influence of light, may lie in the fact that *L. sativus* seedlings undergo hypogeal growth, whereas *P. vulgaris* undergo epigeal growth. In contrast, there occurred a large and progressive increase in the levels of DNA (× 6), RNA (× 6) and protein (× 5) in the embryo-axis (Fig. 1c) throughout the growth period as expected of a tissue undergoing intense cell-division. The dry wt also was enhanced in parallel during this time from 1·0 mg/axis on day 1 to 16·2 mg/axis on day 10, but represented only 20% of the seedlings, even on day 10.

The data pertaining to the variations of amine levels during seedling growth reveal both the qualitative and quantitative changes in the pattern of amine distribution among different parts of the plant embryo (Figs. 2 and 3). The amines which are connected by precursor-product relationship [15] have been examined in separate groups. In the whole seedlings agmatine and putrescine concentrations rose dramatically with growth reaching by day 10 levels which were *ca* 100 and 200-fold higher than the initial values (Fig. 2a). However, the corresponding changes in polyamines followed a different pattern. Though there was a significant net enhancement in the contents of spermidine and spermine by day 10, increases were marginal, being of the order of 25 and 70% of the initial values respectively. Furthermore, both the compounds after a significant initial steep increase underwent gradual diminution during the later stages of embryo development, in agreement with previous observation [12]. The changes of amine levels in the cotyledon tissue (Fig. 2b), were similar to the changes occurring the the whole seedling for agmatine and putrescine. Concurrently, there was a progressive rise in agmatine content throughout the growth period, while the putrescine level increased steeply up to day 3 and attained a steady state thereafter. By day 10, agmatine and putrescine contents were increased by 50 and 100-fold respectively over the values of ungerminated stage accounting for nearly 50% of those accumulating in the whole seedlings (Fig. 2a). The significance of this enhanced elaboration of agmatine and putrescine in cotyledons, despite its being primarily a storage tissue, is unclear. Besides serving in the developmental processes of embryo-axis through translocation mechanisms, it is conceivable that the amines serve an important function in cotyledons themselves. Our earlier studies have shown that *de novo* synthesis of polyamines can be demonstrated in this embryonic tissue [15]. Moreover, synthesis occurs of metabolically active RNA

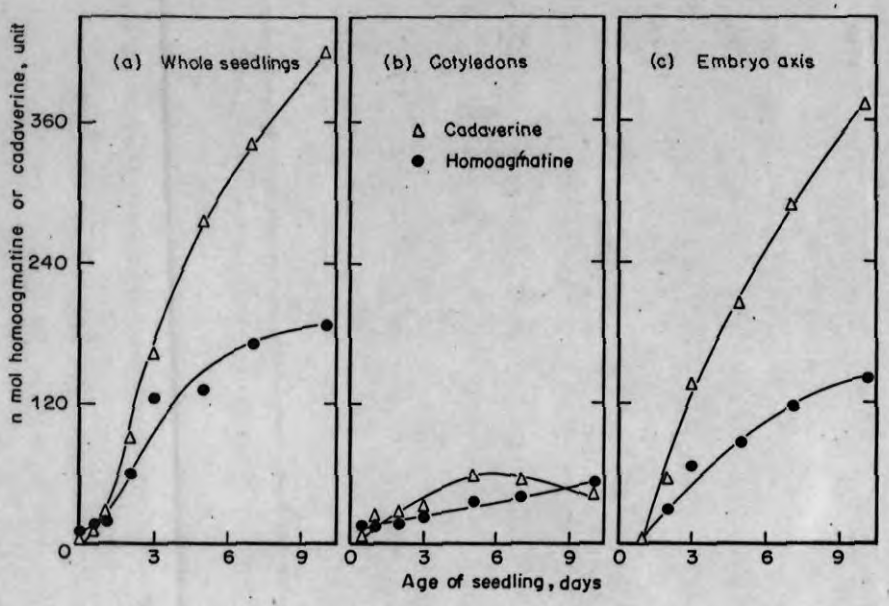

Fig. 3. Variations in the amounts of homoagmatine and cadaverine of *L. sativus* seedlings with growth. Data for homoagmatine was taken from ref. 13. For details see the legend to Fig. 1.

in cotyledons of legumes and endosperm of cereals during germination and growth [20,21] and the data presented in Fig. 1b are suggestive of such synthesis in *L. sativus* cotyledons at later stages of development. It has now been established that putrescine in particular, may have an important role in the maintenance of intracellular pH such as in extreme K^+ deficiency [22]. It is therefore tempting to speculate that the large-scale degradation of macromolecules (especially RNA and DNA) in cotyledons might cause an imbalance in intracellular H^+ concentration and the concomitant putrescine accumulation may be a compensatory mechanism. A different type of polyamine change in cotyledons was evident during this period in that both spermidine and spermine levels (after a lag of 2 days) decreased steeply at first and gradually thereafter attained values which corresponded to 20 and 40% (resp.) of the initial values. Qualitatively similar profiles have been observed with *P. vulgaris* [12] for the polyamine changes during seedling development.

The concurrent alterations in amine levels in the embryo-axis exhibited an entirely different pattern (Fig. 2c) consistent with enhanced elaboration of all the three macromolecules examined (Fig. 1c) as expected of a rapidly growing system. Thus agmatine, putrescine, spermidine and spermine all increased continuously and at day 10, their levels representing 54, 76, 4 and 12-fold increase respectively over those on day 1. These data support the hypothesis that an intimate relationship exi between these amine levels and the macromole lar metabolism in this plant system also.

In Fig. 3 are presented data which reveal a qua titatively different pattern of distribution of cad verine between the two parts of the seedlings du ing the growth. The changes in homoagmati content (data from [13]) is also included for co parison. The observation that in the whole see lings (Fig. 3a) the contents of both the amin were markedly increased during seedling grow by *ca* 19 and 220-fold respectively is in agreeme with our earlier findings [13,14]. While the mag tude of increase in the level of cadaverine w similar to that of putrescine, agmatine content w nearly double that of homoagmatine on day However, the contribution of cotyledons in ter of total contents of homoagmatine and cadaveri in the whole embryo (Fig. 3b), was relatively sma being of the order of 10 and 25% respectively pattern different from that obtained in the case agmatine and putrescine (both *ca* 50%). An anal sis of the corresponding data for the embryo-a showed that in this tissue, the levels of both the amines was greatly increased during developm (cadaverine × 140-fold; homoagmatine × 40-fo accounting for *ca* 90 and 75% of total contents the respective amines in intact embryo. The signi cance of this quantitative difference in the distrib tion of agmatine and putrescine on the one han homoagmatine and cadaverine on the oth

Table 1. Dry wt, protein, RNA, DNA and amine contents in shoot and root during growth of *L. sativus* seedlings

	Amounts/unit*							
	Day 3		Day 5		Day 7		Day 10	
	S	R	S	R	S	R	S	R
Dry wt (mg)	2·40	2·50	4·50	3·20	7·90	4·20	12·60	5·00
Protein (mg)	0·25	0·29	0·33	0·41	0·43	0·56	0·64	0·97
RNA (μg)	29·10	28·10	34·20	37·40	40·10	40·50	66·40	67·50
DNA (μg)	2·80	2·70	4·30	4·70	9·00	6·00	12·60	7·90
Agmatine (nmol)	52·50	41·10	73·10	64·50	93·60	77·60	126·80	101·70
Putrescine (nmol)	64·00	69·70	119·40	101·10	147·10	112·30	204·00	95·80
Spermidine (nmol)	15·30	4·40	14·80	6·60	12·40	10·00	14·40	8·70
Spermine (nmol)	8·40	trace	10·30	trace	13·70	trace	21·80	2·10
Homoagmatine (nmol)	32·60	32·50	62·90	38·80	71·40	66·00	79·50	53·60
Cadaverine (nmol)	78·00	69·30	132·60	85·50	204·40	104·60	274·40	98·50

* Unit is one shoot (S) or one root (R). Values represented are mean values of six independent experiments. S.e. is less than 6%. Day refers to the age of the seedling.

between the two tissues of the growing plant embryo is not clear.

In view of the almost parallel enhancement in the contents of the two groups of aliphatic amines with similar increases in the macromolecular contents of the embryo-axis, it was of interest to extend these investigations to the two parts of this tissue, viz shoot and root with differential growth rate during the seedling development. The seedlings were harvested on 3, 5, 7 and 10 days and the distribution and contents of the above amines and corresponding levels of DNA, RNA and protein in roots and shoots were examined. The results obtained are summarized in Table 1. On day 3, both parts of the embryo-axis attained almost equal growth as reflected in dry wt and their contents of DNA, RNA and protein. The only significant qualitative difference between the two parts was in terms of their polyamine contents, but the reason for this is not clear. As development progressed, the dry wt, DNA and RNA contents of the root also increased in parallel by *ca* 2-fold while concomitant increases in agmatine and spermidine were of similar order of magnitude. The increments in the levels of diamines and homoagmatine (*ca* ×1·5) were less. Spermine appeared in measurable quantity in this tissue only around day 10. During this period, the epicotyl grew faster, in that its dry wt increased 5-fold, DNA level by 3-fold while RNA and protein contents increased *ca* 2-fold. Corresponding net increases in the levels of the various amines were: spermine (×2·5), putrescine and cadaverine (×3), and homoagmatine and agmatine (×2·5). Only spermidine remained more or less constant during this time, presumably due to its enhanced continuous conversion to spermine which increased significantly during this time. Thus it is clear that in general, there is substantial parallelism between the levels of these amines and the macromolecular contents even of different parts of the developing embryo, in complete agreement with observations made with a variety of biological systems [4].

A significant feature of the present investigation is the intriguing observation that a close relationship exists between the site and magnitude of cadaverine accumulation and the contents of nucleic acids and protein in the rapidly growing portion of the plant embryo. This suggests that this diamine, like putrescine and polyamines, might fulfil some important function in the vital processes concerned with the plant embryo development. The fact that even within the growing tissue this diamine is distributed in different parts and its levels altered in association with those of important macromolecules like DNA, RNA and protein reinforces this postulate. While the *in vivo* function of cadaverine in the macromolecular metabolism is still unknown, this amine has been shown to effectively substitute for putrescine and polyamines in increasing melting temperature of DNA, in stabilizing urea-damaged bacteriophage T_5 and in stimulating poly-U directed poly-phenylalanine synthesis in a cell-free protein synthesizing system [4]. Furthermore, cadaverine or putrescine fed to ears of wheat have been shown to increase nucleic acid

contents of seeds and enhance subsequent growth rate of resultant seedlings [23]. While in developing chick embryo, an increase in the levels of cadaverine along with those of putrescine and the polyamines during growth has been noticed earlier [24], to the best of our knowledge *L. sativus* appears to be the first higher plant system in which cadaverine accumulation has been shown to be closely linked to macromolecular contents of a rapidly proliferating tissue, during embryonic development and growth.

EXPERIMENTAL

Materials. Lathyrus sativus seeds were procured from the Plant Breeding Section of Indian Agricultural Research Institute, New Delhi. Putrescine. 2HCl, spermidine. 3HCl, agmatine SO_4, calf thymus DNA, yeast RNA and crystalline bovine serum albumin were purchased from Sigma, St. Louis, Mo. U.S.A. Cadaverine. 2HCl and spermine. 4HCl were obtained from California Foundation for Biochemical Research, Los Angeles. Ion exchange resins were from Bio-Rad Laboratories. All the amines were purified by chromatography on ion exchange resins and crystallized prior to use. RNA and DNA were also purified before use.

Germination and growth of L. sativus *seeds.* Healthy seeds of uniform size were germinated after surface sterilization with 0·5 M NaOCl and the seedlings were grown in dark on moist filter papers in sterile Petri-dishes under aseptic conditions in an incubator at 25–28° as described earlier [13]. The cotyledons were excised by a cut at the point of attachment of the embryo-axis to the tissue and the shoots and roots were separated from the embryo-axis by cutting at the joint of their attachment in the axis. 10–15 Whole seedlings or isolated parts were harvested at specified periods of growth, and used for dry wt and chemical determinations of the macromolecules and the amines.

Acid soluble fraction was prepared according to the method in ref. 13. Briefly, *L. sativus* seeds or seedlings were homogenized with 3 vol. of cold 0·4 M $HClO_4$, cooled overnight at 0–2° centrifuged at 4° and the residue was washed × 2 with 1 vol. of cold 0·4 M $HClO_4$. The $HClO_4$ ppts were used for the estimation of DNA, RNA and protein. The acid extracts were pooled and adjusted to pH 8 with 40% KOH. The precipitated $KClO_4$ was removed by centrifugation. The clear extract represented acid soluble fraction.

Estimation of proteins. The $HClO_4$ ppt. was heated with 10 ml of 0·4 M $HClO_4$ at 90° for 20 min to degrade the nucleic acids, the residue was washed by centrifugation × 2 with 5 ml of $HClO_4$ at 4°. The sediment was then extracted with 5 ml of M NaOH by warming at 90° and centrifuged. The insoluble residue was again extracted as before with 3 ml of 1 M NaOH. The supernatants were pooled, neutralized to pH 7 with HCl and made up to 50 ml. An aliquot was analyzed for protein by the method of Lowry *et al.* [25] using bovine serum albumin as standard.

Nucleic acids were estimated by the procedure of ref. 26. The $HClO_4$ ppt. was washed by centrifugation at room temp. × 2 with each (4 ml) of the following: Cold EtOH saturated with NaOAc, EtOH–$CHCl_3$ (3:1), EtOH–Et_2O (3:1) and Et_2O, to remove lipids. The dry residue was then treated with 6 ml of 0·3 N KOH at 37° for 16–17 hr to degrade RNA, then acidified with $HClO_4$ (1 M) to a final concentration of 0·4 M, cooled overnight and centrifuged. The residue was then washed by centrifugation twice with 2 ml aliquots of cold 0·4 M $HClO_4$. The supernatants combined and made up to a known vol. and A_{260} was measured. Purified yeast RNA taken through the same procedure, served as the standard. Polypeptide contaminants contributing to A_{260} in the RNA extract of the seedlings was measured by the procedure of Lowry *et al.* [25] and suitable correction for this was made in calculating the RNA content of the plant embryo.

For estimating DNA, the residue after alkali digestion and washing with $HClO_4$, was heated with 6 ml of 0·4 M $HClO_4$ at 90° for 15 min to degrade DNA and centrifuged. DNA content was calculated by estimating P content of an aliquot of the above extract by the procedure of ref. 27. Calf thymus DNA taken through the above procedure was used as the standard.

Quantitative estimation of the amines. From the acid-soluble fraction the amines were isolated and quantitated by the procedure described in ref. 28.

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