

Level of endogenous free amino acids during various stages of culture of *Vigna mungo* (L.) Hepper – Somatic embryogenesis, organogenesis and plant regeneration

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A comparative study on endogenous free amino acid levels was conducted in embryogenic and regenerating tissues *vis-à-vis* regenerated shoots of *Vigna mungo* PS1. A number of important amino acids were found to increase during initiation of embryogenesis and regeneration. To study the effects of exogenous application of amino acids in this system, media were supplemented with three amino acids, tryptophan (Trp), serine (Ser) and proline (Pro). We found that in the presence of proline, a stress-associated amino acid, there was an enhancement in shoot-bud regeneration as well as in the production of somatic embryos. We also analysed the endogenous levels of amino acids in these tissues. This communication thus deals with the induction of globular embryo by auxin and its promotion by proline in *V. mungo*.

GROWTH and regeneration *in vitro* is a complex phenomenon and is influenced by a number of genetic and environmental factors¹⁻³. As every species seems to have its own specific requirements, there are several reports about the substances and conditions which help cells to differentiate. Amino acids have been shown to be effective morphogenetic regulators in a number of species⁴⁻⁹. Media have been supplemented frequently with amino acids or hydrolysed proteins in tissue culture to enhance growth of explants, although some amino acids may also be inhibitory¹⁰. Differences in endogenous amino acid pools have been found to affect the rate of protein synthesis during morphogenetic processes such as embryogenesis^{11,12}, seed germination¹³ and organogenesis¹⁴. Claparols *et al.*¹⁵ reported the incidence of endogenous amino acid metabolism when embryogenic calli were externally supplemented with amino acids. An earlier report from this laboratory has shown that in *Brassica juncea* cultures, exogenous application of amino acids like methionine and threonine enhanced cell proliferation, whereas leucine and isoleucine promoted differentiation⁶. Several other

studies have demonstrated stimulation of somatic embryogenesis by addition of L-proline (Pro), L-serine (Ser), L-glutamine (Gln), etc. into the medium^{5,15}. Changes in the free amino acid pools have also been associated with the metabolic state, which controls various growth processes in a homeostatic manner¹⁶. Proline, in particular, has been shown to have specific biological function during morphogenesis and in providing osmotolerance in antisense transgenic *Arabidopsis thaliana*⁹.

The present study was designed to evaluate the endogenous levels of different amino acids associated with a particular state of *in vitro* development, i.e. embryogenic or regenerating tissue and regenerated shoots of *Vigna mungo* (L.) Hepper, one of the most important pulse crops of India. Plant regeneration from *V. mungo* callus has been found to be difficult for a long time¹⁷. However, successful multiplication of nodal buds and shoot-bud formation in mature cotyledon in this system have been reported recently^{18,19}. In order to understand the effect of amino acids on *in vitro* differentiation in this system, media were supplemented exogenously with three of these amino acids, Trp, Ser and Pro. The response of the tissues on these media and the endogenous levels of amino acids in these tissues were also studied. We report, in *V. mungo*, a promotion of embryogenesis by exogenous supply of proline.

Seeds of *V. mungo* var. PS1 were collected from the Pulse Research Laboratory, Indian Agricultural Research Institute, New Delhi, India. Healthy seeds were washed thoroughly with a dilute solution of Teepol (5%) for 10 min and rinsed in running tap water. Thereafter seeds were surface sterilized with mercuric chloride solution (0.1%) followed by repeated washings (3–4 times) with sterile double-distilled water. Seeds were allowed to imbibe water for 18–20 h and were inoculated on basal medium for germination.

For *in vitro* induction of somatic embryogenesis, two types of explants, primary leaves (of 2–4 day-old etiolated seedling) and mature cotyledons were used. Complete cotyledons were placed in a way with the abaxial side touching the medium. Basal medium was composed of MS basal salts²⁰ with B₅ vitamins²¹, referred as MSB in the text. Three auxin-type growth regulators, namely 2,4-D, 2,4,5-T and picloram were initially tried in varying concentrations (0.1–5.0 mg/l) for initiation of somatic embryogenesis. Percentage of explants showing formation of somatic embryos and mean number of embryos formed per responding explant were recorded. After initiation, globular embryos were subcultured on unsupplemented MSB or MSB supplemented with 0.1 mg/l of each of IAA and BAP, either in liquid culture or in culture solidified with 0.7% agar (BDH). The pH of the media was adjusted to 5.8 prior to autoclaving.

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Cultures were incubated at $25 \pm 1^\circ\text{C}$ under a 16/8-h day/night cycle, with an illumination of ca. $60 \mu\text{mol}^{-2} \text{s}^{-1}$ at the level of culture. Each experiment was repeated three times with ten replicates.

To initiate shoot buds, mature cotyledons were cultured in the presence of TDZ (0.01 and 0.05 mg/l) and plantlets were raised from cotyledon callus following the method reported earlier¹⁹.

Regenerating (MSB + 0.01 mg/l TDZ) as well as embryogenic media (MSB + 2 mg/l picloram) were supplemented with three amino acids, Trp, Ser and Pro (1 mg/l), individually. Explants (cotyledon and primary leaves) were inoculated on media containing individual amino acid as well as on control set (without amino acids). Explants were subcultured after every 28 days and maintained for three months to check the effect of amino acids on initiation of shoot regeneration as well as on somatic embryogenesis. Percentage of explants showing formation of somatic embryos and mean number of embryos formed per responding explant were recorded.

All experiments were repeated three times. Data were recorded as the mean \pm standard deviation. Different mean values of different treatments were compared with the control sets, a *t* test was performed and the level of significance was evaluated.

Three types of samples have been used for the analysis of endogenous free amino acids – (i) embryogenic tissue, (ii) regenerating tissue and (iii) shoots from four-week-old regenerated plantlets. Embryogenic tissue was sampled after 18–20 days of growth on MSB with 2 mg/l of picloram and consisted of globular to heart-shaped embryos, while shoot buds (15–18 days old) developed on cultures of mature cotyledons on MSB with 0.01 mg/l of TDZ were used as regenerating tissues.

Plant material (500 mg) was homogenized in 70% ethanol (4 ml/g fresh wt) and boiled for 10 min in air-tight vials for extraction of amino acids, following the method of Bielecki and Turner²². On cooling, the homogenate was centrifuged at 13,000 rpm for 3 min. The pellet was further extracted in water:chloroform:methanol (3:5:12 v/v) mixture. This was followed by centrifugation at 13,000 rpm for 3 min. To the combined supernatant, methanol:water:: (2:1) was added and centrifuged. The upper water-methanol phase was collected, dried and dissolved in 200 μl of deionized H_2O .

Fifty μl of standards and samples containing equal ratio of internal standard solution (0.4 mM methionine sulphone in 0.1 M HCl) were dried in vacuum and derivatized by 7:1:1:1:: phenyl-isothiocyanate: ethyl alcohol: water: triethyl amine (v/v). The mixture was incubated at room temperature for 20 min. The derivatized amino acids were analysed on a PICO-TAGTM column (Waters Div., Millipore Co, MA, USA) at 254 nm.

The solvents used and gradient programmes were according to the instructions of PICO-TAGTM analysis system (Waters Div., Millipore Co, MA, USA). The amino acid composition and quantification were determined by comparison with the retention time of standards by PDA software (Waters Div, Millipore Co, USA).

Mature cotyledons and primary leaf explants responded differently on MSB supplemented with various doses of 2,4-D, 2,4,5-T and picloram. Both the explants demonstrated a potential to form friable callus in the presence of 2,4,5-T as well as picloram. But 5–20% of mature cotyledonary explants showed white, friable and globular callus, followed by the formation of globular embryos in 2,4,5-T and picloram (Table 1). On the other hand, friable callus originated from primary leaf explants continued to remain in an undifferentiated state in any medium used. In the presence of 2,4-D, there was no induction of embryos in any medium from any explant, but the callus formed in this set from different explants showed initiation of roots after 15 days of culture. About 5–10% of mature cotyledons showed formation of globular embryos in the presence of 1–2 mg/l 2,4,5-T (Figure 1 a and b). The best response in terms of embryogenesis was found with cotyledon explants placed on MSB containing 2 mg/l picloram. Approximately 20% of explants showed formation of globular embryos in this set. Different explants thus exhibited differential response to form somatic embryos under a similar set-up in the present experimental system.

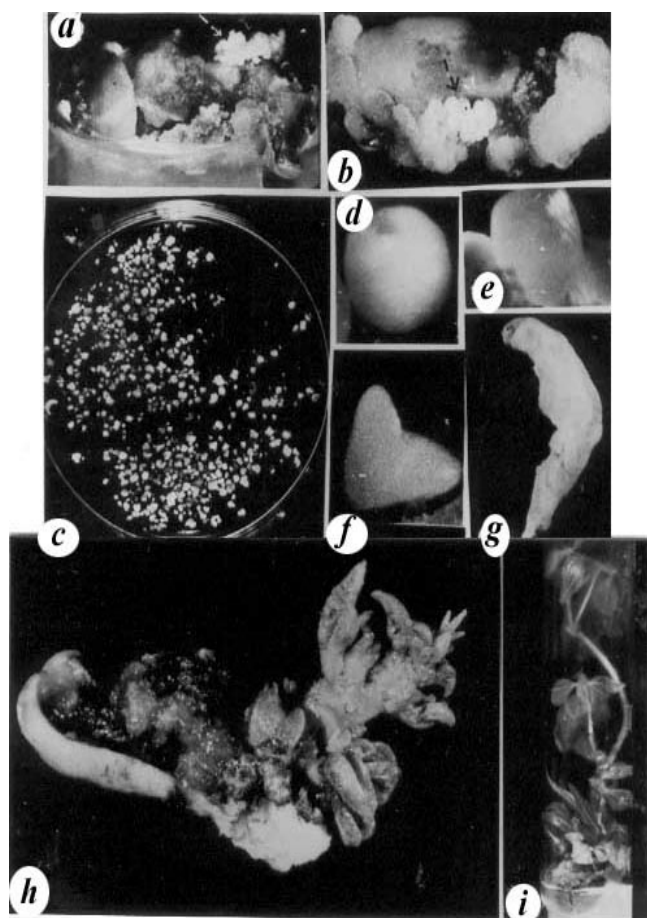
On transfer of embryogenic calli to liquid MSB or MSB supplemented with 1 mg/l of IAA and BAP, globular embryos grew into elongated and heart-shaped embryos and even showed differentiation of root as well as

Table 1. Effect of different auxins* on somatic embryogenesis of *V. mungo* PS1 from mature cotyledons

Auxin	Concentration (mg/l)	Percentage cotyledons forming embryos \pm SD	Mean number of embryo/cotyledon \pm SD
2,4,5-T	Control	sw	–
	0.1	c	c
	1.0	5.2 ± 1.2	4.3 ± 0.8
	2.0	9.8 ± 1.4	5.2 ± 1.1
	5.0	3.1 ± 0.9	1.2 ± 0.3
Picloram	Control	sw	–
	0.1	c	c
	1.0	13.4 ± 2.1	6.6 ± 1.5
	2.0	20.2 ± 1.8	6.9 ± 1.4
	5.0	04.0 ± 1.1	1.4 ± 0.9

*Only results from callus growing on 2,4,5-T and picloram are presented, since callus growing on 2,4-D did not show induction of somatic embryos.

Values are average of three replications each with 60 explants; significant at $P < 0.0001$ level, sw, swelling; c, callusing.



Figures 1. *a, b*, Clusters of globular embryos on cotyledon in picloram-containing medium; arrow shows embryogenic callus; *c*, Liquid culture of MSB showing growing somatic embryos at different stages; *d, e*, Globular embryos grown on cotyledonary leaf cultured on MSB supplemented with 2 mg/l of picloram; *f*, Heart-shaped embryo; *g*, Elongated mature embryo showing germination and differentiation of root and shoot apices in liquid MSB; *h*, Shoot-bud regeneration on mature cotyledon cultured on MSB supplemented with TDZ; and *i*, *In vitro*-regenerated shoots of *V. mungo* PS1.

shoot poles after 15 days of culture (Figure 1 *c-g*). Embryos could be best matured on basal medium without hormone (MSB) and eventually developed into plantlets, but the frequency of germination was found to be low (8–10%). Percentage of explants showing embryogenesis, mean number of embryos formed per cotyledon as well as growth and germination of embryo were found to be better when cotyledons were grown in the presence of picloram. At higher doses of picloram and 2,4,5-T (5 mg/l), there was a reduction in somatic embryo formation. Even development of embryos to further stages was inhibited, as the globular embryos were seen to callus after 20–22 days of culture.

The usual strategy to start embryogenic suspensions in *Daucus* is to expose the explants to a high concentration of auxin, which leads to the formation of proembryogenic mass²³. This mass and the globular embryos are insensitive to auxin as they do not produce auxin,

but the postglobular-stage embryos synthesize auxin and sensitivity is again regained. In contrast, in the present experiment, lower levels of auxin were found to induce somatic embryos from callus. At higher concentrations of auxin and on prolonged culture on auxin-containing medium, there was a reduction in the development as well as maturity of globular embryos. This is indicative of the fact that the culture condition in the present experimental system might maintain the intracellular auxin at a higher level. Similar results have also been observed in other legumes, where it has been shown that higher dosage of auxin reverted embryogenic culture to caulogenesis^{24,25}. Thus transferring embryogenic calli to medium with low auxin and cytokinin or to unsupplemented basal medium showed better maturation as well as germination of globular embryos, in the present case.

The potential of *V. mungo* PS1 to form somatic embryos *in vitro* has been demonstrated here, though it needs further experimentation to improve the processes of maturation as well as germination of these embryos.

In order to study the effect of amino acids on somatic embryogenesis in the present system, three of these individual amino acids, Trp, Ser and Pro, were added separately to embryogenic medium (MSB + 2mg/l picloram) (Table 2). With Trp or Ser, there was an initial increase in the response of explants for formation of globular embryos (71% and 40%, respectively) compared to the control. But 40–60% of these globular embryos did not show further maturation at a later stage and showed rhizogenesis. No shoot formation was recorded. On the other hand, in the presence of Pro, there was approximately an initial three-fold enhancement in embryo production (64%) and 40% of these embryos showed germination as well. There was no rhizogenesis in this set of media. Induction of globular embryo by auxin and its promotion by proline in this system was clearly evident. Further knowledge regarding better maturation and germination of embryos may make this system suitable for improvement of this species by biotechnological methods.

Table 2. Effect of amino acids (Trp, Ser and Pro) on embryo formation in *V. mungo* PS1

Medium	Percentage explant showing embryo formation \pm SD	Mean no. of globular embryos showing germination \pm SD
MSB + 2 mg/l picloram (P1)	20.50 \pm 1.8	15.4 \pm 1.1
P1 + Trp (1 mg/l)	71.66 \pm 2.4	Rooting
P1 + Ser (1 mg/l)	40.56 \pm 1.9	Rooting
P1 + Pro (1 mg/l)	64.85 \pm 1.5	8.0 \pm 0.8

Values are average of three replications each with 60 explants; significant at $P < 0.0001$ level.

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Shoot buds were initially regenerated from excised cotyledon explant in the presence of TDZ through the formation of meristemoid, following the method reported earlier¹⁹. TDZ-induced shoots were transferred to basal medium (MSB) for elongation and proper growth. The shoots were rooted in rooting medium and plantlets were raised, following the same method. To study the effect of individual amino acids on *in vitro* organogenesis in this system, the regenerating medium was supplemented with three individual amino acids, Trp, Ser and Pro (Table 3). There was an increase in the initial response of cotyledons to produce shoot buds (62.8%) compared to control in Trp-supplemented medium. But shoots did not elongate and became necrotic at later stages. Finally, lesser number of shoots could be raised in this set compared to that of the control. However, with Pro, there was an increase in shoot-bud formation as well as shoot regeneration compared to the control.

Table 3. Effect of amino acids (Trp, Ser and Pro) on shoot-bud formation in *V. mungo* PS1

Medium	Percentage cotyledon showing shoot regeneration \pm SD	Mean no. of shoot \pm SD
MSB + 0.01 mg/l TDZ (T1)	50.05 \pm 1.2	6.2 \pm 0.9
T1 + Trp (1 mg/l)	62.88 \pm 1.5	4.4 \pm 0.6
T1 + Ser (1 mg/l)	45.56 \pm 1.1	2.9 \pm 0.1
T1+ Pro (1 mg/l)	75.25 \pm 2.1	9.1 \pm 0.8

Values are average of three replications each with 60 explants; significant at $P < 0.0001$ level.

Table 4. Comparison of free amino acid contents in embryogenic, regenerating cultures and shoots from regenerated plantlets of *V. mungo* PS1

Amino acid	Embryogenic callus	Regenerating callus	Shoots from regenerated plantlets
Phe	3.15	2.61	2.54
Tyr	2.18	0.73	2.02
Trp	1.13	0.14	0.21
Thr	0.19	4.14	2.96
Met	0.74	2.74	1.19
Glu	5.03	5.85	4.39
Asp	3.51	3.47	3.58
Lys	3.78	2.22	6.50
Arg	0.50	0.28	0.92
Cys	10.85	13.96	12.40
Pro	2.59	3.83	2.93
His	5.55	12.03	11.23
Gly	15.49	16.86	17.99
Ser	32.23	13.99	19.68
Ile	4.29	9.08	4.79
Leu	1.72	0.88	0.90
Ala	4.34	5.36	4.64
Val	0.68	1.73	0.52

Values represent % content of each amino acid and each value is a mean of three experiments, $SD \leq \pm 0.05$.

The number of shoots formed per explant was maximum in the presence of Pro and there was also no inhibition of shoot growth at later stages (Figure 1 *h* and *i*). Addition of Ser did not improve shoot regeneration at all.

Requirement of exogenous supply of amino acids for *in vitro* somatic embryogenesis has been reported in a number of plant species^{6,15}. In the present system too, it was found that exogenous application of a specific amino acid, Pro, promoted the process of somatic embryogenesis and regeneration.

It has been proposed that the specific levels of endogenous-free amino acid are important in the regulation of plant growth and therefore their levels are precisely controlled in the cytoplasm^{12,16}. In the present study it was found that endogenous levels of amino acids varied during morphogenesis (Table 4). All important amino acids were found to be present in regenerated shoots as well as in embryogenic cultures. During embryogenesis (from globular embryo onwards), a few amino acids like Phe, Trp, Lys, Arg, Leu, Tyr, Asp and Ser, were present in higher levels compared to regenerated plantlets. However, during regeneration some other amino acids like Thr, Met, Glu, Cys, Pro, Ile, Ala, Val and His showed significantly higher levels. Such differences in endogenous amino acid pool during *in vitro* initiation of morphogenetic processes have also been reported earlier^{12,26}. A rapid increase in the total amount of amino acids has been reported during cell proliferation and early stages of somatic embryo formation in carrot cultures¹². In peanut somatic embryogenesis, similar multifold accumulation of amino acids has been reported during the induction phase, which has been suggested to be due to enhanced biosynthesis rather than reduced catabolism²⁶. Here too, an increase in the endogenous levels of different amino acids could be observed during induction of different morphogenetic processes.

Endogenous-free amino acid levels were analysed in embryogenic as well as regenerating cultures grown on media supplemented with amino acids Ser, Trp and Pro. A marginal increase in their endogenous content was found in regenerating tissue grown on medium supplemented with Pro, Ser and Trp, respectively. However, in case of embryogenesis increase in the endogenous levels of a particular amino acid was not so pronounced (Figure 2 *a-c*).

The need for exogenous supply of proline for induction of regeneration as well as somatic embryogenesis and increase in endogenous Pro content during embryogenesis have been reported in other plant species²⁷⁻²⁹. It was considered to provide organic nitrogen supply and protection against possible stress associated with *in vitro* culture and embryogenesis¹⁵. Similarly in the present system, Pro, which is a stress-associated amino acid, had a promotive effect in the processes of regeneration as well as somatic embryogenesis, when sup-

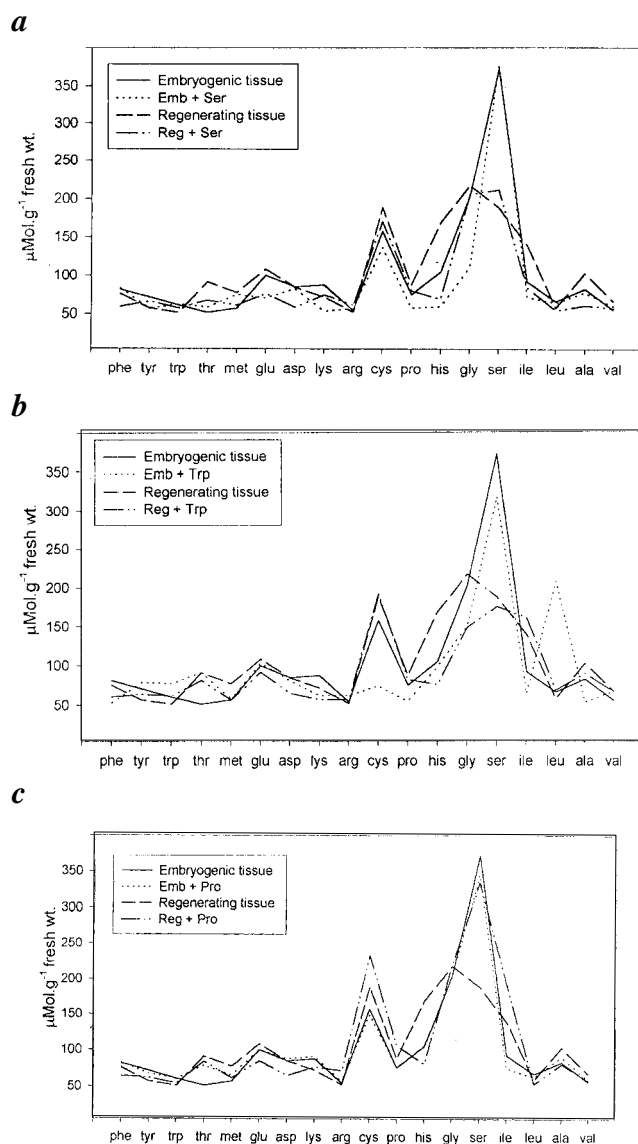


Figure 2. Amino acid profiles in embryogenic and regenerating cultures supplemented with (a) Ser, (b) Trp and (c) Pro.

plied exogenously. The levels of different amino acids present varied during different morphogenetic processes, indicating possibly the requirement of specific amino acids for specific events. However, definitive roles of these amino acids during the regeneration as well as somatic embryogenesis in *V. mungo* PS1 need to be further elucidated, including their mode of action leading to expression of specific genes.

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