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Effect of Culture Conditions on L-Dopa Accumulation in Callus Culture Of *Mucuna pruriens*

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ABSTRACT

Effect of various carbon and nitrogen sources, PGRs and their precursors, on the callus growth and L-dopa production by the stem and leaf derived callus of Mucuna pruriens were studied. Five different carbon sources were glucose, fructose, sucrose, mannose and raffinose. Glucose and sucrose were almost equally effective in both promoting the growth as well as L-dopa content. The four nitrogen sources were phenylalanine, tyrosine, glutathione and casein hydrolysate. Tyrosine was found to be most effective nitrogen source at 25 mgl^{-1}. Leaf derived callus showed more increase in L-dopa than stem. Phenylalanine (10 mgl^{-1}) along with tyrosine (25 mgl^{-1}) could raise the L-dopa content (i.e. % of dry weight) from 1.4% of control to 1.7%. Casein hydrolysate was not very effective in L-dopa production.

The PGRs tried were BA, Kin, Ad.SO_4; IAA, its precursor shikimic acid and an intermediary compound tryptophan; and gibberellic acid and its precursor mevalonic acid. 1.0 mgl^{-1} IAA was most effective in promoting both growth and L-dopa content, whereas gibberellic acid and mevalonic acid inhibited both L-dopa content as well as growth.

Incorporation of best nitrogen source (25 mgl^{-1} tyrosine) + most effective PGR (1 mgl^{-1}IAA) to maintenance medium enhanced the L-dopa production to 1.95%.

Key words: Auxin, Callus, L-dopa, 3-(3,4-dihydroxyphenyl)-L-alanine

INTRODUCTION

L-dopa has found wide application for the symptomatic relief of Parkinson's disease. Clinical work carried out by [1], using *Mucuna pruriens* seed powder (containing L-dopa) and synthetic Carbidopa; have shown that L-dopa from seeds was more active in curing Parkinson's disease. The Ancient Ayurvedic system of Medicine has also described medicinal use of *M. pruriens* for

the treatment of diseases of the central nervous system and also as geriatric tonic [2]. Its active ingredient L-dopa {3-(3, 4-dihydroxyphenyl)-L-alanine} is used as a remedy of mental disorder [3] (British Pharmacopoeia). Since *M. pruriens* is being used as source of L-dopa by many pharmaceutical companies in India, a continuous supply of L-dopa producing *M. pruriens* seeds are very much in demand. *M. pruriens* seeds are usually collected from forests, leading to problem of quality control specially of % of L-dopa content. The other hurdle in collection as well as cultivation of *M. pruriens* is the presence of trichomes on the pods, which gives a very strong itching sensation. Therefore attention has been diverted to *in vitro* culture of *Mucuna* on the same lines as that of the *Stizolobium* [4], which also contains L-dopa [5]. The present work is an attempt to culture the callus of *M. pruriens*. Since leaf and stem are rich in L-dopa content, they have been selected as explant to generate callus. Moreover, there has also been an effort to increase the production of L-dopa by callus cell; by incorporating various organic nitrogen and carbon sources into the medium. Efforts are further directed towards the use of different PGRs and their precursors for increasing the L-dopa content in the static culture cells.

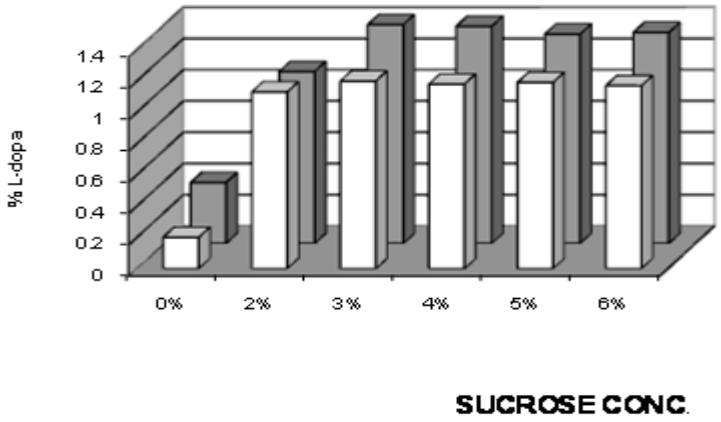
EXPERIMENTAL SECTION

Seeds of *Mucuna pruriens* obtained from Zandu Pharmaceutical Works were germinated on moist saturated filter paper for two weeks. From germinated seedlings 0.5 to 1 cm long stem and leaf segments were taken and surface sterilized using a dip in 70% ethanol followed by 5 min treatment with 2% NaOCl and three washes of 2min each with autoclaved distilled water. Then they were dipped in 2000 μg/ml Ciprofloxacin solution and inoculated.

Callus initiation: was done on MS basal medium [6] supplemented with 2 mgl^{-1} BA (Benzyl Adenine) + 3% sucrose + 0.8% agar at 25 ± 3^0C under 2500 lux light intensity for 16 hrs photoperiod followed by 8 hrs dark period. Initiated cultures were further maintained on MS basal medium supplemented with 2.5 mgl^{-1} 2,4-D (2,4-Dichlorophenoxy acetic acid) under same physical conditions.

To find out most ***suitable concentration of sucrose*** for callus growth as well as for L-dopa content 2 to 6% sucrose was incorporated into the MS medium in the initial set of experiments. Since 3% sucrose showed maximum increase in callus growth and L-dopa content (Fig.-1); it was used for all the experimental trials.

Figure 1. L-dopa content in leaf (gray) and stem (white) derived callus of *Mucuna pruriens* cultured on various concentrations of sucrose for 28 days.



Other ***carbon sources*** tried were glucose and fructose (monosaccharides), mannose (disaccharide) and raffinose (trisaccharide). Concentrations of all the five tried sugars were kept constant at 3%.

Different ***organic nitrogen sources*** that could have taken part in L-dopa biosynthesis cycle were incorporated into the media included phenylalanine (1, 2.5, 5, 7.5, 10 50 and 100 mgl^{-1}), glutathione (1, 5, 10, 20, 30, 40, 50, 100 and 200 mgl^{-1}) and casein hydrolysate (50, 100 and 200 mgl^{-1}).

In addition to these nitrogen sources combinations of (a) 10 mgl^{-1} phenylalanine + 50 mgl^{-1} glutathione; (b)10 mgl^{-1} phenylalanine + 25 mgl^{-1} tyrosine; (c) 50 mgl^{-1} glutathion + 25 mgl^{-1} tyrosine and (d) 10 mgl^{-1} phenylalanine + 50 mgl^{-1} glutathione + 25 mgl^{-1} tyrosine were tried

Various **plant growth regulators** i.e. kinetins, auxins and gibberellins and **their precursors i.e.** Adenine sulfate , tryptophan, shikimic acid and mevalonic acid, were incorporated into the medium for enhancing L-dopa production.

The concentrations of PGRs tried in the test media were BA (0.5, 1,2 and 5 mgl^{-1}), Kin. (0.5, 1, 2 and 5 mgl^{-1}), Adenine sulfate (0.5, 1, 2 and 5 mgl^{-1}), IAA (0.1, 0.5, 1 and 1.5 mgl^{-1}), tryptophan (Kin. (0.1, 0.5, 1, 2 and 1.5 mgl^{-1}), shikimic acid (0.1, 0.5, 1, 2 and 1.5 mgl^{-1}), GA_3 (0.1, 0.5, 1, 2 and 1.5 mgl^{-1}) and mevalonic acid (0.1, 0.5, 1, 2 and 1.5 mgl^{-1}).

100 mg of one month old callus grown on 2, 4-D (2.5 mgl^{-1}) containing medium was inoculated on the above test media. Callus was grown for one month at 25 ± 3^0C, 16 hrs photoperiod followed by 8 hrs dark period at and above 60% RH. For control callus was grown on the callus maintenance medium i.e. MS basal + 2.5 mgl^{-1} 2, 4-D.

After one month fresh and dry weight of callus and L-dopa content, using [7] method, with slight modification, was recorded.

For **analyzing L-dopa content**, to 120 mg of callus powder, 10 ml absolute ethanol was added, mixed thoroughly, kept for 15 min and then centrifuged at 1000 rpm for 5 min. Ethanol layer was discarded. To the sediment 0.1 N HCl was added and mixed thoroughly. This suspension was warmed, then diluted to 100 ml and filtered under vacuum. Filtrate was used for L-dopa estimation.

0.5, 1 and 1.5 ml of the filtrate were pipetted out in test tubes. To each test tube 2 ml 0.2% freshly prepared isoniazid (INH) solution was added and shaken well, allowed to stand for 10 min and then 5 ml of 20% sodium carbonate was added and mixed well. This solution was kept at room temperature for reaction and color development. Final volume was adjusted to 10 ml. Optical Density (OD) of the sample and standard L-dopa solutions were recorded at 476 nm.
For plotting standard curve, 10 mg of L-dopa (Sigma Chemicals) was dissolved in 100 ml distilled water to obtain 0.1 mg/ml L-dopa solution.

RESULTS AND DISCUSSIONS:

Effect of Carbon source on fresh weight*:* As it can be seen from the table 1 both stem and leaf explant derived callus showed best growth at 3% sucrose. Though at 4% there was as good accumulation of biomass (%dry weight) as at 3% sucrose; but fresh weight was significantly more at 3% sucrose.

Table 1.Effect of different concentrations of sucrose on the growth of stem and leaf derived callus of *Mucuna pruriens*, recorded after 28 days of culture. (Values are mean of 10 replica)

Sucrose Conc.	Fresh Weight (gm) of		Dry Weight (gm) of		% Dry Weight. of	
	Stem callus	Leaf callus	Stem Callus	Leaf callus	Stem callus	Leaf callus
0%	0.184 ± 0.001	0.193 ± 0.001	0.010 ± 0.0003	0.010 ± 0.0002	5.43	5.18
2%	1.219 ± 0.017	1.968 ± 0.016	0.096 ± 0.0005	0.158 ± 0.0012	7.87	8.03
3%	3.453 ± 0.010	3.406 ± 0.033	0.308 ± 0.0007	0.306 ± 0.0007	8.92	8.97
4%	2.989 ± 0.027	3.085 ± 0.008	0.267 ± 0.0006	0.277 ± 0.0006	8.93	8.98
5%	2.581 ± 0.017	2.786 ± 0.013	0.320 ± 0.0008	0.249 ± 0.0008	8.91	8.94
6%	1.986 ± 0.017	2.017 ± 0.025	0.166 ± 0.0007	0.178 ± 0.0007	8.36	8.82

Both stem and leaf derived callus showed similar response to various sugars (Table -2). Glucose was found to be the most effective sugar a far as increase in fresh weight was concerned followed by sucrose. Fructose, mannose and raffinose were not very effective; however, they caused more increase in fresh weight than the callus grown on sugar free medium.

Table 2.Effect of different carbon sources on the growth of stem and leaf derived callus of *Mucuna pruriens*, recorded after 28 days of culture. (Values are mean of 10 replicas).

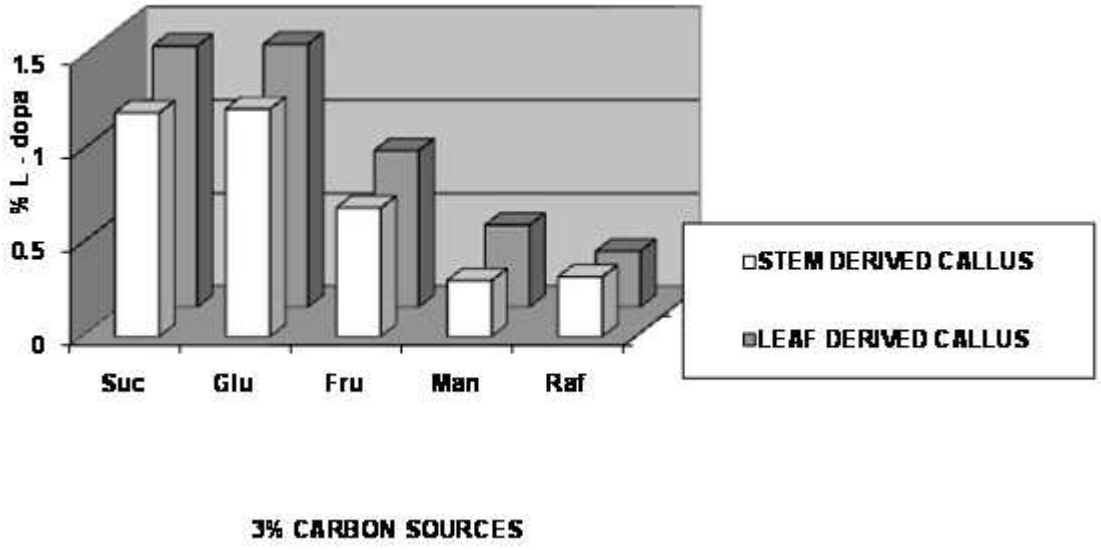
C-Source (3%)	Fresh Weight. (gm) of		Dry Weight. (gm) of		% Dry Weight. of	
	Stem callus	Leaf callus	Stem Callus	Leaf callus	Stem callus	Leaf callus
Sucrose	3.453 ± 0.010	3.406 ± 0.033	0.308 ± 0.0007	0.306 ± 0.0007	8.92	8.97
Glucose	3.754=	3.417=0.01	0.335=0.0011	0.305=0.0010	8.92	8.93
Fructose	2.177 ± 0.088	2.148 ± 0.017	0.161 ± 0.0018	0.158 ± 0.0018	7.39	7.36
Mannose	0.678 ± 0.013	0.762 ± 0.011	0.045 ± 0.0019	0.050 ± 0.0019	6.63	6.56
Raffinose	0.346 ± 0.006	0.642 ± 0.006	0.024 ± 0.0010	0.044 ± 0.0010	6.93	6.85

Effect of carbon source on dry weight: Percentage dry weight of callus grown on different sugars showed that callus grown on glucose and sucrose containing medium had approximately 10% dry weight in both stem and leaf derived callus (Table – 2). Whereas callus grown on fructose, mannose and raffinose had 7.3%, 6.5% and 6.9% dry weight respectively. This suggests that increase in fresh weight of callus in presence of glucose and sucrose was supported by good accumulation of metabolites (the dry matter), whereas in presence of fructose, mannose and raffinose the accumulation of primary metabolite was less.

Effect of carbon source on L-dopa content: Same carbon sources that caused best growth i.e. glucose and sucrose; also caused production of maximum amount of L-dopa (Fig. 1 & 2). Cultures grown in absence of sugar had 0.2% L-dopa in stem derived and 0.39% in leaf derived callus. In the presence of glucose and sucrose, the L-dopa content increased 6 folds in stem derived callus and more than 3 folds in leaf derived callus, over control. Thus confirming that actively growing cells of callus are the sites of L-dopa production [8]. It is a well known fact that sucrose, which is heat labile compound, on autoclaving breaks down into D-glucose and D-

fructose [9] therefore the available active sugar in the medium remains the glucose. Hence similar amount of growth and L-dopa was produced by the cultures grown on sucrose or glucose containing medium.

Figure 2. L-dopa content in leaf and stem derived callus of *Mucuna pruriens* cultured on various 3% carbon sources (Suc = Sucrose, Glu = Glucose, Fru = Fructose, Man = Mannose & Raf = Raffinose) for 28 days.



Effect of Nitrogen source on fresh weight: Fresh weight of callus increased with increasing concentration of phenylalanine, however, at 100 mgl^{-1} there was a decrease in fresh weight. Fresh weight increase was in the range of 32 to 36 folds in 30 days time. Tyrosine showed maximum increase in fresh weight of callus at 50 mgl^{-1} and minimum at 1 mgl^{-1}; fresh weight increase ranged from 32 to 39 folds. Glutathione also showed almost similar increase in fresh weight i.e. 30 to 38 folds. It was maximum at 200 mgl^{-1} glutathione and minimum at 1 mgl^{-1}. Casein hydrolysate showed increasing trend in fresh weight of callus from 13 folds at 50 mgl^{-1} to 21 folds at 200 mgl^{-1} (Table 3).

Effect of Nitrogen source on dry weight*:* Percentage dry weight increased steadily from 7.81% at 1 mgl^{-1} to 8.55 % at 100 mgl^{-1} phenylalanine. Since percentage dry weight was higher at higher concentrations of phenylalanine, it could be concluded that concentration of phenylalanine was not hindering the growth. Tyrosine and glutathione both showed increasing trend in dry weight. In presence of tyrosine dry weight increased from 8.25% to 8.93% with increasing concentration (Table – 3). Whereas dry weight increase in presence of glutathione was from 8.16% to 8.73%. Casein hydrolysate showed different response pattern; at 50 mgl^{-1} percentage dry weight increased to 7.17%, which decreased to 7.02% at 100 mgl-1, and 6.92 at 200 mgl^{-1}. All the results show that dry weigh followed the same trend of response to nitrogen source as that of the fresh weight.

Table 3. Effect of different concentrations of various nitrogen sources (Phe = Phenylalanine, Tyr. = Tyrosine, Glu. = Glutathione and CH = Casein Hydrolysate) on the growth of stem and leaf derived callus of *Mucuna pruriens*, recorded after 28 days of culture. (Values are mean of 10 replicas)

N-Source (mg/l)	Fresh Weight. (gm) of		Dry Weight. (gm) of		% Dry Weight. of	
	Stem callus	Leaf callus	Stem Callus	Leaf callus	Stem callus	Leaf callus
Control	3.208 ± 0.0017	3.215 ± 0.0024	0.241 ± 0.0009	0.222 ± 0.0011	7.51	7.34
Phe 1.0	3.283 ± 0.0026	3.256 ± 0.0033	0.256 ± 0.0013	0.245 ± 0.0012	7.81	7.52
Phe 2.5	3.394 ± 0.0015	3.401 ± 0.0031	0.265 ± 0.0010	0.264 ± 0.0008	7.82	7.77
Phe 5.0	3.422 ± 0.0018	3.458 ± 0.0018	0.278 ± 0.0009	0.270 ± 0.0009	8.13	7.81
Phe 7.5	3.510 ± 0.0019	3.609 ± 0.0038	0.289 ± 0.0007	0.287 ± 0.0010	8.23	7.96
Phe10.0	3.637 ± 0.0020	3.652 ± 0.0025	0.307 ± 0.0008	0.305 ± 0.0007	8.44	8.35
Phe 50.0	3.641 ± 0.0027	3.660 ± 0.0024	0.309 ± 0.0008	0.308 ± 0.0008	8.49	8.41
Phe 100.0	3.628 ± 0.0038	3.613 ± 0.0022	0.310 ± 0.0004	0.305 ± 0.0017	8.55	8.44
Tyr 5.0	3.226 ± 0.0031	3.629 ± 0.0021	0.271 ± 0.0013	0.299 ± 0.0007	8.41	8.25
Tyr 10.0	3.320 ± 0.0021	3.718 ± 0.0024	0.288 ± 0.0011	0.315 ± 0.0005	8.68	8.48
Tyr 25.0	3.530 ± 0.0030	3.874 ± 0.0014	0.308 ± 0.0006	0.344 ± 0.0012	8.76	8.87
Tyr 50.0	3.951 ± 0.0042	3.925 ± 0.0048	0.353 ± 0.0007	0.350 ± 0.0006	8.93	8.91
Glu 1.0	3.291 ± 0.0008	3.045 ± 0.0019	0.268 ± 0.0012	0.249 ± 0.00009	8.16	8.17
Glu 5.0	3.308 ± 0.0015	3.070 ± 0.0031	0.275 ± 0.0005	0.254 ± 0.0016	8.31	8.28
Glu 10.0	3.385 ± 0.0029	3.130 ± 0.0028	0.283 ± 0.0013	0.260 ± 0.0010	8.37	8.32
Glu 20.0	3.433 ± 0.0033	3.185 ± 0.0030	0.292 ± 0.0011	0.269 ± 0.0007	8.52	8.46
Glu 30.0	3.478 ± 0.0024	3.229 ± 0.0030	0.298 ± 0.0006	0.276 ± 0.0008	8.58	8.55
Glu 40.0	3.525 ± 0.0025	3.334 ± 0.0029	0.304 ± 0.0007	0.289 ± 0.0011	8.62	8.67
Glu 50.0	3.692 ± 0.0015	3.472 ± 0.0033	0.320 ± 0.0009	0.302 ± 0.0009	8.67	8.69
Glu 100.0	3.732 ± 0.0025	3.522 ± 0.0019	0.324 ± 0.0004	0.306 ± 0.0007	8.68	8.70
Glu 200.0	3.830 ± 0.0029	3.564 ± 0.0036	0.334 ± 0.0010	0.311 ± 0.0010	8.73	8.74
GH 50.0	1.352 ± 0.006	1.314 ± 0.04	0.097 ± 0.0013	0.093 ± 0.0010	7.17	7.08
CH 100,0	1.932 ± 0.018	1.758 ± 0.006	0.136 ± 0.0011	0.122 ± 0.0012	7.02	6.94
CH 200.0	2.174 ± 0.005	2.080 ± 0.005	0.150 ± 0.0012	0.145 ±0.0009	6.90	6.97

Effect of combination of various Nitrogen sources on fresh weight: Increase in fresh weight was maximum (39 and 38 folds for stem and leaf derived callus respectively), when phenylalanine, glutathione and tyrosine were applied together (Table-4). The next best combination was phenylalanine + tyrosine, which produced 35 folds increase for both stem and leaf derived callus. Whereas phenylalanine + glutathione and tyrosine + glutathione produced slightly less increase in fresh weight (34 fold each in stem derived callus and 34.7 and 34.8 fold respectively in leaf derived callus).

Effect of combination of various Nitrogen sources on dry weight: In leaf derived callus combination of three nitrogen sources caused maximum accumulation of dry weight being 8.58%. In other combinations I was slightly less; being 8.55%, 8.54% and 8.46% for the combinations glutathione + tyrosine; phenylalanine + tyrosine and phenylalanine + glutathione respectively (Table - 4).

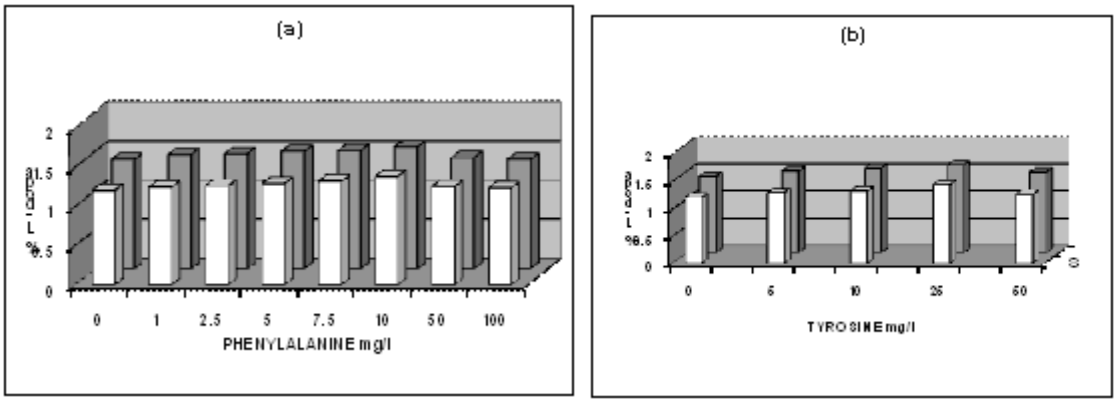
Table - 4. Effect of different combinations of three nitrogen sources (Phe = Phenylalanine, Tyr. = Tyrosine and Glu. = Glutathione) on the growth of stem and leaf derived callus of *Mucuna pruriens*, recorded after 28 days of culture. (Values are mean of 10 replicas).

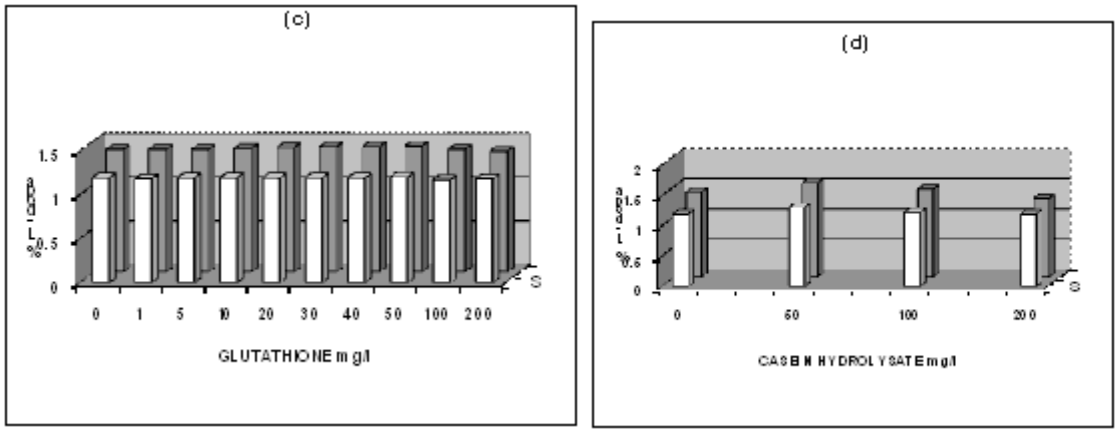
N-Source (mg/l) Phe Tyr Glu	Fresh Weight. (gm) of		Dry Weight. (gm) of		% Dry Weight. of	
	Stem callus	Leaf callus	Stem Callus	Leaf callus	Stem callus	Leaf callus
0 + 0 + 0	3.318 ± 0.0038	3.227 ± 0.0019	0.266 ± 0.0008	0.265 ± 0.0007	8.01	8.21
10 + 0 + 50	3.476 ± 0.0023	3.484 ± 0.0031	0.293 ± 0.0006	0.295 ± 0.0016	8.44	8.46
10 + 25 + 0	3.528 ± 0.0040	3.546 ± 0.0050	0.297 ±0.0008	0.303 ± 0.0009	8.42	8.54
0 + 25 + 50	3.483 ± 0.0018	3.475 ± 0.0027	0.303 ± 0.0004	0.297 ± 0.0005	8.70	8.55
10 + 25 + 50	3.918 ± 0.0025	3.891 ± 0.0026	0.338 ± 0.0009	0.334 ± 0.0015	8.62	8.58

Percentage dry weight followed different trend in stem derived callus, showing maximum dry weight with the combination of glutathione + tyrosine (8.7%). Combination of all the three nitrogen sources was at second place with 8.62% dry weight. Percentage increase in the dry weight of remaining combinations was 8.44% for phenylalanine + glutathione and 8.42% for phenylalanine + tyrosine.

***Effect of Nitrogen source on L-dopa content*:** Stem and leaf derived callus showed increase in L-dopa content with increase in concentration of phenylalanine from 1 mgl^{-1} to 10 mgl^{-1}. It increased from 1.24% to 1.38% in stem derived and 1.46% to 1.56% in leaf derived callus. Concentrations higher than that declined the L-dopa content (Fig. 3a). As it can be seen from Fig 3b, stem and leaf derived callus showed increasing trend in L-dopa content with increase in tyrosine concentration from 4 to 25 mgl^{-1} and then decreased with further increase in tyrosine level to 50 mgl^{-1}. Tyrosine was found to be more effective than phenylalanine for growth as well as L-dopa production. 25 to 50 mgl^{-1} tyrosine was effective in growth of stem and leaf derived callus. Whereas 25 mgl^{-1} tyrosine was obviously the best nitrogen source for L-dopa production, which could produce 1.44% L-dopa in stem derived and 1.61% in leaf derived callus.

Figure 3. L-dopa content in leaf (gray) and stem (white) derived callus of *Mucuna pruriens* cultured on various nitrogen sources for 28 days (a) Phenylalanine (b) Tyrosine (c) Glutathione and (d) Casein hydrolysate



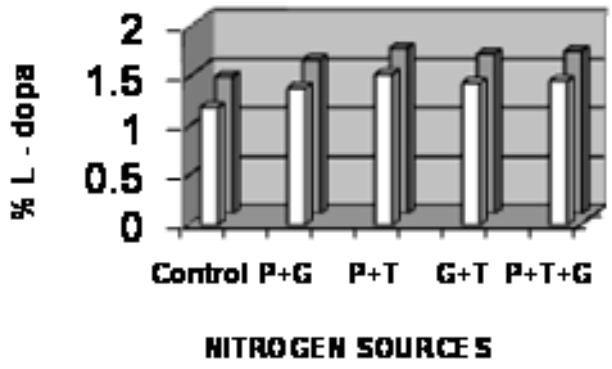


It has been suggested [10, 11] that incorporation of tyrosine during encapsulation of *M. pruriens* cells. Later [12] it successfully converted tyrosine (which is one of the intermediary compound in the metabolic pathway of L-dopa) to L-dopa. *M. pruriens* cells were also used [13] to bioconvert L-tyrosine into L-dopa. Their work can not be directly compared with the present results, however supplying tyrosine in culture medium did help in biosynthesis of more L-dopa in callus, in the present work, suggesting that *M. pruriens* callus cells could use and convert tyrosine present in the medium into L-dopa.

Glutathione failed to increase L-dopa production in stem derived callus above control., maximum being 1.21% at 50 mgl^{-1} (control was 1.20%). Leaf derived callus showed slight increase reaching up to 1.44% at 50 mgl^{-1} (control being 1.40%). Though it had promoted growth (fresh and dry weight) at all the tested concentrations. Since glutathione is a tripeptide and is involved in protein building and L-dopa is a non-proteinaceous amino acid it is quite in order that it did not enhance L-dopa production (Fig 3c).

With increasing concentration of casein hydrolysate, L-dopa showed decreasing trend in both stem and leaf derived callus, suggesting that this complex mixture of amino acids is not very useful for increased L-dopa production (Fig. 3d).

Figure 4. L-dopa content in leaf (gray) and stem (white) derived callus of *Mucuna pruriens* cultured on medium supplemented with 10 mg/l phenylalanine (P) + 25 mg/l tyrosine (T) + 50 mg/ml glutathione (G); for 28 days.



Effect of combination of various Nitrogen sources on L-dopa content: Encouraging results of increase in L-dopa content by incorporating phenylalanine, tyrosine and glutathione guided for

an attempt to incorporate these nitrogen sources together in the medium. For both stem and leaf derived callus maximum L-dopa content was noted in the cells grown on the combination of tyrosine + phenylalanine followed by tyrosine + glutathione and minimum in phenylalanine + glutathione (Fig. 4). Combination of all the three nitrogen sources gave 1.47% L-dopa in stem and 1.63% in leaf derived callus.

Leaf derived callus produced more L-dopa than stem derived callus.
In conclusion it could be said that tyrosine was the best nitrogen source for production of L-dopa, as all the tyrosine containing combinations produced maximum L-dopa.

Table 5.Effect of various Plant Growth regulators (BA= Benzyl Adenine, Kin = Kinetin, AS = Adenine Sulfate, IAA = Indole Acetic Acid, Try = Tryptophan, SA = Shikimic Acid, GA = Gibberellic Acid and MA = Mevalonic Acid) on the growth of stem and leaf derived callus of *Mucuna pruriens*, recorded after 28 days of culture. (Values are mean of 10 replicas).

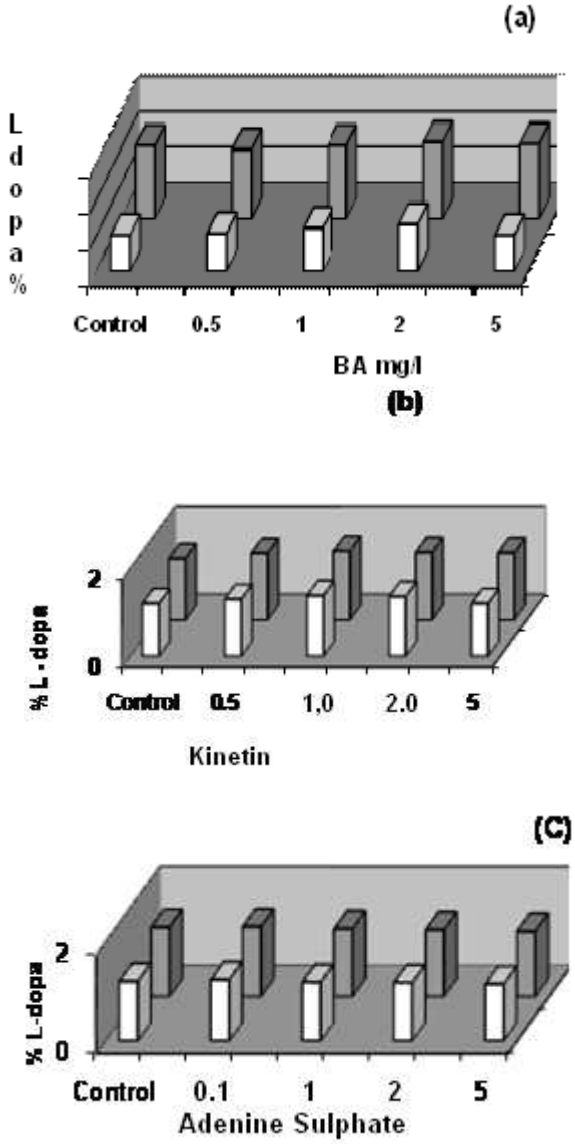
PGRs mg/l	Fresh Weight.(gm) of		Dry Weight. (gm) of		% Dry Weight. of	
	Stem callus	Leaf callus	Stem Callus	Leaf callus	Stem callus	Leaf callus
Control	2.082 ± 0.0016	2.130 ± 0.0020	0.167 ± 0.0008	0.171 ± 0.0006	8.02	8.03
BA 0.5	2.067 ± 0.0024	2.192 ± 0.0012	0.168 ± 0.0006	0.178 ± 0.0007	8.13	8.12
BA 1.0	2.517 ± 0.0024	2.228 ± 0.0020	0.207 ± 0.0007	0.183 ± 0.0013	8.22	8.21
BA 2.0	1.823 ± 0.0019	2.344 ± 0.0025	0.229 ± 0.0011	0.192 ± 0.0011	8.11	8.19
BA 5.0	1.877 ± 0.0020	2.485 ± 0.0033	0.231 ± 0.0007	0.203 ± 0.0017	8.00	8.17
Kin 0.5	1.198 ± 0.0024	2.177 ± 0.0015	0.188 ± 0.0007	0.177 ± 0.0009	8.18	8.13
Kin 1.0	1.325 ± 0.0025	2.234 ± 0.0015	0.192 ± 0.0009	0.184 ± 0.0011	8.26	8.24
Kin 2.0	2.359 ± 0.0026	2.318 ± 0.0019	0.196 ± 0.0008	0.194 ± 0.0008	9.30	8.35
Kin 5.0	2.836 ± 0.0024	2.411 ± 0.0025	0.232 ± 0.0011	0.200 ± 0.0010	8.21	8.30
AS 0.5	2.443 ± 0.0016	2.254 ± 0.0030	0.196 ± 0.0007	0.181 ± 0.0005	8.02	8.03
AS 1.0	2.583 ± 0.0020	2.312 ± 0.0015	0.210 ± 0.0007	0.187 ± 0.0005	8.13	8.09
AS 2.0	2.800 ± 0.0012	2.353 ± 0.0021	0.229 ± 0.0009	0.192 ± 0.0011	8.18	8.16
AS 5.0	2.885 ± 0.0024	2.496 ± 0.0017	0.238 ± 0.0011	0.205 ± 0.0005	8.25	8.21
IAA 0.1	2.252 ± 0.0012	2.716 ± 0.0023	0.183 ± 0.0007	0.219 ± 0.0009	8.13	8.06
IAA 0.5	2.283 ± 0.0020	2.755 ± 0.0020	0.189 ± 0.0011	0.227 ± 0.0019	8.28	8.24
IAA 1.0	2.350 ± 0.0021	2.856 ± 0.0017	0.199 ± 0.0005	0.243 ± 0.0008	8.35	8.40
IAA 1.5	2.387 ± 0.0021	2.774 ± 0.0016	0.191 ± 0.0007	0.233 ± 0.0007	8.47	8.51
Try 0.1	2.257 ± 0.0020	2.446 ± 0.0019	0.179 ± 0.0009	0.196 ± 0.0006	7.93	8.01
Try 0.5	2.355 ± 0.0025	2.510 ± 0.0010	0.190 ± 0.0005	0.241 ± 0.0007	8.07	8.01
Try 1.0	2.359 ± 0.0024	2.514 ± 0.0021	0.192 ± 0.0009	0.202 ± 0.0006	8.14	8.04
Try 1.5	2.454 ± 0.0021	2.606 ± 0.0021	0.201 ± 0.0008	0.212 ± 0.0008	8.19	8.14
SA 0.1	2.214 ± 0.0019	2.236 ± 0.0019	0.177 ± 0.0011	0.179 ± 0.0007	7.99	8.01
SA 0.5	2.252 ± 0.0020	2.245 ± 0.0015	0.181 ± 0.0011	0.186 ± 0.0011	8.04	8.06
SA 1.0	2.317 ± 0.0022	2.356 ± 0.0019	0.187 ± 0.0008	0.191 ± 0.0009	8.07	8.11
SA 1.5	2.404 ± 0.0021	2.417 ± 0.0021	0.195 ± 0.0009	0.198 ± 0.0007	8.11	8.19
GA 0.1	1.808 ± 0.0016	1.387 ± 0.0020	0.137 ± 0.0010	0.104 ± 0.0014	7.58	7.49
GA 0.5	1.571 ± 0.0011	1.365 ± 0.0019	0.117 ± 0.0015	0.101± 0.0012	7.45	7.40
GA 1.0	1.520 ± 0.0015	1.359 ± 0.0028	0.111 ± 0.0007	0.098 ± 0.0011	7.30	7.21
GA 1.5	1.263 ± 0.0017	1.327 ± 0.0020	0.091 ± 0.0010	0.095 ± 0.009	7.21	7.16
MA 0.1	1.484 ± 0.0016	1.282 ± 0.0021	0.112 ± 0.0004	0.096 ± 0.0003	7.55	7.49
MA 0.5	1.225 ± 0.0019	1.248 ± 0.0015	0.090 ± 0.0005	0.092 ± 0.0011	7.53	7.37
MA 1.0	1.153 ± 0.0018	1.242 ± 0.0016	0.081 ± 0.0009	0.089 ± 0.0015	7.11	7.17
MA 1.5	1.091 ± 0.0020	1.232 ± 0.0025	0.076 ± 0.0012	0.087 ± 0.0012	6.97	7.06

Effect of PGRs on fresh weight: Control callus grown on MS medium supplemented with 2,4-D showed 20 fold increase in fresh weight in stem derived and 21 folds increase in leaf derived

callus after 4 weeks of culture. Addition of 5 mgl^{-1} either BA, Kin or adenine sulfate to control medium showed 28 fold increase in fresh weight of stem derived callus, whereas leaf derived callus showed 24 fold increase at he same concentration of BA and Kin and only 22 fold increase at the same concentration of adenine sulfate (Table – 5).

Effect of PGRs on dry weight: Control callus showed similar increase in dry weight as in fresh weight; i.e.21 folds and 20 folds for stem and leaf derived callus respectively. Unlike fresh weight the effective concentration of BA and Kin for maximum increase in dry weight was 1 and 2 mgl^{-1} respectively. However most effective concentration of adenine sulfate for increase in dry weight was the same as for the fresh weight i.e. 5 mgl^{-1} (Table 5).

Figure 5. L-dopa content in leaf and stem derived callus of *Mucuna pruriens* cultured on various concentrations of (a) BA (b) Kinetin and (c) Adenine sulfate for 28 days



Effect of PGRs on L-dopa content*:* L-dopa content increased steadily in stem derived callus cultured on medium containing with increase in BA concentration, being 1.21% at 0.5 mgl^{-1} to 1.26 at 2.0 mgl^{-1}. Thereafter it decreased to 1.20% at 5 mgl^{-1} BA. Leaf derived callus followed the same pattern with maximum L-dopa being 1.43% at 2 mgl^{-1} BA and minimum 1.39% at 0.5

mgl^{-1} BA. BA treatment was not found to be much effective in increasing L-dopa content much above control (Fig. 5a).

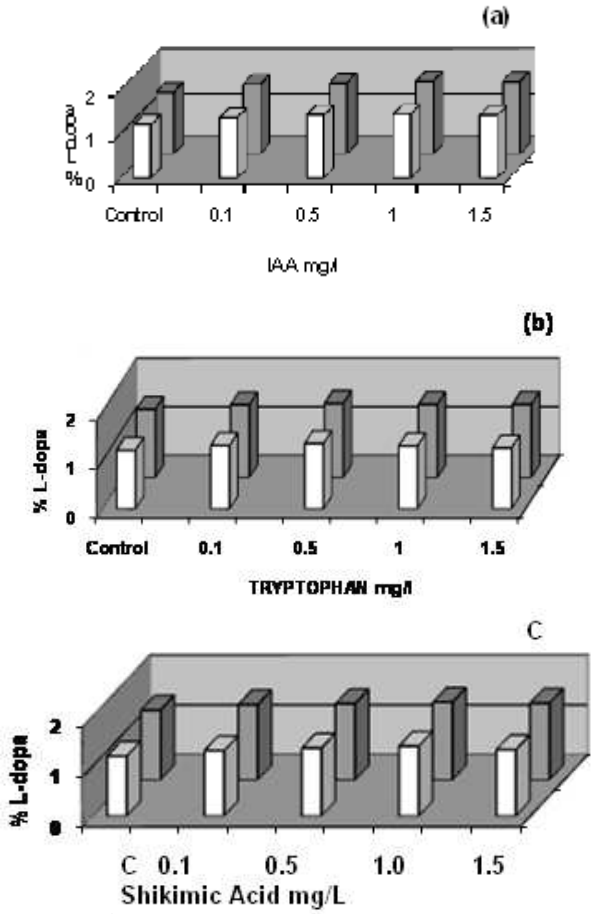
Kin could enhance L-dopa content in callus more than BA. In leaf derived callus cultured on control medium supplemented with Kin, L-dopa content was maximum (1.57%) at 1.0 mgl^{-1}. L-dopa content was 1.52%, 1.54% and 1.53% at 0.5, 2 and 5 mgl^{-1} Kin respectively. Stem derived callus also had the same trend (Fig. 5b).

Adenine sulfate could hardly increase the L-dopa content over control as stem and leaf derived callus showed 1.23% and 1.43% L-dopa respectively at 0.5 mgl^{-1} Adenine sulfate (Fig. 5c). Then it decreased with increase in adenine sulfate concentration.

These results show that in callus grown on cytokinin containing medium the fresh weight, dry weight and L-dopa content of the callus are not linked together. The culture, which showed maximum increase in fresh weight, did not have maximum dry matter or L-dopa.

Effect of auxin IAA and its precursor shikimic acid and tryptophan an intermediary compound for biosynthesis of both L-dopa and IAA were also studied.

Figure 6.L-dopa content in leaf and stem derived callus of Mucuna pruriens cultured on various concentrations of (a) IAA (b) Tryptophan and (c) Shikimic acid, for 28 days



IAA was most effective at 1 mgl^{-1} (Table 5) for both fresh weight and dry matter content of stem and leaf derived callus. However L-dopa production was maximum and almost same at 1 and 1.5 mgl^{-1} IAA (Fig. 6a). Tryptophan was most effective for increase in fresh weight and dry matter

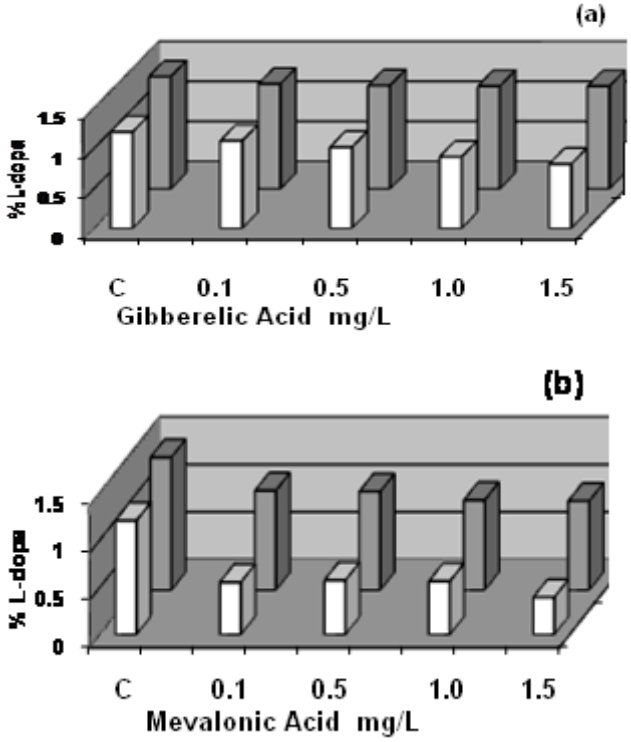
content at 1.5 mgl^{-1}, but increased L-dopa production was noted in callus at 0.5 mgl^{-1} tryptophan. However, 1 and 1.5 mgl^{-1} tryptophan also considerably increased the L-dopa content (Fig. 6b).
It was interesting to note that effective concentration of shikimic acid was also 1.5 $mgl^{-1}$for growth (fresh and dry weight). However, the most effective concentration of shikimic acid for L-dopa production was 1 mgl^{-1}, though 1.5 mgl^{-1} shikimic acid also considerably increased the L-dopa content (Fig. 6c).

Unlike cytokinins, the effect and effective concentrations of auxin (IAA) and its intermediary compound (tryptophan) and precursor (shikimic acid) were the same on growth and L-dopa content. All three were most effective at the same concentration. IAA (1mgl^{-1}) stimulated maximum L-dopa production (1.65%). However, *M. pruriens* cell suspension culture when they provided Kin, BA, IAA or 2, 4-D at various concentrations failed [14]to increase L-dopa production. They did not get significant cell growth either. The difference may be due to the culture type i.e. in present work static callus culture is used whereas they worked with cell suspension culture.

Effect of gibberellic acid and its precursor mevalonic acid on growth and L-dopa content in stem derived callus showed a decreasing trend in fresh weight with increase in concentration. At the lowest concentration it showed an 18-fold increase in fresh weight whereas at the highest concentration increase was only 12 folds. Unlike stem derived callus, the fresh weight of leaf derived callus did not show any change with increase in GA_3 concentration. It was 13-fold increase at all the tried concentrations (Table – 5).

Incorporation of mevalonic acid produced similar effects as GA_3 i.e. in stem derived callus fresh weight decreased with increase in concentration, whereas leaf derived callus showed same response to all tested concentrations.

Figure 7.L-dopa content in leaf and stem derived callus of *Mucuna pruriens* cultured on various concentrations of (a) Gibberellic Acid and (b) Mevalonic Acid, for 28 days.



Both GA_3 and mevalonic acid caused a very significant decrease in percentage dry weight as compared to control. Since GA_3 is known to be involved in carbohydrate metabolism, especially hydrolysis of stored starch, it does not allow accumulation of more dry matter.
Increasing concentration of GA3 adversely affected the L-dopa content in both stem and leaf derived callus (Fig 7a). Mevalonic acid was found to be a greater suppressant of L-dopa content than GA_3 (Fig 7b).

The data presented above shows that incorporation of 1 mgl^{-1} IAA to callus maintenance medium could produce maximum L-dopa (1.65%) and 25 mgl^{-1} tyrosine was the most effective nitrogen source in stimulating L-dopa production (1.61%). Moreover augmentation of 2, 4-D (2.5mgl^{-1}) to MS basal medium with 3% sucrose was found to not only stimulate L-dopa production but also maintain good callus growth. Therefore a mixture of 25 mgl^{-1} tyrosine + 1mgl^{-1} IAA + 2.5 mgl^{-1} 2,4-D was incorporated into MS medium and leaf derived callus was grown on this medium for4 weeks. The callus on 28th day could produce as much as 1.95-% L-dopa.

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