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CELL SUSPENSION CULTURE OF *Mucuna pruriens* FOR PRODUCTION AND IMPROVEMENT OF L-3, 4-DIHYDROXY PHENYLALANINE CONCENTRATION

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Abstract
The production and improvement of L-3, 4-dihydroxy phenylalanine (L-Dopa) content in *Mucuna pruriens* was carried out through cell suspension culture. Thirty days old healthy, friable and soft calli derived from leaf and stem of two varieties of *M. pruriens*: (IIHR Selection 3 and Arka Dhanvantari) were used for the cell suspension in Murashige and Skoog's liquid medium supplemented with IAA (1.0mg/L), NAA (1.5mg/L) and BAP (1.5mg/L) with treatment of elicitors: chitin and pectin (100, 150, and 200mg/L), precursor: L-tyrosine (5, 10, and 15mg/L) and ascorbic acid (250mg/L). The highest percentage of packed cell volume (PCV) (42.4 ± 0.1) was observed in leaf-derived callus of AD variety with treatment L4: MS + NAA (1.5mg/L) + Pectin (100mg/L) + ascorbic acid (250mg/L). From the HPLC analysis, treatment T3c: MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L) recorded highest peak area percentage of 31.19 at retention time (RT) of 2.36 against control (1.39) at 2.35 RT. L-Dopa concentration was observed to increase with increase in elicitor and precursor treatment. This shows that L-Dopa can be produced from natural source in desired concentration through cell suspension culture.

Keywords: *Mucuna pruriens,* cell suspension culture, L-Dopa, plant growth regulator, elicitor and precursor

Introduction

Mucuna pruriens var utilis is a leguminous plant which consists of about 150 different species of climbing vines and shrubs (Umberto, 2000). It is found mainly in tropical Africa, India and the Caribbean where it grows as twinning annual plants with length of 15-20meters (Sahaji, 2011). This plant synthesizes L-Dopa, a neurotransmitter precursor, which is used in the treatment of Parkinson's disease and other mental disorders (Kotisree *et al.,* 2018). As a non-protein amino acid, L-DOPA also has insecticidal and allelochemical activity and can be used for pest and weed control (Claudia *et al.,* 2018). According to ancient ayurvedic literature, secondary metabolites form leaf and seed of *Mucuna pruriens* is a potent aphrodisiac and also effective in the treatment of menstruation disorders, diabetes, edema, constipation, tuberculosis etc. The demand for *Mucuna pruriens* products in India and international drug markets increased many folds after the discovery that it contains of L-3, 4-dihydroxy phenylalanine (Farooqi, 1999). To accommodate the large demand for L-Dopa, *in vitro* production of the compound using cell suspension cultures is now practiced extensively and the accumulation of L-Dopa in cell cultures of *Mucuna pruriens* has been reported by several researchers Lahiri *et al.* (2011).

L-Dopa obtained from natural sources especially *Mucuna pruriens* has been recorded to be more potent in the treatment of health issues when compared with the ones obtained through synthetic production (Ramya and Thaakur 2007). The incorporation of precursors in cell suspension cultures result to increase in the production of target secondary metabolites (Dheeranapattana *et al.,* 2009). The aim of this study was to investigate the influence of plant growth regulators: (IAA, NAA and BAP), elicitor molecules: (pectin and chitin), and precursor: (L-tyrosine) on production and improvement of L-DOPA in cell suspension cultures of two varieties of *M. pruriens*: (IIHR Selection 3 and Arka Dhanvantari)

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Materials and methods

Source of plant samples and chemicals

Seeds of two varieties of *Mucuna pruriens var utilis* [IIHR selection 3 (S3), and *Arka Dhanvantari* (AD)],

were collected from the Indian Institute of Horticultural Research, (IIHR) Bengaluru India (Fig 1). The plants were raised and maintained in the greenhouse in pots of 20 x 30cm size and watered at two weeks interval for further analysis (Fig 2). Analytical grade chemicals used for preparing the stock solutions and subsequent media preparation, plant growth regulators, sucrose, vitamins, precursor, elicitors etc, were purchased from Sigma-Aldrich chemicals and Himedia laboratories India.

Media and culture conditions

Murashige and Skoog's medium with 3% (w/v) sucrose was used as basal medium at pH of 5.7 ± 0.1, followed by gelling with 0.8% of agar per liter for the solid medium, and no agar for the MS liquid medium used for cell suspension culture. All the cultures were incubated in a growth chamber maintained at a temperature of 25 ± 2°C, under cool fluorescent light (1500 - 2000lux) with a 16 hours/8 hours light/dark cycle.

Callus induction

Callus was initiated using leaf and stem explants of S3 and AD varieties of *M. pruriens*, cultured on MS medium supplemented with 2, 4-D, IAA, kinetin, NAA and BAP (at 0.5, 1.0, 1.5, 2.0 and 2.5mg/L) alone or in combination as recorded in Table 1.

Maintenance of callus

Calli obtained from leaf and stem explants of the two varieties of *M. pruriens* were maintained in MS medium supplemented with two different concentrations of auxins (2, 4-D 1.5mg/L and NAA 2.0mg/L), cytokinin (BAP 1.5mg/L and kinetin 1.5mg/L) and combination of NAA + 2, 4-D at 1.5mg/L with ascorbic acid (250mg/L) in all the treatments to control phenol exudation.

Suspension culture

Cell suspensions were initiated with thirty (30) days old healthy, friable and soft calli maintained in 2, 4-D (2.0mg/L) and NAA (1.5mg/L) which gave equal yield of optimal callus proliferation. Callus of 1g fresh weight was aseptically transferred to 100ml conical flasks containing 50ml of MS liquid medium supplemented with IAA (1.0mg/L), NAA (1.5mg/L) and BAP (1.5mg/L), 3% sucrose for proliferation and ascorbic acid (250mg/L) to reduce phenol exudation. In the control, no plant growth regulator was added to the liquid MS medium. Suspensions were established by shaking the cultures on a rotary shaker (Scigenics Biotech, India) at 100rpm at 24°C.

Treatment of cell suspensions with elicitors and precursor

Treatment of cell suspensions with elicitors: chitin and pectin (100, 150, and 200mg/L) and precursor: L-tyrosine (5, 10, and 15mg/L) was done after 7 days of cell growth. In the control, no elicitor or precursor was added. Ascorbic acid at 250mg/L was added to the MS liquid medium in all the treatments to control phenol exudation.

Packed cell volume (PCV)

To monitor cell growth in the suspension culture, the packed cell volume was evaluated at each passage. 1.5ml of each treatment of the suspension culture was dispensed aseptically into eppendorf tubes and centrifuged at 5000rpm for 20min. The volume of the pellets formed was recorded, and the packed cell volume calculated thus;

$$(PCV)\ (\%) = \frac{\text{Volume of the pellet}}{\text{Volume of suspension taken}} \times 100$$

The packed cell volume was measured at three different passages (Days: 7, 14 and 21) to ascertain the treatment that gave the highest production of L-Dopa using the peak areas given by HPLC analysis.

L-Dopa improvement using PGR and Elicitor treatment

The combinations of PGR and elicitors used at various combinations for L-Dopa improvement are furnished thus;

L 1: MS + NAA (1.5mg/L) + Chitin (100mg/L) + ascorbic acid (250mg/L)

L2 MS + BAP (1.5mg/L) + Chitin (150mg/L) + ascorbic acid (250mg/L)

L 3 MS + IAA (1.0mg/L) + Chitin (200mg/L) + ascorbic acid (250mg/L)

L 4: MS + NAA (1.5mg/L) + Pectin (100mg/L) + ascorbic acid (250mg/L)

L5 MS + BAP (1.5mg/L) + Pectin (150mg/L) + ascorbic acid (250mg/L)

L6 MS + IAA (1.0mg/L) + Pectin (200mg/L) + ascorbic acid (250mg/L)

L-Dopa improvement using PGR and precursor treatment

The combinations of PGR and precursor used at various combinations for L-Dopa improvement are furnished thus;

L 7 MS + NAA (1.5mg/L) + L-tyrosine (5mg/L) + ascorbic acid (250mg/L)

L 8: MS + BAP (1.5mg/L) + L-tyrosine (10mg/L) + ascorbic acid (250mg/L)

L 9: MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L)

L10: (Control) Cell suspension culture with no plant growth regulator, elicitor or precursor treatment + ascorbic acid (250mg/L).

Extraction and quantification of L-Dopa

Preparation of L-Dopa standard stock solution for HPLC analysis: Ten (10) mg of L-Dopa standard was dissolved in 100ml of distilled water to obtain 0.1mg/ml of L-Dopa solution.

Preparation of cell suspension sample for HPLC analysis: Extraction of L-Dopa was done as described by Myhrman (2002) with appropriate modifications. The suspended cells were separated from the medium, washed with sterile distilled water to remove any adhering medium to the cell surface. Approximately, 1g of cell was taken and crushed using pestle and mortar by

adding 10ml of distilled water. The extract was boiled for 10min, cooled and centrifuged for about 10min at 5000xg. Supernatant was collected, boiled, cooled and centrifuged again to get clear solution which was used for HPLC analysis.

HPLC analysis: L-Dopa content in the extract was estimated by HPLC using waters, model 2487, pump 1515 with 2487 dual absorbance UV detection, manual sampler injector using C-18 column and a guard pre-column that was packed with material; as in the main column. Isocratic elution was carried out using water: methanol: phosphoric acid [975.5:19.5:1 (v/v)], as described by Saddhuraju and Becker (2003). Separation was performed at room temperature (25°C) and after the injection of 20µL, the column was operated with the flow rate of 1.2ml/min. Absorbance was monitored at 282nm and peak heights and areas determined. Sample was eluted between 3 and 4min. The amount of L-Dopa present in the tissue extracts was calculated with the help of standard curve for L-Dopa (Sigma, St. Louis, MO., USA). Amount of L-Dopa present in tissue extract was expressed as milligram per gram tissue dry weight.

Statistical analysis
The experiments were conducted in six replicates. Data generated were subjected to one-way analysis of variance (ANOVA) to assess treatment differences and interaction using the SPSS version 11.0.

Results and Discussion
In vitro culture of Mucuna pruriens
Figures 3 and 4 show the results of callus induction of the two varieties of *M. pruriens* using leaf and stem explants. For leaf-derived callus in AD variety, the maximum callus induction frequency (100 ± 0.0) was observed in MS + NAA at 2.5mg/L, while in S3 variety, three treatments recorded maximum callus induction frequency (100 ± 0.0) *viz*: MS + 2, 4-D at 2.0mg/L, MS + NAA at 0.5 mg/L and MS + NAA at 2.5mg/L. For stem-derived callus, the maximum callus induction frequency (91.7 ± 0.3) was observed in S3 variety from NAA 2.0 and 2.5mg/L.

Suspension culture
The cell suspension cultures of leaf and stem-derived calli of *M. pruriens* for L-Dopa production and improvement is shown in Figure 5. The effect of plant growth regulators in combination with elicitor and precursor treatment on percentage of packed cell volume (PCV) of leaf-derived callus and stem-derived callus of the cell suspension culture over three passages are shown in Tables 2a & b and 3a &b respectively. For AD leaf-derived callus cell suspension, at day 7 (Passage: 1), the highest percentage of PCV was 15.57 ± 0.6 from treatment L6**:** IAA (1.0mg/L) + Pectin (200mg/L) + ascorbic acid (250mg/L), while at days 14 and 21 (Passages: 2 and 3), the highest packed cell volume percentage of AD was 30.4 ± 0.2 and 42.4 ± 0.1 respectively from treatment L4 MS + NAA (1.5mg/L) + Pectin (100mg/L) + ascorbic acid (250mg/L). For S3, at day 7 (Passage: 1), the highest percentage of PCV in leaf callus cell suspension was 14.6 ± 0.1 from treatment L9 MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L). At day 14 (Passage: 2), the highest percentage of packed cell volume was 28.3 ± 0.2 from treatment L6 MS + IAA (1.0mg/L) + Pectin (200mg/L) + ascorbic acid (250mg/L), while at day 21 (Passage: 3), the highest percentage of PCV in leaf-derived callus cell suspension was 55.1 ± 0.1 from treatment L9 MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L).

Improvement of L-Dopa concentration
The effect of elicitor and precursor treatment of the cell suspension culture on L-Dopa production and improvement as recorded through HPLC analysis carried out after three passages (21days) is shown in Table 4. The peaks for standard L-Dopa in 1000, 50 and 1ppm were dictated at 2.33, 2.36 and 2.35 retention time respectively. The highest amount of L-Dopa concentration was observed in the L9 treatment MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L) which gave a peak area percentage of 31.19 at retention time of 2.36.

For *in vitro* culture of *M. pruriens,* callus was initiated using the leaf and stem explants cultured on MS medium supplemented with 2, 4-D, IAA, kinetin, NAA and BAP (at 0.5, 1.0, 1.5, 2.0, 2.5mg/L respectively) alone, or in combination. Looking at the interactions between the five different treatments of plant growth regulators used for callus induction in the two varieties of *M. pruriens*; 2, 4-D and NAA gave the maximum callus induction frequency. For leaf-derived callus in AD variety MS + NAA at 2.5mg/L gave 100 ± 0.0 as the highest callus induction frequency, while, in S3 variety three (3) treatments gave maximum callus induction frequency (100 ± 0.0) *viz*: MS + 2, 4-D at 2.0mg/L, NAA at 0.5mg/Land NAA at 2.5mg/L. For stem-derived callus in AD variety MS + 2; 4-D at 2.5mg/L gave 83.3 ± 0.8 as the highest callus induction frequency, while, in S3 variety, two (2) treatments MS + NAA at 2.0 and 2.5mg/L gave 91.7 ± 0.3 respectively as the highest callus induction frequency.

When the auxins and cytokinin were used in combination for callus induction, maximum callus induction frequency (100 ± 0.0) was recorded in the leaf-derived callus of both AD and S3 variety from the treatment: MS + NAA (3.0mg/L) + BAP (0.5mg/L). While, for stem-derived callus, the treatment: MS + 2, 4-D (2.0mg/L) + BAP (0.5mg/L) gave maximum callus induction frequency (100 ± 0.0) for both AD and S3 variety. Kavitha and Vadivel (2005) indicated that MS basal medium supplemented with 2, 4-D at 2.0mg/L and NAA at 2mg/L were found to be the best medium for inducing callus in various explants, namely: stem bits, leaf bits and root bits in their study on *in vitro* production of L-Dopa from *Mucuna pruriens* (L.) DC. Subsequently, callus proliferation was studied on cotyledon, leaf and stem explants of *Mucuna pruriens* Bak cultured on MS medium supplemented with 0.5 – 2.5mg/L of 2,4-D, IBA, NAA and BAP alone or in

combination by Shalini *et al.*(2007) and maximum callus proliferation was observed in both leaf and stem derived calli.

Cell suspension culture was established with one month old healthy, friable and soft calli. The effect of plant growth regulators in combination with elicitor and precursor treatment on packed cell volume (PVC) percentage of the calli over three passages was determined. In passage 1, the highest PCV percentage in AD leaf callus cell suspension was 14.7 ± 0.1 from treatment L5: BAP (1.5mg/L) + Pectin (150mg/L) + ascorbic acid (250mg/L), while, in passages 2 and 3, the highest PCV percentage of AD was 30.4 ± 0.2 and 42.4 ± 0.1 respectively from treatment L4: NAA (1.5mg/L) + Pectin (100mg/L) + ascorbic acid (250mg/L). For S3, in passages 1, 2 and 3, the highest PCV percentage in leaf callus cell suspension was 14.6 ± 0.1, 28.6 ± 0.9 and 55.1 ± 0.1 respectively from L_9: IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L).

In stem derived callus cell suspension at days 7, 14 and 21, the highest packed cell volume percentage from AD variety was 14.7 ± 0.1, 22.6 ± 0.4 and 49.6 ± 0.2 respectively as observed in treatment L9: MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L). While in S3 variety, at day 7 (Passage 1), the highest packed cell volume percentage of stem callus cell suspension was 15.6 ± 0.2 from L2: MS + BAP (1.5mg/L) + Chitin (150mg/L) + ascorbic acid (250mg/L). At day 14 (Passage 2), the highest packed cell volume percentage was 36.1 ± 0.3 from L9: MS + IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L) and at day 21, the highest packed cell volume percentage was 49.3 ± 0.1 from L6: MS + IAA (1.0mg/L) + Pectin (200mg/L) + ascorbic acid (250mg/L). Cell suspension culture is one of the most popular methods for *in vitro* production of secondary metabolites. It has been reported that the incorporation of tyrosine during encapsulation of *M. pruriens* cells successfully converts the tyrosine (which is one of the intermediary compounds in the metabolic pathway of L-Dopa) to L-Dopa (Pras, 1998; Pras *et al.,* 1998). Therefore, addition of L- tyrosine in the cell suspension cultures of *M. pruriens* used in present study was helpful for the biosynthesis of increased L-Dopa concentration in the callus cell suspension after three passages, suggesting that *M. pruriens* callus cells could convert L-tyrosine present in the medium into L-Dopa.

HPLC analysis was carried out after three passages (21days) of the cell suspension culture to determine the amount of L-Dopa produced. The production and enhancement of L-Dopa in the cell suspension culture samples were observed from the retention time and peak area percentage of the chromatographs. In T1 treatment group, T1c: IAA (1.0mg/L) + Chitin (200mg/L) + ascorbic acid (250mg/L) gave the highest peak area percentage of 29.99 at 2.33 retention time. In T2 treatment group, T2c: IAA (1.0mg/L) + Pectin (200mg/L) + ascorbic acid (250mg/L) gave the highest peak area of 24.63 at 2.33 retention time while in T3 group T3c: IAA (1.0mg/L) + L-tyrosine (15mg/L) + ascorbic acid (250mg/L) showed the highest peak area percentage of 31.19 at retention time of 2.36. For control group T5: (Cell suspension culture + ascorbic acid (250mg/L) - PGR - elicitor or precursor), the peak area percentage was 1.39 at 2.35 retention time.

L-Dopa content was observed to increase with increase in concentration of the elicitors as compared with control. This is similar to the findings of Raghavendra *et al.* (2012) who recorded the increase of L-Dopa up to 12 folds when elicitors and precursors were used for L-Dopa production in cell suspension cultures of *M. pruriens*. In chitin-treated cell cultures at 200mg/L, the increase was a 5.17 fold, while in pectin-treated cell cultures at 250mg/L, a 3.13 fold increase of L-Dopa was recorded. Similar observations were also reported by Ashish and Dixit (2008). Also, Lahiri *et al.* (2011) reported 2-3 fold increase in L-Dopa production in *M. pruriens in vitro* cultures when the media was incorporated with BAP and sucrose.

Several attempts to increase the production of desired secondary metabolites from various medicinal plants by incorporating precursors in suspension media have been studied (Caretto *et al.,* 2010; Lee *et al.,* 2007; Liu *et al.,* 2007). Production of anthocyanin and phenylethanoid glycosides was enhanced by incorporating L-phenylalanine and related precursors to the cell suspension cultures of strawberry and *Cistanche deserticola* (Edahiro *et al.,* 2005; Quyang *et al.,* 2005). According to Raghavendra *et al.,* (2012), several-fold increase in L-Dopa concentration was observed in the suspension cultures of *Mucuna pruriens* and *Mucuna prurita* when elicitor and precursor treatment was given to the suspensions. It was also observed that L-Dopa concentration was comparatively higher in the cell cultures that received precursor treatment than those that received elicitor treatment. Rahmani-Nezhad *et al.* (2018) recorded that L-Dopa content in cell suspension cultures of *Phaseolus vulgaris* increased to several folds after elicitor treatment and precursor feeding when compared to the control, also leading to an observed simultaneous increase in tyrosinase and polyphenol oxidase levels.

Conclusion

Cell suspension culture has been known to be a very useful technique in the production of various secondary metabolites including L-Dopa. This study has showed that L-Dopa concentration can be significantly increased even up to 15 folds when precursors and elicitors are incorporated in the suspension culture of *M. pruriens,* and thus could be applied successfully in large-scale production of L-Dopa. Also, this study has demonstrated the various combinations of phytochemicals that can be used to obtain maximum callus induction in *M. pruriens* which can adapt to any variety. MS medium supplemented with 0.5-2.5mg/L of 2,4-D, IBA, NAA and BAP alone or in combination is proposed as an optimum medium to enhance maximum callus induction in *M. pruriens.*

References

Ashish, B. and Dixit, V. K. (2008). Enhanced artemisinin production by cell cultures of *Artemisia annua*. *Current Trends in Biotechnology and Pharmacy,* 2: 341–348.

Caretto, S., R. Nisi, A. Paradiso, A. and De Gara, L. (2010). Tocopherol production in plant cell cultures. *Molecular Nutrition and Food Research*, 54: 726-730.

Claudia, A., Oviedo-Silva, M., Elso-Freudenberg, and Mario, A. (2018). L-DOPA Trends in Different Tissues at Early Stages of *Vicia faba* Growth: Effect of Tyrosine Treatment. *Applied Sciences,* 8: 24-31.

Dheeranapattana, S., Wangprapa, M. and Jatisatienr, A. (2009). Effect of sodium acetate on stevioside production of *Stevia rebaudiana*. *Acta Horticulturae (Internatinal Society for Horticultural Science)*. 786: 269-272.

Edahiro, J. I., Nakamura, M., Seki, M. and Furusaki, S. (2005). Enhanced accumulation of anthocyanin in cultured strawberry cells by repetitive feeding of L-phenylalanine into the medium. *Journal of Bioscience and Bioengineering,* 99: 43-47.

Farooqi, S. (1999). Dynamics of kinetically controlled binary adsorption in a fixed bed. AIChE *Journal of Chemical Engineering,* 37 (2): 299-301.

Kavitha, C. and Vadivel, E. (2005). In vitro production of L-DOPA from Mucuna pruriens (L.) DC. *Journal of Biochemical Cellular Archives,* 5: 161-168.

Kotisree, L., Madhumita, J. M. and Sandip, M. (2018). Biochemical marker-based comparative genomic characterization of *in vivo* Varieties and *in vitro* Regenerates of *Mucuna pruriens* L., an important medicinal plant. *International Journal of Botany Studies,* 3 (3): 01-10.

Lahiri, K., Mukhopadhyay, M. J. and Mukhopadhyay, S. (2011). Enhancement of L-DOPA production in micropropagated plants of two different varieties of Mucuna pruriens L., available in India. *Plant Tissue Culture Biotechnology,* 21:115–125.

Lee, M. H., Cheng, J. J, Lin, C.Y., Chen, Y. J. and Lu, M. K. (2007). Precursor feeding strategy for the production of solanine, solanidine and solasodine by a cell culture of *Solanum lyratum. Process Biochemistry,* 42: 899–903.

Liu, J, Y., Guo, Z. G. and Zeng, Z. L. (2007). Improved accumulation of phenylethanoid glycosides by precursor feeding to suspension culture of *Cistanche salsa*. *Biochemical Engineering Journal,* 33: 88–93.

Myhrman, R. (2002). Detection and removal of L-DOPA in the legume Mucuna. In Flores, M., Eilitta, M., Myhrman, R., Carew, L., and Car sky, R. (Eds.), Mucuna as a food and feed: Current uses and the way forward. Proceedings in an international workshop (pp. 142–163). Tegucigalpa, Honduras: CIDICCO.

Pras, E., Livneh, A., Balow-Jr, J. E., Pras, E., Kastner, D. L., Pras, M. and Langevitz, P. (1998). Clinical differences between North African and Iraqi Jews with familial Mediterranean fever. *American Journal of Medical Genetics,* 75 (2): 216-219.

Pras, N. (1998). Biotechnological production of catechols: by conversion spectrum related kinetic aspects of entrapped cells of *Mucuna pruriens* L. *Pharmaceutisch Weekblad Scientific Edition,* 11 (1): 30 – 31.

Quyang, J., Wang, X. D., Zhao, B. and Wang, Y. C. (2005). Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of *Cistanche deserticola*. *Process Biochemistry,* 40: 3480–3484.

Raghavendra, S., Kumar, V., Ramesh, C. K. and Moinuddin Khan, M. H. (2012). Enhanced production of L-DOPA in cell cultures of *Mucuna pruriens* L. and *Mucuna prurita* H. *Natural Product Research.* 26 (9): 792–801.

Rahmani-Nezhad, S., Shima D., Mina S., Maliheh, B. T., Adel, G. and Abbas, H. (2018). Evaluating the accumulation trend of l-dopa in dark-germinated seeds and suspension cultures of *phaseolus vulgaris* l. by an efficient UV-Spectrophotometric method. *Quim Nova.,* 41 (4): 386-393.

Ramya, K. B and Thaakur, S. (2007). Herbs containing L- Dopa: An update. *Ancient Science of Life*, XXVII (I) 50-55.

Saddhuraju, P. and Becker. K. (2003). Antioxidant properties of various solvent extracts of total phenolic constituents from three different agro-climatic origins of drumstick tree (*Moringa oleifera lam*) leaves. *Journal of Agricultural Food chemistry,* 51: 2144-55.

Sahaji, P. S. (2011). Acute oral toxicity *of Mucuna pruriens* in albino mice. International Research *Journal of Pharmacy,* 2(5):162-163.

Shalini P., Anita M. and Pandey, R. K. (2007). *In-vitro* regeneration and callus formation from different parts of seedling of *Mucuna pruriens* bak: a valuable medicinal plant. *International Quarterly Journal of Life Sciences,* 2 (1): 63-66.

Umberto, Q. (2000). CRC World Dictionary of Plant Names. 3 M-Q. CRC Press. Pp. 1738.

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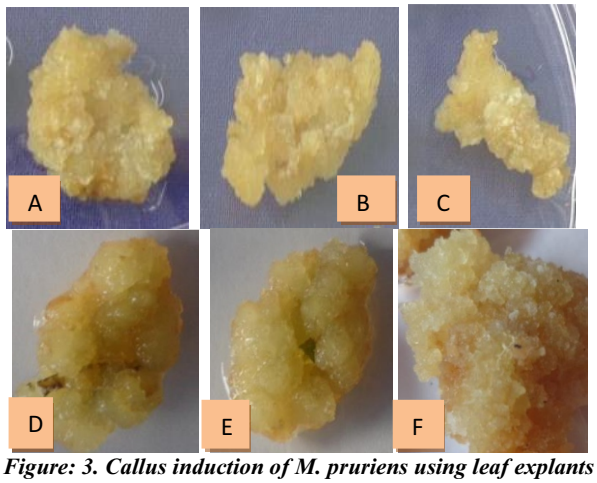
Conflict of interest: The authors of this research work hereby declare no conflict of interest.

Figure 1: Source of crude extracts for HPLC and in vitro analysis.
A- Seeds of M. pruriens var utilis (IIHR selection 3)
B- Seeds of M. pruriens var utilis (Arka Dhanvantari)

Figure 2: Source of leaf/stem explants
A- 15 days old (IIHR selection 3) plants in the greenhouse
B- 95 days old (Arka Dhanvantari) plants with matured pods in the greenhouse



A, B & C: Leaf-derived calli of *M. pruriens* (S3) at 21 days old.

D, E & F: Leaf-derived calli of *M. pruriens* (AD) at 21 days old.

Figure: 3. Callus induction of M. pruriens using leaf explants

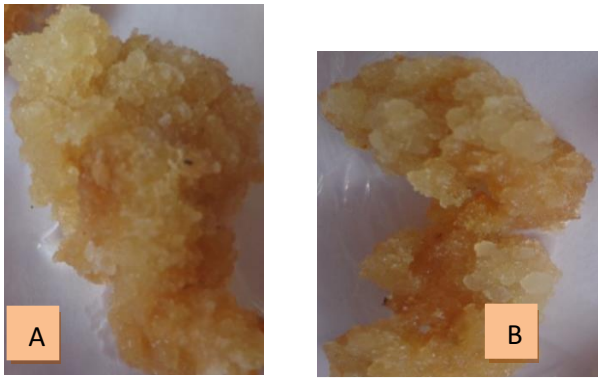


Figure 4: Callus induction of *M. pruriens* using stem explants.
A and B: Stem-derived callus of M. pruriens (S3 and AD respectively) at 21 days after inoculation

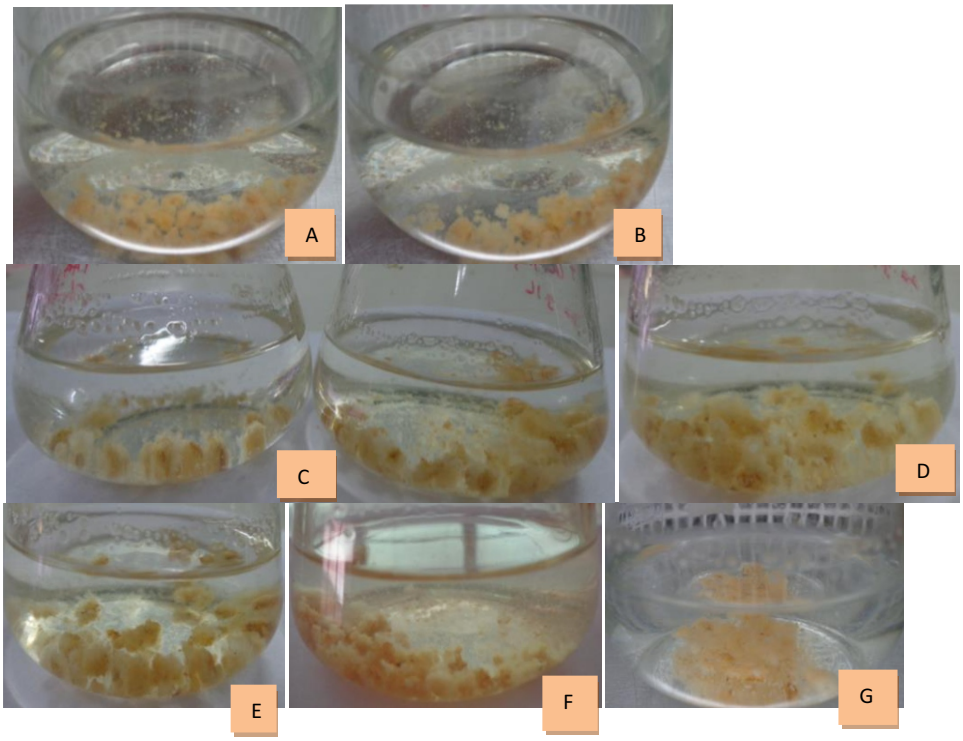


Figure 5: Cell suspension cultures of leaf and stem derived callus of M. pruriens

A and B: Cell suspension culture at 7th day after initiation.
C: Cell suspension culture at passage 1.
D and E: Cell suspension culture at passage 2.
F and G: Cell suspension culture at passage 3.

Table 1: Combination of plant growth regulators (auxins + cytokinin) used for callus induction in MS medium

Combinations of plant growth regulators and concentrations (mg/L)							
NAA + BAP (T1)		**2, 4-D + BAP (T2)**		**NAA + KIN (T3)**		**2, 4-D + IAA (T4)**	
1.0	0.5	1.0	0.5	1.0	0.5	1.0	0.5
2.0	0.5	2.0	0.5	2.0	0.5	2.0	0.5
2.5	0.5	2.5	0.5	2.5	0.5	2.5	0.5
3.0	0.5	3.0	0.5	3.0	0.5	3.0	0.5

Table 2a: Effect of plant growth regulators in combination with elicitor and precursor treatments on packed cell volume (PCV) of leaf-derived callus cell suspension over three passages for variety AD

Treatment	Packed cell volume (cm^3)			$Mean_{Treatment}$
	7 days	14 days	21 days	
L1 [a] NAA (1.5 mg/L) + Chitin (100 mg/L) + ascorbic acid (250 mg/L)	14.2 ± 0.2	21.4 ± 1.0	29.2 ± 0.4	21.6±0.5
L2 [b] BAP (1.5 mg/L) + Chitin (150 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.3	22.5 ± 0.3	41.1 ± 1.2	25.8±0.6
L3 [b] IAA (1.0 mg/L) + Chitin (200 mg/L) + ascorbic acid (250 mg/L)	13.9 ± 0.3	23.8 ± 0.6	36.9 ± 0.8	24.9±0.6
L4 [c] NAA (1.5 mg/L) + Pectin (100 mg/L) + ascorbic acid (250 mg/L)	14.3 ± 0.3	**30.4 ± 0.2**	**42.4± 0.1**	**29.0**±0.2
L5 [b] BAP (1.5 mg/L) + Pectin (150 mg/L) + ascorbic acid (250 mg/L)	14.7 ± 0.1	24.5 ± 0.9	34.2 ± 1.3	29.2±0.8
L6 [b] IAA (1.0 mg/L) + Pectin (200 mg/L) + ascorbic acid (250 mg/L)	**15.5 ± 0.6**	27.8 ± 0.6	35.4 ± 0.3	26.2±0.5
L7 [b] MS + NAA (1.5 mg/L) + L-tyrosine (5 mg/L) + ascorbic acid (250 mg/L)	14.3 ± 0.4	28.4 ± 0.3	32.5 ± 0.3	25.1±0.3
L8 [a] BAP (1.5 mg/L) + L-tyrosine (10 mg/L) + ascorbic acid (250 mg/L)	14.0 ± 0.3	22.9 ± 0.1	29.2 ± 0.3	22.0±0.2
L9 [a] IAA (1.0 mg/L) + L-tyrosine (15 mg/L) + ascorbic acid (250 mg/L)	14.1 ± 0.3	22.0 ± 1.0	30.5 ± 1.2	22.2±0.8
L10 [d] Cell suspension culture + ascorbic acid (250 mg/L) - PGR - elicitor and precursor	13.3 ± 0.0	13.3 ± 0.0	13.3 ± 0.0	13.3±0.0
$Mean_{periods}$	14.21±0.5	23.7±0.6	32.5 ±0.6	

Values are mean ± standard error of 12 explants per replicate, (n=6)

Table: 2b: Effect of plant growth regulators in combination with elicitor and precursor treatments on packed cell volume (PCV) of leaf-derived callus cell suspension over three passages for variety S3

Treatment	Packed cell volume (cm^3)			$Mean_{Treatment}$
	7 days	14 days	21 days	
L1 [a] NAA (1.5 mg/L) + Chitin (100 mg/L) + ascorbic acid (250 mg/L)	14.1 ± 0.3	21.8 ± 0.6	34.9 ± 0.5	23.6±0.5
L2 [b] BAP (1.5 mg/L) + Chitin (150 mg/L) + ascorbic acid (250 mg/L)	13.4 ± 0.1	21.2 ± 0.8	29.7 ± 0.0	21.4±0.3
L3 [b] IAA (1.0 mg/L) + Chitin (200 mg/L) + ascorbic acid (250 mg/L)	13.6 ± 0.1	20.4 ± 1.2	30.3 ± 0.7	21.4±0.7
L4 [b] NAA (1.5 mg/L) + Pectin (100 mg/L) + ascorbic acid (250 mg/L)	13.5 ± 0.1	19.9 ± 0.6	29.7 ± 1.2	21.0±0.6
L5 [a] BAP (1.5 mg/L) + Pectin (150 mg/L) + ascorbic acid (250 mg/L)	14.2 ± 0.3	26.4 ± 1.7	34.0 ± 1.1	24.9±1.0
L6 [a] IAA (1.0 mg/L) + Pectin (200 mg/L) + ascorbic acid (250 mg/L)	14.3 ± 0.3	28.3 ± 0.2	38.6 ± 0.6	27.1±0.4
L7 [b] MS + NAA (1.5 mg/L) + L-tyrosine (5 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.3	22.2 ± 0.3	31.5 ± 0.0	22.5±0.2
L8 [b] BAP (1.5 mg/L) + L-tyrosine (10 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.3	21.8 ± 0.8	33.2 ± 0.8	22.9±0.6
L9 [c] IAA (1.0 mg/L) + L-tyrosine (15 mg/L) + ascorbic acid (250 mg/L)	**14.6 ± 0.1**	**28.6 ± 0.9**	**55.1 ± 0.1**	**32.80.4**
L10 [d] Cell suspension culture + ascorbic acid (250 mg/L) - PGR - elicitor and precursor	13.3 ± 0.0	13.3 ± 0.0	13.3 ± 0.9	13.3±0.3
$Mean_{periods}$	13.9±0.7	22.39±0.6	33.0±0.5	

Values are mean ± standard error of 12 explants per replicate, (n=6)

Table 3a: Effect of plant growth regulators in combination with elicitors and precursor on packed cell volume (PVC) of stem-derived callus cell suspension over three passages for variety AD

Treatment	Packed cell volume (cm^3)			Mean Treatment
	7 days	14 days	21 days	
L1[a] NAA (1.5 mg/L) + Chitin (100 mg/L) + ascorbic acid (250 mg/L)	13.4 ± 0.1	17.2 ± 0.8	21.3 ± 0.7	17.3±0.5
L2 [b] BAP (1.5 mg/L) + Chitin (150 mg/L) + ascorbic acid (250 mg/L)	13.3 ± 0.1	19.3 ± 0.8	29.3 ± 0.0	20.6 ±0.3
L3 [b] IAA (1.0 mg/L) + Chitin (200 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.2	20.7 ± 1.3	33.3 ± 0.6	22.6 ±0.7
L4 [a] NAA (1.5 mg/L) + Pectin (100 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.3	14.0 ± 0.3	19.9 ± 2.5	15.9 ±1.0
L5 [b] BAP (1.5 mg/L) + Pectin (150 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.3	20.0 ± 1.4	30.7 ± 0.6	**21.5** ±0.8
L6[c] IAA (1.0 mg/L) + Pectin (200 mg/L) + ascorbic acid (250 mg/L)	13.9 ± 0.4	**23.3 ± 0.1**	39.0 ± 1.2	25.4 ±0.6
L7 [b] NAA (1.5 mg/L) + L-tyrosine (5 mg/L) + ascorbic acid (250 mg/L)	13.5 ± 0.1	19.8 ± 0.6	33.2 ± 0.5	22.2 ±0.4
L8 [b] BAP (1.5 mg/L) + L-tyrosine (10 mg/L) + ascorbic acid (250 mg/L)	14.1 ± 0.3	22.2 ± 0.7	32.2 ± 0.3	22.8 ±0.4
L9[d] IAA (1.0 mg/L) + L-tyrosine (15 mg/L) + ascorbic acid (250 mg/L)	**14.7 ± 0.1**	22.6 ± 0.4	**49.6 ± 0.2**	29.0 ±0.2
L10 [c] Cell suspension culture + ascorbic acid (250 mg/L) - PGR - elicitor and precursor	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.6	6.7 ±0.2
$Mean_{periods}$	13.1 ±0.2	18.6 ±0.7	29.5 ±0.5	

Values are mean ± standard error of 12 explants per replicate, (n=6)

Table 3b: Effect of plant growth regulators in combination with elicitors and precursor on packed cell volume (PVC) of stem-derived callus cell suspension over three passages for variety S3

Treatment	Packed cell volume (cm^3)			$Mean_{Treatment}$
	7 days	14 days	21 days	
L1 [a] NAA (1.5 mg/L) + Chitin (100 mg/L) + ascorbic acid (250 mg/L)	12.0 ± 0.7	28.8 ± 4.4	39.3 ± 0.2	26.7±1.8
L2[a] BAP (1.5 mg/L) + Chitin (150 mg/L) + ascorbic acid (250 mg/L)	**15.6 ± 0.2**	34.0 ± 0.3	38.4 ± 0.1	29.3±0.2
L3 [b] IAA (1.0 mg/L) + Chitin (200 mg/L) + ascorbic acid (250 mg/L)	13.9 ± 0.3	34.0 ± 0.4	40.4 ± 0.1	29.4±0.3
L4 [c] NAA (1.5 mg/L) + Pectin (100 mg/L) + ascorbic acid (250 mg/L)	13.4 ± 0.1	21.7 ± 0.7	37.5 ± 0.2	24.2±0.3
L5 [c] BAP (1.5 mg/L) + Pectin (150 mg/L) + ascorbic acid (250 mg/L)	13.4 ± 0.0	20.5 ± 0.1	39.8 ± 0.6	24.6±0.2
L6[a]IAA (1.0 mg/L) + Pectin (200 mg/L) + ascorbic acid (250 mg/L)	13.8 ± 0.4	21.0 ± 0.8	**49.3 ± 0.1**	28.0±0.4
L7 [c] NAA (1.5 mg/L) + L-tyrosine (5 mg/L) + ascorbic acid (250 mg/L)	13.9 ± 0.4	19.8 ± 0.6	37.8 ± 0.4	23.8±0.5
L8 [c] BAP (1.5 mg/L) + L-tyrosine (10 mg/L) + ascorbic acid (250 mg/L)	13.7 ± 0.3	19.6 ± 0.8	39.3 ± 0.7	24.2±0.6
L9[b] IAA (1.0 mg/L) + L-tyrosine (15 mg/L) + ascorbic acid (250 mg/L)	13.9 ± 0.2	**36.1 ± 0.3**	47.1 ± 0.2	32.4±0.2
L10 [d] Cell suspension culture + ascorbic acid (250 mg/L) - PGR - elicitor and precursor	6.7 ± 0.0	6.7 ± 0.0	6.7 ± 0.6	6.7±0.2
$Mean_{periods}$		13.03±0.3	24.22±0.8	37.56±0.3

Values are mean ± standard error of 12 explants per replicate, (n=6)

Table 4: HPLC analysis of the effect of elicitor and precursor treatment of cell suspension culture on L-Dopa production and improvement

Treatment	RT (min)	Peak area	Area%
Standard (1000 ppm)	2.33	10911.92	100
Standard (50 ppm)	2.36	4126.72	100
Standard (1 ppm)	2.35	46.53	100
T1a: NAA (1.5 mg/L) + Chitin (100 mg/L) + ascorbic acid (250 mg/L)	2.35	56.39	5.14
T1b: BAP (1.5 mg/L) + Chitin (150 mg/L) + ascorbic acid (250 mg/L)	2.36	89.03	16.19
T1c: IAA (1.0 mg/L) + Chitin (200 mg/L) + ascorbic acid (250 mg/L)	**2.33**	**113.88**	**29.99**
T2a: NAA (1.5 mg/L) + Pectin (100 mg/L) + ascorbic acid (250 mg/L)	2.33	95.45	8.21
T2b: BAP (1.5 mg/L) + Pectin (150 mg/L) + ascorbic acid (250 mg/L)	2.36	101.24	19.59
T2c: IAA (1.0 mg/L) + Pectin (200 mg/L) + ascorbic acid (250 mg/L)	**2.33**	**1125.66**	**24.63**
T3a: NAA (1.5 mg/L) + L-tyrosine (5 mg/L) + ascorbic acid (250 mg/L)	2.36	915.45	18.21
T3b: BAP (1.5 mg/L) + L-tyrosine (10 mg/L) + ascorbic acid (250 mg/L)	2.35	1033.90	26.09
T3c: IAA (1.0 mg/L) + L-tyrosine (15 mg/L) + ascorbic acid (250 mg/L)	**2.36**	**2707.91**	**31.19**
T4: Dried callus powder with no treatment	2.55	1249.18	80.98
T5: Control: Cell suspension culture + ascorbic acid (250 mg/L) - PGR - elicitor or precursor	2.35	123.42	1.39