INNO SPACE

SJIF Scientific Journal Impact Factor

CERTIFICATE OF INDEXING (SJIF 2014)

This certificate is awarded to

BIONANO FRONTIER

(ISSN: 2320-9593 (E) / 0974-0678 (P))

The Journal has been positively evaluated in the SJIF Journals Master List evaluation process
SJIF 2014 (Scientific Journal Impact Factor Value for 2014) = 4.856

SJIF (A division of InnoSpace)

Innovative Space of Scientific Research - Morocco

"Plant Tissue Culture and Phytochemical Analysis of *Mucuna pruriens* var *hirsuta*"

Mr Ashutosh. L. Upadhye and Prof (Mrs) J.P. Jadhav

Department of Biotechnology

Shivaji University, Kolhapur

Abstract

A large number of neurological degenerative diseases are threatening to human beings and many of them are presently not curable. One of them is Parkinson's disease (PD), a progressive disorder of the nervous system primarily affecting the motor system of the body. There is currently no treatment to cure PD. All therapies are designed to increase the amount of dopamine. The most effective therapy for PD is L-DOPA (3-(3, 4-dihydroxyphenyl)-L-alanine), which is converted to dopamine in the brain. The chemical production of this L-DOPA (3-(3, 4-dihydroxyphenyl)-L-alanine) results in racemic mixture which interferes with the normal functioning of the metabolism. The most suitable way is to procure this chemical from natural source. The *Mucuna pruriens* var *hirsuta is* an important legume cover crop. Almost all the parts of the plant are reported to contain L-3, 4-dihydroxy phenylalanine (L-Dopa) in considerable higher concentration formed as a product from pathway for tyrosine metabolism. The plant is known to be phytochemically rich in Anti-nutritional, Antioxidants and other phytochemical such as Saponins, Tannins, and Alkaloids etc. Hence Plant Tissue Culture of this plant is necessary in order to obtained higher L-DOPA yield along with other phytochemical. The present study deals with phytochemical analysis and plant tissue culture of the plants.

Key words: L-DOPA, Parkinson's disease, Anti-nutritional, Antioxidants.

Introduction

A large number of neurological degenerative diseases are threatening to human beings and many of them are presently not curable. One of them is Parkinson's disease (PD), a progressive disorder of the nervous system primarily affecting the motor system of the body. It is characterized by progressive loss of muscle control, which leads to trembling of the limbs and head while at rest, stiffness, slowness, and impaired balance. As symptoms worsen, it may become difficult to walk, talk, and complete simple tasks. There is currently no treatment to cure PD. Several therapies are available to delay the onset of motor symptoms and to ameliorate motor symptoms. The most effective therapy for Parkinson's

disease is L-DOPA (3-(3, 4-dihydroxyphenyl)-L-alanine), which is converted to dopamine in the brain. L-DOPA (3-(3,4-dihydroxyphenyl)-L-alanine), a dopamine precursor, either alone or in combination with an aromatic amino acid decarboxylase inhibitor (carbidopa, benserazide) is the most effective drug for the treatment of Parkins's Disease, since dopamine fails to pass through the blood brain barrier. The **demand for L- DOPA is about 250 tons per year** which emphasizes the need for exploration of a cost effective and safe alternative, comprising mainly of naturally occurring compounds. Most of the L-DOPA sold **commercially** is **synthesized from vanillin and hydantoin by a chemical process** that involves eight reaction steps. Use of biological sources, for drug synthesis, is always desirable and advantageous because the **chemical synthesis of DOPA results in racemic DL-mixture, which is inactive** and further separation of enantiomerically pure L-DOPA from this mixture, is very difficult and cumbersome. D-DOPA interferes with the activity of DOPA decarboxylase, the enzyme involved in the production of dopamine in the brain.

Plants have been exploited as an alternate source for the isolation of L- DOPA and in a screening survey; more than 1000 species in 135 plant families have been screened. Among them, genus ***Mucuna*** (Leguminosae) was found to contain the **maximum level of L-DOPA** which has been successfully exploited commercially. Among the various species of *Mucuna*, *M. holtonii* (6.4% of dry weight) and *M. pruriens* (5.21% of dry weight) have shown promising L- DOPA content in their seeds. The conversion of L-tyrosine into L-DOPA and further conversion of L- DOPA into dopaquinone which further gets converted to melanin. The photochemical properties of **melanin** make it an excellent, photoprotectant. It absorbs harmful UV-radiation and transforms the energy into harmless heat through a process called **"ultrafast internal conversion"**. This property enables melanin to dissipate more than 99.9% of the absorbed UV radiation as heat. As the demand for L- DOPA is constantly increasing, it is a need of time to search several efficient sources of this drug. Although plentiful research has been done to evaluate the prospective of *M. pruriens*; less efforts have been directed towards evaluating potentiality of other new sources.

The present work embodies our results on ***M. pruriens*** **var** ***hirsuta*** demonstrating this plant as a promising source of L- DOPA, it also shows good anti-oxidant and anti-nutritional properties and the callus induction, multiple shooting and seed germination. Since the repeated use of the seeds and plant parts is not feasible hence plant tissue culture (PTC) is used as best technology for the invitro plant cultivation and in vitro production of L-DOPA. The varieties of *Mucuna pruriens* found are *Mucuna pruriens* var. *Hirsuta, Mucuna pruriens* var. *Pruriens, Mucuna pruriens* var. *Sericophylla, Mucuna pruriens* var. *Utilis*.

Materials and Methods

1. Collection of the sample

The seeds of *Mucuna pruriens hirusuta* were collected from Kolhapur district, Maharashtra, India. The herbarium accession number of the plant is **MPS 2273** deposited at Department of Botany, Shivaji University, Kolhapur, India. The seeds were cleaned and were properly stored at room temperature.

2. Seed material and surface sterilization

Mature seeds collected from well-dried pods of six-month-old *M. pruriens* var. *utilis* plant, grown at departmental garden of Botany Department, Shivaji University, and Kolhapur were used as seed source. The seeds were initially washed with detergent for 10 min followed by wash under running tap water for 30 min. The seeds were then surface sterilized in a mixture of 0.1% mercuric chloride for 5 min and rinsed four times with sterile distilled water.

3. Establishment of callus cultures of *Mucuna pruriens*:

3.1 Preparation of M.S media

Murashige and Skoog (MS, 1962) medium with adjuvant and growth regulators were prepared as per the composition. Stock solutions of macronutrients (20 X), micronutrients (200 X), iron salt (200 X), vitamins and myo-inositol (200 X) were prepared separately and stored at 4°C. Stock solutions of PGRs were prepared by adding few drops of solvent in the required amount of growth regulator to dissolve. After dissolution, the required concentration was made by addition of double distilled water and stored in refrigerator in sterile bottles. For medium preparation a calculated amount of aliquots were added from these stock solutions. Carbohydrate sucrose was weighed and added in required quantity (3%) and allowed to dissolve. Polyvenyl pyrilidon (PVP) at a concentration of 500 mg ml-1 was added to the medium to control phenolic secretion in the medium. Unless mentioned, pH of all the medium was adjusted to 5.6-5.8 using 1NNaOH or 1N HCl after mixing all the constituents except the gelling agent and prior to the autoclaving. The volume was made up with double distilled water. Gelling agent(agar agar or Clarigel, Himedia, India) was then added and heated on water bath or steamed for the agar to melt. Molten medium was dispersed into sterile culture tubes bottles (40 ml of media) after thorough mixing. The medium was autoclaved at 15 psi and120°C for 20 min.

3.2 Plant material for callus induction

Seeds of *Mucuna pruriens* were collected during the summer from the plants grown in local area and were used as a source of explants. Plant materials were dipped in the liquid soap (Labolene) for about 15 min and were then washed thoroughly under running tap water and then with distilled water. The washed seeds were treated under aseptic conditions with 70% ethanol (v/v) for about 2 min, washed with sterile distilled water and then treated with 0.1% (w/v) $HgCl_2$ solution (surface sterilization) for about 10min. $HgCl_2$ treated seeds were washed repeatedly with sterile distilled water to remove traces of $HgCl_2$ and blot-dried using sterile tissue paper

in sterile petriplates. This surface sterilized plant material was then kept in sterile bottle used for the further inoculation.

3.3 Optimization of the Medium for callus culture of *Mucuna pruriens* var *hirusuta*

Surface sterilized seeds of *Mucuna pruriens* were inoculated on the full strength Murashige and Skoog medium supplied with variable concentration of Auxins and Cytokine and supplied with complex growth regulatory substances like Coconut milk and amino acids such Glutamine. The Auxins mainly used was 2-4-dichlorophenoxyacetic acid and the Cytokinins used was BAP (Benzyl amino purine).

The concentration of Auxin optimized was 2 mg/1000ml.

The concentration of Cytokinins optimized was 2 mg/1000ml.

Coconut Milk added was 15% v/v.

Glutamine concentration was optimized as 500 mg/1000ml.

3 % Sucrose was added in the medium.

3.4 Optimization of the medium for the shooting for *Mucuna pruriens* variety *hirusuta*

Surface sterilized seeds of *Mucuna pruriens* were inoculated on the full strength Murashige and Skoog medium supplied with variable concentration of Cytokinins optimized for the shooting. The Cytokinins used for the shooting was BAP (Benzyl Amino Purine).

3.5 Optimization of the medium for the seed germination for *Mucuna pruriens* variety *hirusuta*

Analytical grade chemicals obtained from Himedia laboratories and hormones and vitamins from Sigma-Aldrich chemicals were used for preparing the stock solutions and subsequent media preparation. Murashige and Skoog's (1962) salt with 3% (w/v) sucrose was used as basal medium excepting seed germination medium, which completely lacked sucrose. After adding the growth regulators, the pH of the medium was adjusted to 5.7 ± 0.1 followed by gelling with 0.8% of agar in case of solid medium. The media was autoclaved at 121°C and 1.06 kg/cm2 pressure for 20 min. All the cultures were incubated in a growth chamber maintained at a temperature of 25 ± 2°C, relative humidity, 70-80% and photoperiod of 16:8 h duration under photon flux density provided by day light fluorescent tubes.

4. Preparation of the Samples

The seeds of ***Mucuna pruriens*** var ***hirusuta*** was powered with seed coat and subjected for various treatments and one sample 'M' was taken as crude *Mucuna* extract for comparison.

(A) Soaking

Whole seeds were soaked in distilled water and 0.02% sodium bicarbonate solution (pH 8.6) (NaHC03, 1 g: lO ml) for 12 and 18 h at room temperature. The water was drained off, then the seeds were dried at 55"C, powdered in a Wiley Mill to 60 mesh size and analysed as above for L-DOPA, Protein content, Tannin content and Total phenolic contents.

(B) Cooking

Separate batches of seeds were cooked in distilled water 3 hours on a hot plate, the seeds rinsed, dried at 55ºC, powdered in a Wiley Mill to 60 mesh size and the contents of L-DOPA, Protein content, Tannin content and Total phenolic contents determined as above.

(C) Autoclaving

The seed samples were autoclaved at 15 lb pressure (121 "C) in distilled water for 20 min. Then the seeds were rinsed with distilled water, dried and powdered in a Wiley Mill to 60 mesh size. L-DOPA, Protein content, Tannin content and Total phenolic contents content were analysed as described below.

5. Phytochemical Analysis of *Mucuna pruriens* var *hirsuta*

5.1 Estimation of L- DOPA

L-DOPA was estimated by Arnow's method (Arnow 1937). In brief, 1ml each of the following reagents was added to 1ml of test sample0.5M HCl, 15% (w/v) sodium molybdate, 15 % (w/v) sodium nitrite, and 1M sodium hydroxide. The acid was added to inactivate the residual free enzyme, stopping further biotransformation Addition of sodium hydroxide turns the yellow colour of the reaction mixture to an orange-red colour due to the diazotization of the amino group of DOPA, and the concentration of L-DOPA formed was detected at 460 nm in a spectrophotometer from the standard curve of

standard samples	Processing date 06/07/13
A	with soaking
B	24 hours D/W
C	18 hours D/W
D	18 hours NaHCO3
E	24 hours NaHCO3
M	Crude seed extract

L-DOPA.

5.2 Analysis of Total Phenolic Content (TPC)

The TPC of ***Mucuna pruriens* var *hirsuta*** was determined spectrophotometrically using the Folin-Ciocalteu assay. An aliquot of 50µl and 100µl extract was mixed with 1.8 ml of Folin- Ciocalteu reagent which was previously diluted 10-fold with distilled water. The solution was allowed to stand at 25°C for 5 min before adding 1.2 ml of 15% aqueous sodium carbonate solution. Reaction was continued for 90 min at room temperature, after which absorbance was measured at 765 nm using a spectrophotometer. This was compared to standard curve of Gallic acid concentrations.

5.3 Analysis of Total Flavonoids Content (TFC)

Total flavonoid contents of all extracts were measured by colorimetric method. ***Mucuna pruriens hirusuta*** extracts (1 ml and 0.5ml) were mixed with methanol (1.5 ml), to which 10% Aluminum Chloride (0.1 ml), 1 M potassium acetate (0.1 ml) and distilled water

(2.8 ml) were added. The solution was vortexes and allowed to stand for 30 min at room temperature. The absorbance of reaction mixture was measured at 415 nm using a UV–Vis spectroscopic analysis. The total Flavonoids content was quantified according to the standard curve prepared using quarcetin and the concentration of Flavonoids was reported as mg of quarcetin equivalents per g of extract.

5.4 Determination of total carbohydrate by Anthrone method

Total carbohydrates were detected using Anthrone method by using Glucose as standard. Different plant extracts (1ml) were added to the Anthrone reagent and kept in boiling water bath for 10 min and colour developed was monitored at 630 nm. Concentrated sulphuric acid hydrolyses glycosidic bonds to give monosaccharides which are then hydrated to furfural derivative. This furfural derivative then reacts with Anthrone to form blue green coloured complex.

5.5 Determination of protein content by Lowry method

Total protein was estimated by Lowry method in which protein reacts with Folin- Ciocalteau reagent to give coloured complex (Lowery et al, 1951) whose optical density was measured at 660nm. Colour so formed is due to the reaction of alkaline copper with the protein and reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

5.6 Determination of Tanin content

1 gm of the samples was added with 50 ml of distilled water and kept in shaking condition for 30 min. Centrifugation was done at 5000 rpm for 10 min the supernatant was taken in another tube by filtration. 5 ml of the extract was added with 1 ml F.C Reagent and then 2.5 ml of Saturated Sodium Chloride was added. Volume was made to 50 ml in a flask and incubated at room temperature for 90 min. The optical density was measured at 760 nm.

$$\text{Tanin (mg/100 gm)} = \frac{\text{Sample reading} - \text{Blank}}{\text{Standard}}$$

5.7 Reducing sugar content

The method used for the estimation of reducing sugar was DNSA method. Glucose was used as standard. 1 ml of the supernatant extract of the treated and crude seed was taken and 2.5 ml of DNSA reagent was added and kept in boiling water bath for 10 min. Optical density was measured at 550 nm.

5.9 Phytic acid content determination in *Mucuna pruriens hirusuta*

1 ml of the supernatant of the treated and crude sample was pipette out in a test tube. 1 ml of Wade's reagent (0.03% $FeCl_3.6H_2O$) and 0.3% (Sulphosalicyclic acid in D/W). The content was mixed and vortex for 5 seconds and the supernatant is taken for determination of optical density at 500 nm.

5.10 Estimation of Vitamin C by colorimetric method

The 1 ml supernatant of the treated and crude extract was added with 1 ml of DNPH (Dinitro-phenyl hydrazine) then 2 ml of Thiourea was added and the content was incubated for 3 hours at 37º. Then 80% H_2SO_4 2 ml was added. Optical density of the coloured compound was measured at 530 nm.

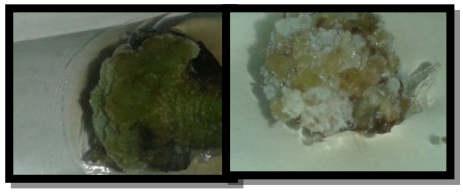
Result and Discussions

1 Callus induction

The following Callusing medium was optimized by various combinations of growth factors and complex growth regulating substances for callusing of Mucuna pruriens hirusuta.

Murashige and Skoog = 4.41 gm/l
BAP = 2 mg/litre
2, 4-D = 2 mg/litre
Coconut milk = 15%
PVP = 1%
Sucrose = 3%
Clarigel = 0.25%
P_H = 5.8

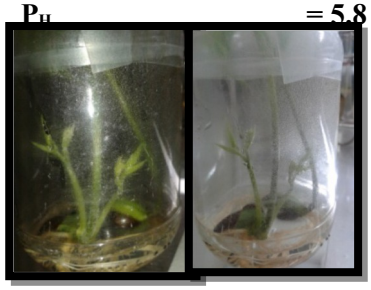
The growing of callus was observed as follows:



2. Development of multiple shoots

The following Shooting medium was optimized by various combinations of growth factors and complex growth regulating substances for callusing of Mucuna pruriens hirusuta.

Murashige and Skoog = 4.41 gm/litre
BAP = 3 mg/litre
Sucrose = 3%
Clarigel = 0.25%
P_H = 5.8



3. Seed germination of Mucuna pruriens hirusuta. The sterile seeds were inoculated on half strength M.S medium and incubated for at a temperature of 25 ± 2ºC, relative humidity, 70-80% and photoperiod of 16:8 h duration under photon flux density provided by day light fluorescent tubes

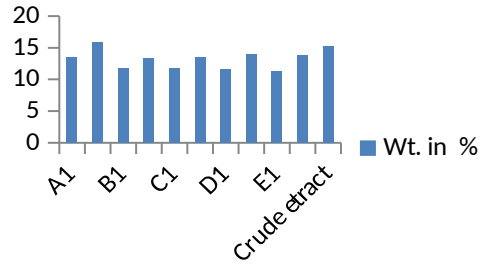
4.Phytochemical analysis

4.1 Determination of the Total Phenolic content (Folin-Ciocaltue Asssay)

Total Phenolic content of the samples was estimated and after treatment it was found that the content decreases.

Standard Querecetin = 0.5 mg/ml

Sample	Incubation Time	Wt. in %	conc./100 gm of seed
A1	0	13.46	13.46
A2	0	15.7666	15.766666
B1	24	11.69332	11.69332
B2	24	13.27332	13.27332
C1	18	11.81332	11.81332
C2	18	13.5	13.5
D1	18	11.55332	11.55332
D2	18	13.9	13.9
E1	24	11.29332	11.2933
			2
E2	24	13.8266	13.82666
Crude etract	no treatment	15.254	15.254

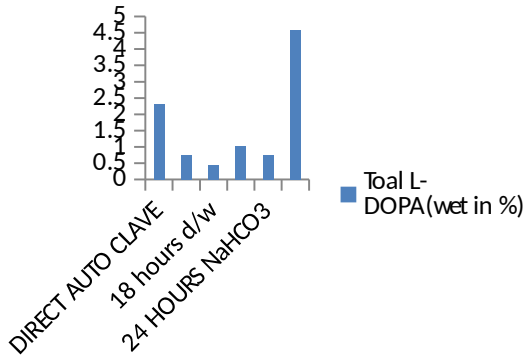


4.2 Estimation of L-DOPA content

L-DOPA was estimated by Arnow's method (Arnow 1937) the concentration of L-DOPA formed was detected at 460 nm in a spectrophotometer from the standard curve of L-DOPA. Maximum L-DOPA concentration was found in crude extract and it decreased as

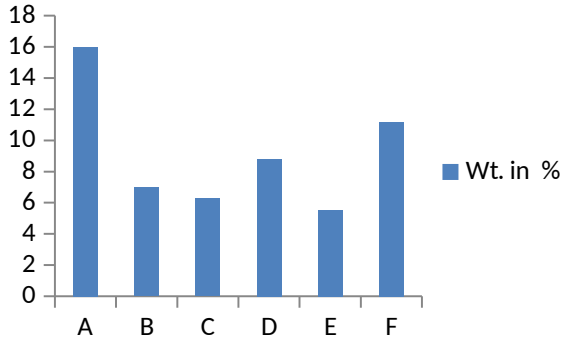
samples was treated. **Standard L-DOPA= 1 mg/ml**

Sample	Incubation Time	conc./100 gm of seed	Wt. in %
A1	0	29.84	2.984
A2	0	28.86	2.886
B1	24	7.22	0.722
B2	24	7.44	0.744
C1	18	4.34	0.434
C2	18	5.44	0.544
D1	18	10.42	1.042
D2	18	9.14	0.914
E1	24	7.48	0.748
E2	24	7.42	0.742
M2 CRUDE	Direct extract untreated	46.1	4.61
M3 CRUDE	Direct extract untreated	40.68	4.068



developed was monitored at 630 nm. **Standard glucose = 100µg/ml**

Sample	Incubation Time	conc/100 gm of seed	Wt. in %
A	DIRECT AUTO CLAVE	0.1600818	16.00818
B	24 hours d/w	0.0696818	6.96818
C	18 hours d/w	0.0628818	6.28818
D	18 hours NaHCO3	0.0878818	8.78818
E	24 HOURS NaHCO3	0.0554818	5.54818
M	Direct extract untreated	0.1116818	11.16818



4.3 Estimation of total sugar content by Anthrone Method

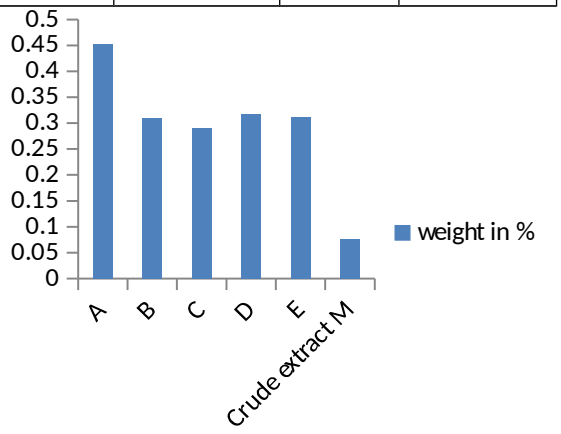
Total carbohydrates were detected using Anthrone method by using Glucose as standard. Different plant extracts (1ml)were added to the Anthrone reagent and kept in boiling water bath for 10 min and colour

4.4 Determination of the Tanin in the sample :- The amount of Tanin was estimated and it was found that as the treatment was given to the seeds the tanin content increased.

Standard Tannic acid (ug/5ml)

Sample	Incubation Time	conc / 100 gm of seed powder	weight in %
A	with out soaking	0.4528	0.4528

B	24 hours D/W	0.3088	0.3088
C	18 hours D/W	0.2912	0.2912
D	18 Hours **NaHCO3**	0.3168	0.3168
E	24 hours NaHCO3	0.3112	0.3112
Crude extract	no treatment	0.0768	0.0768

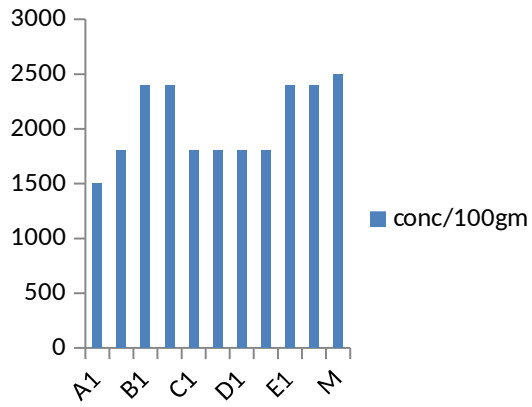


4.5 DNSA Test For Reducing sugar

The method used for the estimation of reducing sugar was DNSA method. Glucose was used as standard. 1 ml of the supernatant extract of the treated and crude seed was taken and 2.5 ml of DNSA reagent was added and kept in boiling water bath for 10 min. Optical density was measured at 550 nm.

Standard Glucose = 500 µg/ml

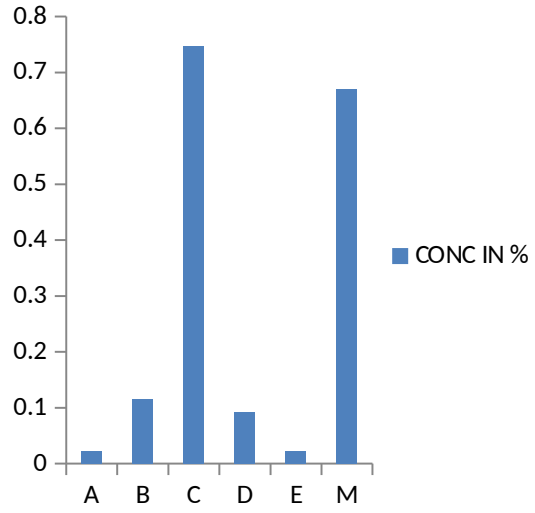
Sample	Incubation Time	conc/100gm	Wt. in %
A1	0	0	0
A2	0	0	0
B1	24	2400	2400
B2	24	2400	2400
C1	18	1800	1800
C2	18	1800	1800
D1	18	1800	1800
D2	18	1800	1800
E1	24	2400	2400
E2	24	2400	2400
M	24	2500	2500



4.6 Estimation of vitamin C

The concentration of the Vitamin C decreases after the treatment of the seeds. **Standard vitamin c= 1 mg/ml**

Sample	Incubation Time	conc/100 gm of seed	CONC IN %
A	DIRECT AUTO CLAVE	0.02309468	0.02309468
B	24 hours d/w	0.11547344	0.11547344
C	18 hours d/w	0.74671048	0.74671048
D	18 hours NaHCO3	0.09237876	0.09237876
E	24 HOURS NaHCO3	0.02309468	0.02309468
M	Direct extract untreated	0.66974596	0.66974596



6. Conclusion

The plant Mucuna pruriens var hirsuta is a very important plant with high levels of L-DOPA found to be 4.61%. It also contains wide variety of useful phytochemical and also can be used as cattle fodder. Due to high content of L-DOPA its plant tissue culture can be done in order to obtain high and enhanced L-DOPA yield.

7. References

- In vitro clonal propagation of *Mucuna pruriens* var. *utilis* and its evaluation of genetic stability through RAPD markers, N. Sathyanarayana*, T. N. Bharath kumar, African Journal of Biotechnology Vol. 7 (8), pp. 973-980, 17 April, 2008.
- Effect of soaking, cooking and autoclaving on Phytic acid and oligosaccharide contents of the tribal pulse, *Mucuna monosperma*, K. Vijayakumari, * P. Siddhuraju & K. Janardhanan, Food Chemistry, Vol. 55, No. 2, pp. 173-177, 19.
- Antibacterial and antioxidant activities of hydro alcoholic stem bark extract of Schotia latifolia Jacq, Sunday Oyewole Oyedemi, Asian Pacific Journal of Tropical Medicine (2011)952-958.
- Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods, Okezie I. Aruoma, Mutation Research 523–524 (2003) 9–20.
- A study on the In vitro Antioxidant Activity of Juniper (Juniper communis L) fruit extracts, Dr.Mahfuz Elmestas, Analytical letters, 39:47-65; 2006.

- Phenolic content and Antioxidant Activity germinated and cooked pulses, Hardeep sing Gujaral, Mamta Angurala, International journal of Food properties, 14:1366-1374, 2011.
- Total Phenolic content and antioxidant of two different solvent extracts from processes legumes of *Pisum sativum*, Srinivasan Nithiyanatham, Subramanian Selvakumar, Journal of food composition and analysis.