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IN VITRO MICROPROPAGATION OF DESMODIUM TRIQUETRUM DC., MYANMAR MEDICINAL PLANT

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Abstract— **Desmodium triquetrum DC possess immense pharmaceutical value. Preliminary phytochemical examinations were carried out before in vitro micropropagation. A rigorous attempt has been made for development of micropropagation procedure for this species, involving five steps, namely: in vitro seed germination, shoot multiplication, callus induction, rooting and hardening. In this study, direct shoot development and callus formation were achieved from different nodes and leaf disc of in vitro seedling of Desmodium triquetrum. Murashige and Skoog's (MS) medium supplemented with different concentrations of cytokinin and in combination with auxin were used for shoot multiplication, callus induction and root formation. Polyphenolic exudation from the cultured explant was controlled by the incorporation of citric acid to the medium. The rooted plantlets were transplanted to a plastic bags containing soil mixture and grown in a green house conditions for adaptation to natural environment.**

Index Terms— **Desmodium triquetrum, micropropagation, cytokinin, auxin, citric acid. .**

I. INTRODUCTION

Medicinal plants played an important role in the treatment of diseases and health disorders for thousands of years and are still important in traditional medicine systems around the world [1]. World Health Organization has listed 21000 plant species used around the world for medicinal purpose. Many herbal remedies individually or in combination have been recommended in varies medical treatments for the cure of different diseases.

Desmodium triquetrum DC (Leguminosae, Subfamily-Papilionaceae) is one of the popular medicinal plants in Myanmar. It is a subshrub, growing up to 3 m tall with erect stems which are almost woody. Branches are triangular in cross section, velvety. Leaves are alternately arranged, and the leaf stalk has prominently wings. Leaves are linear-oblong, ovate or heart-shaped, with a tapering tip. Flowers arise in many-flowered racemes in leaf axils. Flowers are small, shaped like pea flowers, pale violet. Legumes are hairy, 5-8 jointed and distributed through lower Myanmar, central and eastern Himalayas, South India and Sri Lanka.

The leaves of this plant contain tannins, alkaloids, hyperforin, trigonelline, tanning materials, and silicic acid. Fruit leaves contain saponin sitting and flavonoids, while the roots contain saponins, flavonoids, and tannins [2]. The leaves are used as a substitute for tea by hill tribes in upper Assam. All parts except the root (herb) may be used. This herb is efficacious for: prevents fainting from the heat (heat stroke), fever, colds; inflammation of the tonsils (tonsillitis), mumps (parotitis), melt pus (piorea); acute inflammation of the kidney (acute nephritis), swollen (edema); inflammation of the intestine (enteritis), dysentery; hookworm infection (hookwonn), tapeworm infection in the liver; vaginal discharge due to trichomonas (trichomonal vaginitis); vomiting in pregnancy, malnutrition in children; jaundice (jaundice hepatitis); poisoned fruit of pineapple; tuberculosis of bones and lymph glands; scleroderma; hemorrhoids and rheumatism [3]. The fresh leaves are applied to wounds and abscesses that do not heal well. They are used internally as a galactagogue. A paste of the bruised leaves with kamala is applied to in dolent sores and itchi. The fresh juice of the plant is given to children to cough. In Ceylon, it is used in dysentery in the gold coast it is recommended both as a laxative and as a cure for dysentery [4].

At present, this invaluable plant species in Myanmar, as in other countries is unfortunately dwindling due to over collection, and the increasing expansion activities of the growing population and their ambitions. Some of these species became quite scare. A sufficient supply of the plant raw material contains a consistent quality of valuable natural products becomes difficult with increasing the need to consume such natural products. Therefore, laboratories worldwide are trying to produce secondary metabolites from plant tissue cultures for commercial application as an alternative or addition to plants produced in fields or greenhouses [5].

For increased human needs of medicines, plant tissue culture is widely used for micropropagation of medicinal plants

to produce enough amounts of drugs and secondary metabolites. Using this technology, the natural products can be provided at any time of the year without waiting for the suitable season to collect the plant and controlling the environmental conditions of plant growth [6]. In addition to that it could be obtained of the plants in a short time and a small place [7].

Therefore, this study aimed to develop reliable and reproducible protocol for the *in vitro* propagation and conservation of *Desmodium triquetrum* DC.

II. Materials and Methods

The experiment was conducted at the Pharmaceutical Research Department (PRD) and the Plant Tissue Culture Laboratory (PTC), Biotechnology Research Department in Kyaukse, Myanmar. The present study indicates the presence of active compound and the rapid multiplication of *Desmodium triquetrum* DC by optimizing the effect of the concentration of different plant regulators.

A. Collection of Plant Material

Leaves and dried pods of *Desmodium triquetrum* DC were collected from *in vivo* grown plants, Department of Biotechnology, Technological University (Kyaukse).

B. Preparation for Preliminary Phytochemical Tests

The collected leaves were air-dried and ground into powder with the aid of machine. After preparing sample powder, the identification for types of compound, containing in the plant sample was made by employing phytochemical tests.

C. Culture Condition

MS medium [8] solidified with 0.64% agar with 3% sucrose. Basal medium was supplemented with various concentrations of BAP (6-Benzylaminopurine) alone and combined with different concentrations of NAA (1-Naphthalene acetic acid) for axillary shoot multiplication and callus proliferation. For rooting, the *in vitro* raised shootlets were transferred to ½ MS medium with or without plant growth regulator. The medium is adjusted to pH 5.7-5.8 using 0.1 M NaOH or 0.1 M HCl and dispensed in culture bottles before autoclaving at 121˚C for 20 min. All cultures were maintained at 25±2˚C.

D. Surface Sterilization of Explants

Seeds were used as initial explant for establishment. Before surface sterilization, pods were kept under flowing tap water for one hour, and treated with liquid detergent for five minutes. And they were disinfected by immersion in 200 mg/l (Ciprofloxacin) for one hour, followed by shaking in 70% ethanol for 3 min. Under aseptic condition, a final treatment with 0.1% $HgCl_2$ was given for ten minutes. After finishing every steps of surface sterilization, the explants were thoroughly washed with distilled water for several times.

E. Initiation, Multiplication and Callus Induction of Explants

The surface sterilized seed pods were dried onto filter paper. Seed coats were removed with sterile scalpel prior for germination. The seed were germinated on hormone free MS medium. Cotyledonary nodes, first and second leaf nodes together with hypocotyl, epicotyl, shoot and leaf disc from 45 day-old in vitro grown plants were culture on (MS) medium with 5 mg/l BAP. A total of 21 treatments containing plant growth regulators were used for shoot multiplication and callus induction.

F. Root formation, Hardening and Transplantation

Shoots of 2-3 cm in height with two or three leaflets derived were transferred to ½ MS medium containing 0.1-1.0 mg/l NAA for root formation. All the shoots produced roots within 10-30 days of culture. The rooted plants were washed with water to remove media then transferred to plastic bags containing sand and soil mixture, and covered with plastic bag for one weeks to maintain humidity and subsequently exposed to low air humidity for increasing period and finally bags are removed.

G. Data Analysis

Each experiment had three replicates for *in vitro* shoot multiplication, callus induction and rooting. Each replicate had five propagules. The response of explants with different concentrations and combinations of cytokinin and auxin with basal MS medium were recorded at 28 days and 49 days after inoculation. The obtained data were analyzed statistically using SPSS.17 software (IBM Corporation SPSS, North America). The significance of difference among the means was calculated using Duncan's Multiple Range Test.

III. Results and Discussions

Firstly, phytochemical analysis qualitative tests were carried out for the presence of active phytochemicals in the aqueous, ethanol, leaves extracts of *Desmodium triquetrum* DC. This analysis revealed the presence of alkaloids, glycosides, flavonoids, saponins, amino acids and the absence of cyanogenic glycosides (TABLE I) in D. *triquetrum* DC. After finding its potential safety, an *in vitro* propagation technique was used for *Desmodium triquetrum* DC through seeds culture.

Three seeds were germinated in each culture bottle. After germination, 45 day-old hypocotyl, epicotyl and shoot pieces which carrying two cotyledonary nodes, first and second nodes were dissected out from seedling with sterile scalpel and then cultured on MS media containing 5.0 mg/l BAP. Callus was observed from the cut end of leaf, internodal segment when 5mg/l BAP alone or in combination with NAA were used. Axillary shoot, developed after 28 days of culture, were dissected out individually for further multiplication. This process was continued repeatedly every 28 days of culture to get adequate amount for experiments. Citric acid 10.0 mg/l was added to the medium to control the exudation of phenol.

Cotyledonary nodes, first and second leaf nodes are the main sources for direct regeneration, and the leaf pieces and hypocotyl are the main source for callus induction for

Desmodium triquetrum DC. Similar results have been reported in [9, 10, 11, 12]. According to the literature, BAP is one of the most effective hormones for obtaining multiple shoots from cotyledonary nodes. However, many other factors like genotype, composition of the nutrient media and physical growth factors such as light, temperature, moisture etc. are

TABLE I. PRELIMINARY PHYTOCHEMICAL EXAMINATION OF *DESMODIUM TRIQUETRUM*

No.	Constituent	Reagent used	Observation	Tested medicinal plant
1.	Alkaloids	(1) Dragendroof's reagent (2) sodium picrate (3) Wagner's reagent	Orange ppt. Yellow ppt. Reddish-brown	+ + +
2.	Glycoside	10% lead acetate	White ppt.	+
3.	Reducing sugar	Benedict's solution	Brick red ppt.	-
4.	Phenolic compound	$FeCl_3$ solution	Green colour	-
5.	Flavonoids	Benzene, $FeCl_3$	Pink colour	+
6.	Saponin glycosides	Distilled water	frothing	+
7.	Cyanogenic glycoside	Conc:H_2SO_4, sodium picrate	Brick red colour	-
8.	α-amino acids	Ninhydrin reagent	Purple colour	+
9.	Carbohydrate	10% α-napthol+ Conc: H_2SO_4	Red ring	-
10.	Acid or basic or neutral	Bromocresol green	Blue- basic Green-acidic No colour change-neutral	Basic
11.	Tannin	$FeCl_3$ solution, Conc:H_2SO_4	Yellowish-brown	+

TABLE II. TTE EFFECT OF DIFFERENT CONCENTRATIONS OF PLANT GROWTH REGULATORS ON SHOOT MULTIPLICATION AND ELONGATION

Treatments (mg/l)		After 28 d		
BAP	NAA	A	B	C
Con.	0	1±0 f	0 h	0.5±0 e
1	0	1.67±0.58 def	4.25±1.56 cd	1.57±0.12 a
2	0	1.33±0.38 ef	3±0.25 cdef	1.33±0.14 abc
3	0	2.08±0.38 cde	2.33±0.29 efg	1.13±0.11 c
4	0	1.67±0.63 def	0 h	0.8±0 d
5	0	1.85±0.54 cde	0 h	0.8±0 d
1	0.1	1.67±0.14 def	4.5±0.43 c	1.33±0.14 abc
2	0.1	1.83±0.14 cde	2.33±0.14 efg	1.2±0.2 c
3	0.1	2.58±0.14 bc	1±0.73 gh	0.67±0.28 de
4	0.1	3±0.43 b	0 h	0.7±0 de
5	0.1	2.67±0.29 bc	0 h	0.7±0 de
1	0.5	2.08±0.14 cde	4.33±0.38 cd	1.53±0.06 ab
2	0.5	2.08±0.14 cde	4.17±0.14 cd	1.4±0.2 abc
3	0.5	4.5±0.43 a	6.58±0.52 b	1.27±0.11 bc
4	0.5	5.25±0.25 a	8.42±1.04 a	1.23±0.05 c
5	0.5	4.67±0.76 a	2.67±2.47 defg	0.77±0.21 de
1	1	2.17±0.52 cde	3.37±0.23 cde	1.42±0.14 abc
2	1	1.33±0.29 ef	1.58±0.52 fgh	0.86±0.21 d
3	1	2.58±0.72 bc	1.25±1.25 gh	0.68±0.16 de
4	1	2.23±0.50 bcd	1.5±1.39 fgh	0.75±0.25 de
5	1	1.92±0.58 cde	0.42±0.72 h	0.67±0.15 de

Each value represents the mean ±SD of five replications. Mean values ±SD followed by the same letter within columns are not significantly different at P=0.05 by Duncan's multiple range test.

Con, control; A, average no. of shoots/explant; B, average no. of node/explant; C, average length of shoot/explant

TABLE III. TTE EFFECT OF DIFFERENT CONCENTRATIONS OF PLANT GROWTH REGULATORS ON SHOOT MULTIPLICATION AND ELONGATION

Treatments (mg/l)		After 28 d		
BAP	NAA	A	B	C
Con.	0	1±0 f	3.7±0.26 cdef	4.7±0.26 a
1	0	3.68±1.41abcde	9.95±4.95 a	5±0.86 a
2	0	3.08±0.52 cde	10±2.05 a	5±0.86 a
3	0	4.5±1.39 abcd	7.83±2.24 ab	3.07±0.40 bc
4	0	3.25±0.25 bcde	5.42±0.28bcde	3.17±0.28 b
5	0	4.6±0.36 abc	6.5±1 bc	2.9±0.1 bc
1	0.1	3.25±1.15 bcde	5.58±0.80bcde	2.53±0.50 bcd
2	0.1	3.92±1.44abcde	3.5±0.5 cdef	2.1±0.36 d
3	0.1	3.23±0.50 bcde	1.92±2.32 f	1.18±0.72 e
4	0.1	5.08±1.42 a	2.33±1.46 ef	0.9±0.17 e
5	0.1	5±0.5 a	2.33±2.84 ef	0.77±0.21 e
1	0.5	2.92±0.14 de	5.67±0.63 bcd	2.63±0.15 bcd
2	0.5	2.5±0.43 e	6.08±1.01 bcd	3.03±0.06 bc
3	0.5	4.75±0.66 abc	7.92±1.01 ab	2.37±0.11 cd
4	0.5	5.33±0.14 a	10.42±0.52 a	2.93±0.11 bc
5	0.5	4.83±0.76 ab	8.33±1.46 ab	2.73±0.25 bcd
1	1	2.5±0.43 e	5.92±0.76 bcd	2.17±0.29 d
2	1	3.33±0.52 bcde	2±0.5 f	1.05±0.18 e
3	1	3.75±0.90abcde	1.87±1.64 f	1.02±0.23 e
4	1	3.85±1.42abcde	2.92±1.18 def	0.95±0.28 e
5	1	3.28±o.64 bcde	0.83±1.44 f	0.68±0.13 e

. Each value represents the mean ±SD of five replications. Mean values ±SD followed by the same letter within columns are not significantly different at P=0.05 by Duncan's multiple range test.

Con, control; A, average no. of shoots/explant; B, average no. of node/explant; C, average length of shoot/explant

important for *in vitro* micropropagation [13]. Data on shoot multiplication and callus induction efficiency were recorded after 28 days and 49 days of culture.

Within 28 days, there was no significant callogenic response from explants observed in all BAP concentration of experiments. Therefore, individual treatment of BAP showed low response for callus induction. Plant growth regulator (PGR)-free basal MS medium did not show any callus formation from explants. Similar results have been reported no callogenic response from explant on PGR-free medium [14].

After 49 days inoculation, nodal explants from *D. triquetrum* plants were cultured on Murashige and Skoog's basal medium with BAP alone or BAP in combination with NAA, enhanced shoot multiplication. Hormone free MS medium induced shoot formation without proliferation.

Therefore, it is indicated that the cytokinin, BAP is the most essential growth regulator for effective shooting of the study

TABLE IV. EFFECT OF VARIOUS CONCENTRATIONS OF BAP AND NAA ON CALLUS INDUCTION OF DESMODIUM TRIQUETRUM ON MS MEDIUM

Treatments		Fresh callus weight (g), after seven weeks culture initiation
BAP (mg/l)	NAA (mg/l)	
1	0.5	6.29
2	0.5	10.83
3	0.5	7.12
4	0.5	8.52
5	0.5	9.28
1	1.0	10.39
2	1.0	4.81
3	1.0	6.99
4	1.0	8.32
5	1.0	4.29

TABLE V. DIFFERENT CONCENTRATIONS OF AUXIN ON ROOT INDUCTION OF *DESMODIUM TRIQUETRUM* ON MS MEDIUM

Treatments	Average No. of root/explant	Average total root length (cm)
NAA (mg/l)		
Control	3.33±2.31 b	1.23±0.25 b
0.1	2.66±1.53 b	3.5±0.87 a
0.25	3±1 b	2.1±1.15 ab
0.5	2.33±1.53 b	3.17±0.76 a
0.75	6.33±2.08 a	3.5±0.5 a
1	7±0 a	3.5±0.87 a

Each value represents the mean ±SD of five replications. Mean values ±SD followed by the same letter within columns are not significantly different at P=0.05 by Duncan's multiple range test

species, *D. triquetrum.* It is of common fact that cytokinin is the major growth hormone [15, 16, 17, 18, 19, 20]. Although all BAP and NAA containing media showed shoot multiplication, shoot number significantly increased by increasing the BAP concentration; the highest shoot number were 5.08±1.42 at 4 mg/l BAP with 0.1 mg/l NAA, 5±1.42 at 5 mg/l BAP with 0.1 mg/l NAA and 5.33± 0.14 at 4 mg/l BAP with 0.5 mg/l NAA.

The higher shoot length of about 5 cm was achieved in the MS medium fortified with BAP 1 and 2 mg/l and control. In addition, BAP 1-2 mg/l and 4 mg/l BAP with 0.5 mg/l NAA produced highest nodes number (10) per plant. Increased

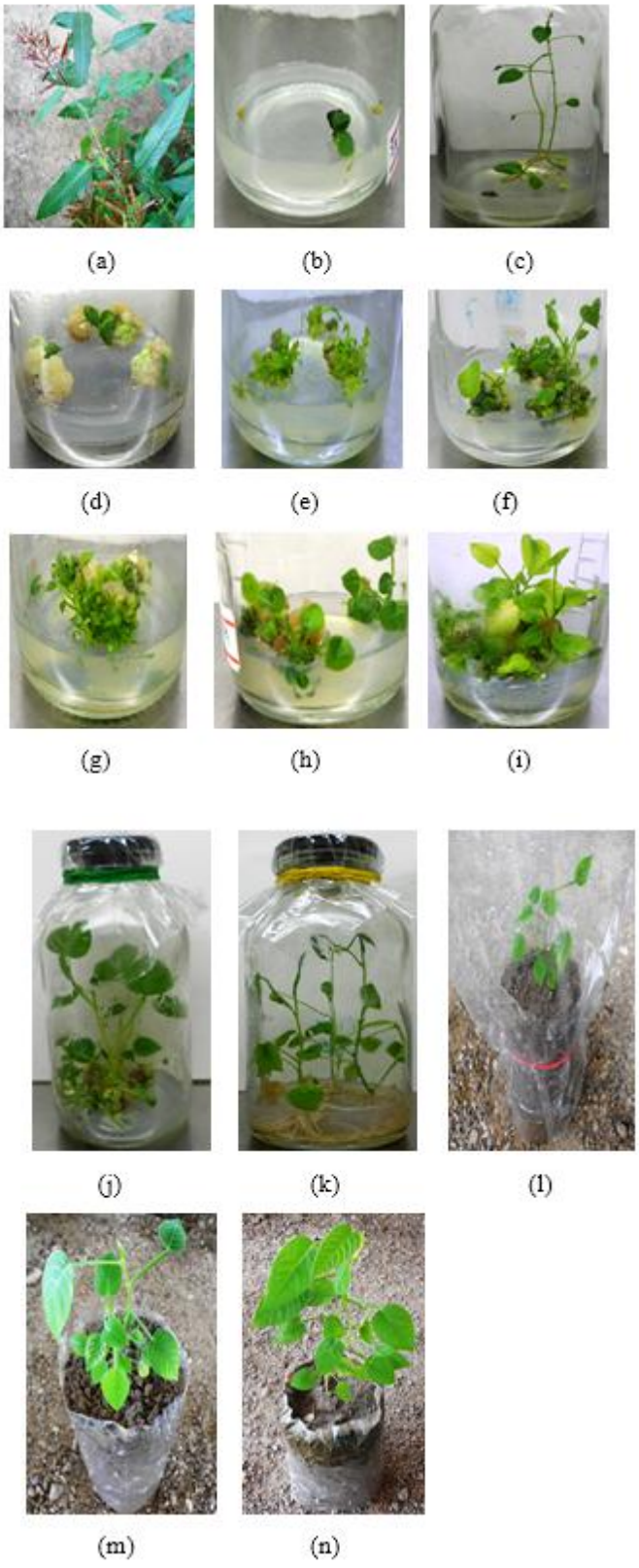

Fig. 1. Explant collection, culture establishment and shoot multiplication and callus induction in *Desmodium triquetrum* DC.; (a) mother plant, (b) the in vitro culture establishment, (c) seed germination, (d) callus induction, (e)-(j) stages of shoot multiplication, (k) root formation on ½ MS medium, hardening stages (l) at 7 days, (m) at 14 days, (n) at 30 days in green house condition

concentration of BAP led to a decrease in shoot length (TABLE III). BAP at varying (1-5) mg/l in combination with 0.5 and 1.0 mg/l NAA induced callus formation when supplemented in MS medium. Effective callus formation from the explants was obtained in the MS medium containing BAP 2 mg/l + NAA 0.5 mg/l and BAP 1 mg/l + NAA 1.0 mg/l (TABLE IV).

The rooting attributes of *D. triquetrum* while subculturing the secondary explants, shoots were well pronounced in the ½MS medium supplemented with the auxin, NAA alone at concentrations from 0.1 to 1.0 mg/l. Root formation was observed within 10- 30 days. The number of roots and average root length were recorded for each concentration after 30 days of culture (TABLE V). The number of roots per shoot was also observed to be higher (7 roots/shoot) in the MS medium containing 1.0 mg/l NAA for the study species, *D. triquetrum*. Similarly, the root length was greater (3.5 cm) during the sub-culturing of *in vitro* cultured shoots for roots on MS medium with NAA at 1.0 mg/l. Plant with at least two roots and minimum 3 cm total root length were transferred to soil and acclimated to the green house conditions.

IV. Conclusion

The impact of different concentrations of auxin and cytokinin were evaluated for shoot multiplication, callus induction and root formation. The statistical analysis showed that BAP 4 mg/l BAP with 0.1 mg/l NAA was optimal for shoot multiplication, BAP 2 mg/l fortified with NAA 0.5 mg/l was the best for callus proliferation. ½MS medium containing at NAA 0.75 or 1-mg/l showed maximum rooting response. The *in vitro* propagation protocol can be standardized highly useful in raising quality planting materials of *Desmodium triquetrum* DC. for commercial plantation programmes and germplasm conservation.

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