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In vitro Seed Germination, Callus Induction and Regeneration of *Tephrosia villosa*(L.) Pers - An Important Ethanomedicinal Plant

Ranjitha V.[1], Kalimuthu K.[2*], Vajjiram Chinnadurai [3] and A. Vanitha[4]

[1]Plant Tissue Culture Division, PG and Research Department of Botany, Government Arts College (Autonomous), Coimbatore-641018, India.
Email - ranjithamsc2015@gmail.com, Ph No-9952516244
Email –chinnaa.v1987@gmail.com ,Ph No-8637628883
Email –avanithagreen@gmail.com, Ph No-9789408477

ABSTRACT

Tephrosia villosa is an annual or perennial medicinal legume. The standardization of micro propagation protocol is useful for mass multiplication of selected plant and germplasm conservation. In addition, it could also facilitate crop improvement methods. For this we conducted research to develop a tissue culture protocol for leaf explants of *Tephrosia villosa*. Basal medium and basal medium with various BAP concentrations were used. The best *in vitro* seed germination percentage (95 ± 0.12) was observed in full strength MS basal medium for *in vitro* seed germination. The callus were induced from leaf explant on Murashige and Skoog (MS) medium containing BAP, NAA, KIN and TDZ. Best growth of callus however occurred on MS+BAP (8.88µm) + TDZ (1.13µm), MS+BAP (6.66µm) + NAA (0.98µm), BAP (8.88µm) + NAA (0.98µm) and BAP (4.44 µm) + NAA (0.98µm) + KIN (2.32 µm).Among the four different combination of growth regulators induced excellent callus. The highest percentage of shoot sprouting frequency (8.96 ± 0.13), highest number shoot formation during sub culture (14.93 ± 0.07) was observed in MS +BAP (2.22µm) + NAA (0.98mm) and TDZ (2.32µm) combination. Regenerated shoots were rooted on the same media combination. The mean number of roots 6.15 ± 0.08 and 7.07± 0.10 mean root length was observed. The well-developed shoots with roots were successfully acclimatized in the shade house with fogger system in selected planting substrates. Among the different planting substrates used the best survival percentage 93.83 was observed on hardening media composed of decomposed coir waste, garden soil and vermiculture. This one step less complicated protocol is useful for germplasm conservation, mass multiplication and extraction of active principles from *in vitro* raised plants without disturbing the wild population.

KEYWORDS: Legume, Regeneration, *in vitro* Callus, Hardening.

[*]Corresponding Author:
Dr. K. Kalimuthu,
Plant Tissue Culture Division, PG and Research Department of Botany,
Government Arts College (Autonomous), Coimbatore - 641 018, Tamilnadu, India.
Email: k_kalimuthu@rediffmail.com. MobileNo:+91 9843366622

INTRODUCTION

Leguminaceae is one of the most important family of the plant kingdom with environmental friendly source of nitrogen fixation, which reduces the risk of eutrophication and contamination of water. The establishment of high quality propagules is one of the most important problems in the woody legumes[1]. The genus *Tephrosia* belongs to the family Leguminaceae and consists of approximately 400 species from herbs to small shrubs distributing in Asia[2].About twenty-four species of *Tephrosia* were recorded in India[3]. Most of the *Tephrosia's* are herbs to undershrub and are grown as weeds. The genus is well known for its richness in prenylated flavonoids and is considered to possess insect repellent, parricidal, pesticidal, antimicrobial and anticancer properties[4]. Many plants from this genus have been used traditionally for the treatment of diseases like rheumatic pains, syphilis, dropsy, stomach ache, diarrhoea, asthma, abortifacient, respiratory disorders, laxative, diuretic, and inflammation etc.[5, 6].

Tephrosia villosa local name is punaikalvetalai a woody and multipurpose medicinal plant collected from Nambiyur, Erode district, Tamilnadu. It is an annual or perennial herb, 0.3-1.3m tall. *Tephrosia villosa* is widely used in traditional Indian medicine as a treatment for dropsy and diabetes[7]. It is also used as green manure in Coffee and *Hevea* Plantations and as a shade crop in Tea Plantations[8].Roots, leaves, fruitsand twinges of *Tephrosia villosa* showed significant activity against *C.quinquefasciatus* larvae[9].*Tephrosia villosa* leaves showed reduction in glucose level and pancreatic cell regeneration in alloxan induced diabetes in presence of 20(29)-lupen-3-one a compound. Four new rotenoids were isolated from seeds and dehydroxyrotenoid and lupenone were isolated from whole plant[10,11].There are no previous reports on micro propagation of *Tephrosiavillosa*, therefore the present research has been focused *in vitro* seed germination, callus induction and regeneration.

MATERIAL AND METHODS

The plants of *Tephrosia villosa* (L.) pers. were collected from Nambiyur, Tamilnadu, India and their identify was further authenticated by Botanical Survey of India (BSI) Southern Region, Coimbatore, India. Reference number is BSI/SRC/5/23/2016Tech/207. Leaf segments were collected and washed with Tween 20 (5% v/v) for 5 minutes and then surface sterilized with 70% alcohol for 30 seconds followed by mercuric chloride (0.1% w/v) solution for 3

minutes. The segments were washed thoroughly with sterile distilled water before cutting into appropriate size explants.

The culture mediumwas fortified with 30g/l sucrose, solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving. All the cultures were incubated in sterile culture room at 25± 2 °C with 16/8 h photoperiod under white florescent light (60µE2/S irradiance) and with 60 -70 % relative humidity. All the cultures were sub-cultured on the fresh medium after 15 to 20 days. MS basal medium and MS basal medium with various concentration plant growth regulator Benzyl amino purine (BAP) in the *in vitro* seed germination of *Tephrosia villosa.*For culture establishment, MS medium was supplemented with various cytokinin viz. benzyl amino purine (BAP) alone or with kinetin (KIN) and thidiazuron (TDZ) in combination. *In vitro* raised shoots were excised and transferred to the same medium for rooting.

The rooted shoots were washed to remove the adhering gel and planted in net pot containing a mixture of decomposed coir waste and compost (1:1). After 15 days, plantlets were transferred to pots containing organic manure, red soil and forest humus (1:1:1). The survival rate of regenerated plants was recorded one month after transfer to pots. Each experiment was repeated at least three times with 10 replicates for each treatment. Data were analysed and recorded.

RESULTS

Seed germination

The relative effectiveness of water agar medium, MS basal medium and MS basal medium with various concentration plant growth regulator Benzyl amino purine (BAP) in the *in vitro* seed germination of *Tephrosia villosa* is summarized in table 1 and figure 1. Among the basal medium and basal medium with various BAP concentration used, the best *in vitro* seed germination percentage (95 ± 0.12) was observed in full strength MS basal medium, followed by MS medium and BAP (8.88µm) concentration with 79 ± 0.81 percentage. MS medium with germination percentage 2 ± 0.9 was observed in water agar medium. Increasing in BAP concentration from 2.22 to 8.88µm the seed germination percentage also increased from 30 ± 1.31 to 79 ± 0.81. All the BAP concentration produces callus formation after seed germination.

Table 1 - Germination percentage of *Tephrosia villosa* seeds under different media condition

S. No	Treatments	Germination %
1	Water Agar Medium	02 ± 0.9
2	½ MS	55 ±1.13
3	Full MS	95 ± 0.12
4	½ MS +BAP (2.22 µM)	30 ±1.31
5	½ MS +BAP (4.44 µM)	38 ± 0.18
6	½ MS BAP (6.66 µM)	47 ± 1.39
7	½ MS BAP (8.88 µM)	54 ± 0.78
8	Full MS +BAP (2.22 µM)	55 ± 1.57
9	Full MS BAP +(4.44 µM)	67 ± 0.85
10	Full MS BAP +(6.66 µM)	74 ± 0.13
11	Full MS BAP +(8.88 µM)	79 ± 0.81

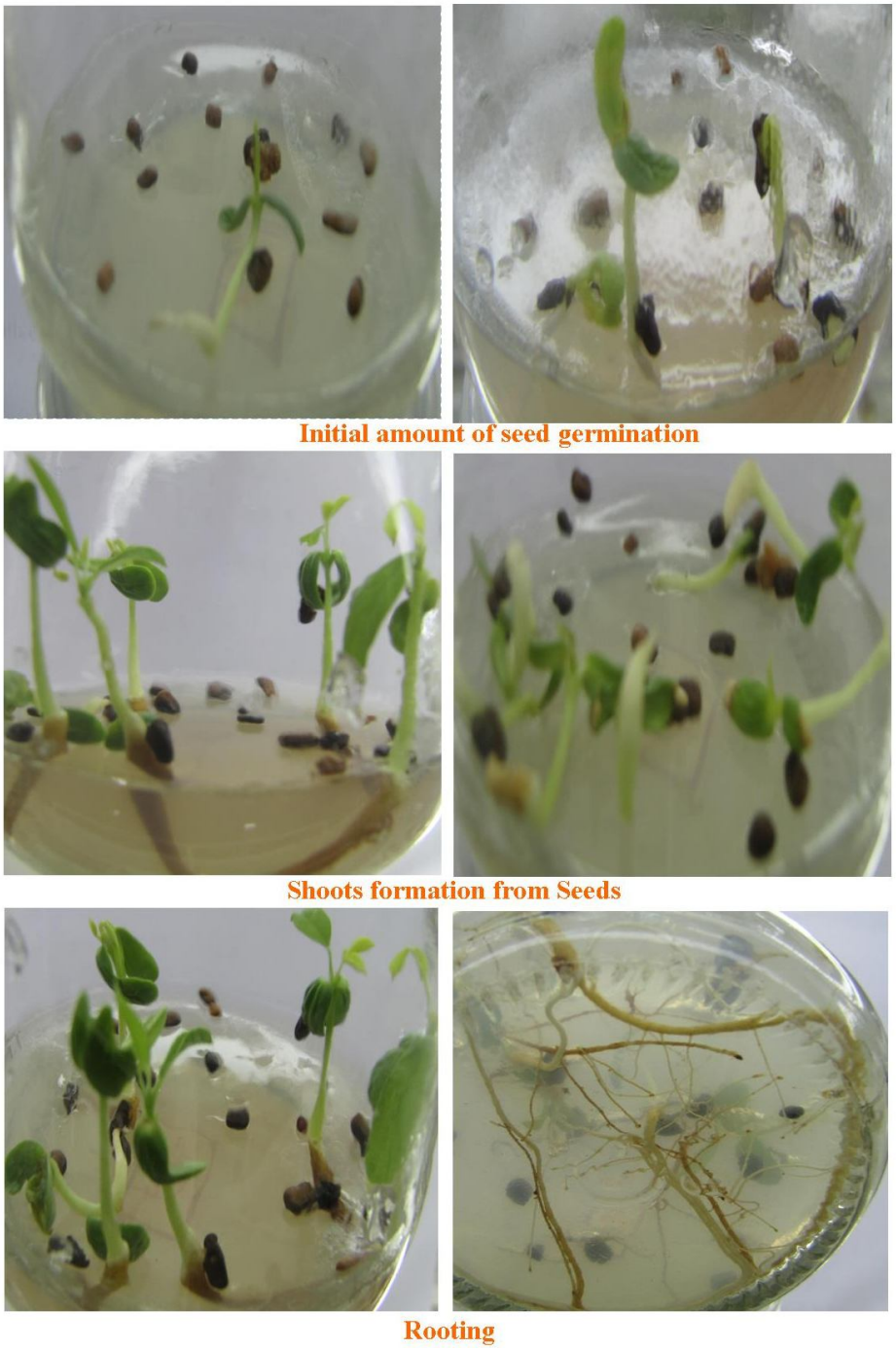

Figure 1 - *In vitro* seed germination of *Tephrosia villosa* on MS Basal medium

Callus culture

The morphogenetic response of leaf explants to growth regulators (BAP, NAA, KIN and TDZ) are summarized in table 2. No callus and shoot formation was observed in a medium without growth regulators. The combination of BAP, NAA, KIN and TDZ induced callus from

leaf explant of *T. villosa* and the morphology of the callus was green, yellow and brown, friable and nodular in nature. There was a wide range of variation on callus induction from less callus (+) to excellent callus (+++). Best growth of callus however occurred on MS+BAP (8.88µm) + TDZ (1.13µm), MS+BAP (6.66µm) + NAA (0.98µm), BAP (8.88µm) + NAA (0.98µm) and BAP (4.44 µm) + NAA (0.98µm) + KIN (2.32 µm). Callus formation first started at cut ends or along the surface of the explants after 8 days of culture, and after 21 days the entire segment was turned into a mass of green, soft and friable callus (Figure 2).

Table 2 - Effect of BAP, KIN, NAA and TDZ on callus initiation and multiple shoot induction from leaf explants of *Tephrosia villosa* cultured on MS medium

S. No	Plant growth hormones (µM/l)					Nature of callus	Shoot sprouting frequency %	Shoot No/explant	Shoot No/explants Subculture	Shoot length (cm)	Mean number of Roots	Mean root length (cm)
	BAP	NAA	KIN	TDZ								
1	2.22	-	-	-	+	BF	3.0±0.10	3.85±0.07	5.15±0.07	2.98±0.09	-	-
2	4.44	-	-	-	+	BF	2.05±0.07	4.05±0.07	4.06±0.11	2.93±0.12	-	-
3	6.66	-	-	-	+	BF	3.06±0.08	5.1±0.09	5.13±0.08	3.41±0.09	-	-
4	8.88	-	-	-	+	BF	2.01±0.11	2.96±0.21	4.06±0.11	3.05±0.07	-	-
5	11.10	-	-	-	+	BF	4.9±0.11	6.05±0.07	6.15±0.07	2.86±0.08	-	-
6	2.22	-	-	1.13	+	BF	4.15±0.07	5.15±0.07	6.13±0.08	3.85±0.07	-	-
7	4.44	-	-	1.13	++	BF	3.06±0.08	4.05±0.07	5.15±0.07	2.91±0.10	2.85±0.08	2.05±0.07
8	6.66	-	-	1.13	++	BF	3.98±0.13	5.15±0.07	7.05±0.07	2.65±0.07	2.11±0.09	3.03±0.11
9	8.88	-	-	1.13	+++	GF	5.13±0.08	6.05±0.07	8.93±0.08	3.11±0.09	3.95±0.07	3.1±0.09
10	11.10	-	-	1.13	++	BF	4.05±0.07	6.1±0.09	8.05±0.07	3.15±0.07	2.85±0.08	2.25±0.07
11	2.22	0.98	--	-	++	BF	4.16±0.08	5.1±0.09.	7.05±0.07	2.25±0.07	2.05±0.07	2.08±0.10
12	4.44	0.98	-	-	++	BF	2.04±0.12	2.96±0.21	8.06±0.06	3.05±0.07	3.11±0.09	2.5±0.07
13	6.66	0.98	-	-	+++	GF	3.9±0.11	7.1±0.09	8.91±0.05	4.85±0.09	5.95±0.07	3.0±0.10
14	8.88	0.98	-	-	+++	GF	5.85±0.07	5.15±0.07	9.06±0.06	4.15±0.07	4.85±0.07	3.05±0.11
15	11.10	0.98	-		++	GF	5.86±0.08	6.11±0.09	7.9±0.08	3.75±0.07	8.85±0.07	3.73±0.20
16	2.22	0.98	2.32	-	+	YF	8.96±0.13	8.05±0.07	14.93±0.07	4.8.85±0.09	6.15±0.08	7.0±0.10
17	4.44	0.98	2.32	-	+++	GF	5.88±0.09	5.2±0.09	11.91±0.05	4.15±0.08	3.9±0.11	3.08±0.10
18	6.66	0.98	2.32	-	++	YF	4.56±0.18	6.05±0.07	9.95±0.07	2.95±0.08	3.08±0.10	2.25±0.07

19	8.88	0.98	2.32	-	++	YF	4.66±0.15	5.14±0.33	6.17±0.13	3.66±0.33	4.46±0.17	3.86±0.13
20	11.10	0.98	2.32	-	++	GF	3.76±0.17	4.96±0.16	5.96±0.26	3.96±0.32	3.96±0.34	4.96±0.47
Basal Medium		-	-	-	-	-	-	-	-	-	-	-

+ Less callus, ++ Moderate callus, +++ Excellent callus

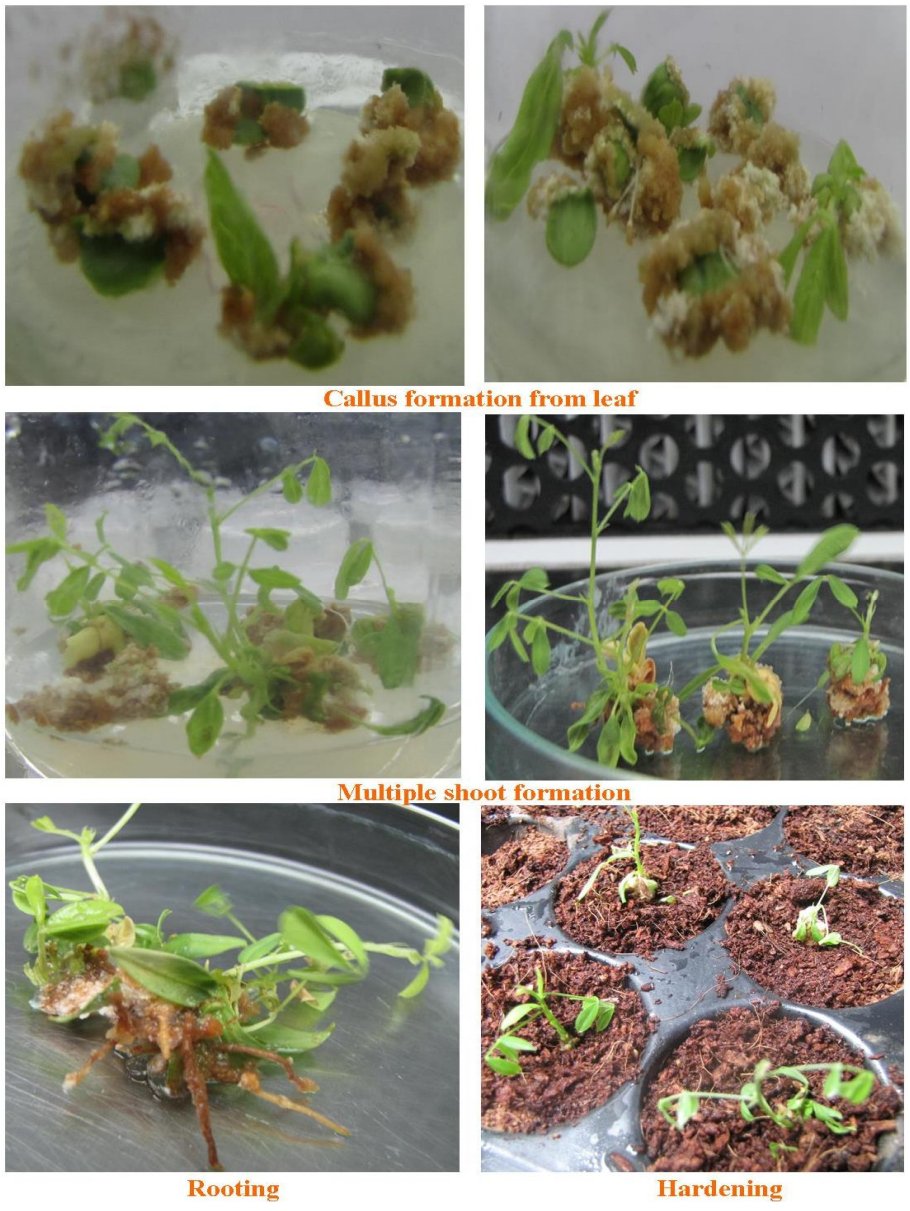

Figure 2 - Callus initiation, shoot, root formation and hardening of *T. villosa*.

The combination of BAP + NAA and TDZ had the organogenic ability for certain extent. Among the four different combination of growth regulators induced excellent callus the highest percentage of shoot sprouting frequency (8.96 ± 0.13), highest number of shoot formation from callus, (8.05 ± 0.07) highest number of shoot formation during sub culture (14.93 ± 0.07) was observed in MS +BAP (2.22µm) + NAA (0.98mm) and KIN (2.32µm) concentration. The second best combination was BAP (4.44µm) + NAA (0.98µm) and KIN (2.32µm) with 5.88 ± 0.09 shoot sprouting frequency, 5.2 ± 0.09 shoots formation in initial stage and 11.91 ± 0.05 shoots during subculture. All other combination and concentration also produced callus and induced shoots and roots but not at the level of previous combination (Figure 2). During sub culture the shoot formation followed by root formation also observed in the same combination. The mean number of roots 6.15 ± 0.08 and 7.07± 0.10 mean root length was observed respectively.

Acclimatization

The well-developed shoots with roots were successfully acclimatized in the shade house with fogger system in selected planting substrates (Table 3) for 15days (Figure 2). Among the different planting substrates used the best survival percentage 93.83 was observed on hardening media composed of decomposed coir waste, garden soil and vermiculture. Partially hardened plantlets than transferred to the poly bag containing red soil sand and composition the ratio of 1:1:1 after fifteen days the hardened plants were transferred to pots.

Table 3 - Evaluation of different planting substrates for acclimatization of *in vitro* plantlets of *Tephrosia villosa*

S.No	Planting substrates	No. of plants Transferred	No. of plants survived	Survival (%)
1	Garden soil	50	28	55.83
2	Vermiculite	50	30	60.83
3	Decomposed coir waste	50	40	80.50
4	Hardening media- (decomposed coir waste: garden soil: vermiculite)	50	47	93.83

DISCUSSION

The combination of BAP, NAA and KIN induced greater amount of callus from the leaf explants of *T. villosa* and the morphology of the callus was green, friable and nodular in nature. The caulogenic effect of auxin along with cytokinin observed in the present study is in consonance with other reports[12, 13, 14].The best growth of callus however occurred on MS+BAP (2.22µM) +NAA (0.98 µM) and KIN (2.32 µM). The shoot formation occurred on the same medium or the friable callus were subculture on MS+BAP+NAA+KIN combination.The promotry effect of cytokinin on shoot proliferation is reported, while BAP, KIN and NAA in combination was found to be superior [15, 16,12].Around 80% of cultures reported with 8.05 shoots and during subculture 14.93 shoots was observed on MS Medium supplemented with BAP (2.22 µM) + NAA (0.98) + KIN (2.32 µM).The root formation occurred on the same medium or individual shoots were separated and subcultured on the same medium within a week time. Formation of roots on the same shoot proliferation medium observed is in consonance with other reports [17]. But in *Crotalaria* species the root induction was observed more in MS+IBA when compared with MS + NAA[12].The rooted shoots were successfully transplanted to net pot containing hardening media and the humidity was maintained at approximately 90% by keeping the net pots inside the shade house. After primary hardening the plantlets were transferred to polythene bags containing red soil, sand and compost.

Plant research often involves growing plants in a controlled environment condition. There may be plants with genetically modified or may be plants of which we need many copies all exactly alike. These thingscan be achieved through tissue culture of small pieces of tissue from the plant of intertests.Tissue culture techniques are often used for commercial production of plants as well as for various research programmes. By using *in vitro* techniques a wide range of plants have been successfully propagated. It has also allowed material to be stored in *in vitro*gene banks for future use.

Tephrosiaviilosa seeds were inoculated in MS basal medium with or without growth regulators to know the effectiveness of the medium. The seeds were hydrated by water absorption from the basal medium and radicals sprout out through the seed coat.The seed showing the typical Leguminaceae members germination in the cotyledonary leaves developed above the medium.Similar resultswereobtainedin the leguminaceae members like *Vigna subterranean*[18].

The best and successful *in vitro* seed germination (95±0.12%) was obtained from full Strength MS basal medium and low seed germinationwere observed in MS basal medium with various growthregulators when compared to full strength medium. This may be due to the effect of growth regulator concentration and combinations. Very low percentage of seed germination was observed in water agar medium. This may be due to lack of nutrients in this medium. In*Moringaoleigera* ½ MS media produced the higher seed germination compared to full strength MS medium. Now a days the medicinal plant species are gaining importance and are promoted for commercial cultivation due to increasing demand in the market. Many industries likePharmaceutical, Cosmetics, Food and Herbal industries are mainly depends on wildmedicinal plants. *T.villosa* is one such important medicinal plant which possesses achieveprinciples. Such as 20(29) lupen 3-one etc.Also four new rotenoids andlupenone were isolated from seeds and whole plant[10, 11].

CONCLUSION

This study suggested a one-step effective protocol for the tissue culture technique of an important IUCN redlisted medicinal plant, *Tephrosia villosa*. The protocol was developedfor economical and can be applied for large-scale micropropagation for germplasm conservation without disturbing natural habitat.

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