



Research article

In vitro propagation of *Pueraria tuberosa* (Roxb. ex Willd.) DC.

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Abstract: *Pueraria tuberosa*, commonly known as Indian kudzu or 'Vidari' is an important medicinal plant belonging to the family Fabaceae. The tubers of this plant are an important constituent in many Ayurvedic formulations as a restorative tonic, immune booster and antiageing. Annual demand of Vidari by the Ayurvedic industry is 135 tonnes and industry is facing a severe scarcity of this raw material. Micropropagation technology offers large-scale production of disease-free, quality planting materials for pharmaceutical industries. A successful protocol for the *in vitro* propagation of *P. tuberosa* has been achieved by using nodal segments as explants. Multiple shoot induction and proliferation was obtained in Murashige and Skoog medium supplemented with 0.5 mg.L⁻¹ BAP, 0.5 mg.L⁻¹ KN and 2% glucose. MS medium with 1.5 mg.L⁻¹ KN favoured maximum shoot elongation. A maximum number of roots with the highest percentage of rooting was observed on half strength MS media supplemented with 0.5 mg.L⁻¹ IBA. Elongated shoots in half strength basal MS medium induced roots in 14 days of culture. Regenerated shoots with well-developed roots were successfully hardened with 80% survival.

Keywords: Multiple shoots - Nodal explants - Antioxidants - Vidari.

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INTRODUCTION

Pueraria tuberosa (Roxb. ex Willd.) DC. known as 'Mile a minute vine' is a rapidly growing perennial woody climber, distributed throughout tropical parts of India, mostly in moist regions, monsoon-forests and coastal tracts (Chopra *et al.* 1956). The rejuvenating drug 'Vidari' is prepared from its tuberous roots which act as a galactagogue, stimulant and emollient (Warrier *et al.* 1995). It has been reported to have an extensive range of medicinal properties and exhibits effective role in the treatment of leprosy, spermatorrhoea, hepatosplenomegaly, tuberculosis and cough. In ethnomedicine, the edible tubers are used to treat various ailments such as chest pain, rheumatism and fever (Jain 1991). *Pueraria* species are also popular for its hypoglycemic (Raghuwanshi & Jain 2012), fibrinolysis enhancing (Verma *et al.* 2009), antioxidant (Pandey *et al.* 2007), hypolipidemic (Tanwar *et al.* 2008), and antimicrobial (Ratnam & Raju 2009) properties. Some of the isoflavonoids present in the *P. tuberosa* are puerarin, daidzein, genistein and genistin (Goyal & Ramawat 2007). Studies proved that genistein reduces systolic blood pressure and enhances aortic relaxation to acetylcholine (Vera *et al.* 2007). The isolated antioxidant tuberosin exhibited a variety of biological responses including influence on inflammatory pathways (Pandey & Tripathi 2010). Dietary antioxidants lower the risk of heart diseases and neurodegenerative diseases caused by the free radicals or reactive oxygen species (ROS) generated during metabolism (Sulaiman *et al.* 2014).

The tubers of *P. tuberosa* are widely used in various formulations in the Indian system of Ayurvedic medicine (Goyal & Ramawat 2007). The widespread harvesting of the medicinal plants as a source of the drug has restricted its reproduction, regeneration and survival. The growth of Pharmaceutical industries accompanied by unscientific and destructive collection, threaten the existence of many rare species. Micropropagation protocols offer an alternate method to propagate medicinal plants and produce the compounds of interest in a short period of time, without sacrifice of natural populations.

MATERIALS AND METHODS

Plant sample and experiment designing

The nodal segments of *Pueraria tuberosa* were collected from 10 months old plant maintained in pots. Nodes were washed in running tap water followed by soap water treatment for 15 minutes. Nodal segments (2 cm length) were immersed in Bavistin (15 g.L⁻¹), cefotaxime (200 mg.L⁻¹) and tetracycline (200 mg.L⁻¹) for 40 minutes and thoroughly washed with distilled water. These explants were surface sterilized with 0.1% (w/v) HgCl₂ for four minutes followed by wash with sterile distilled water. The explants were cultured on Murashige and Skoog (Murashige & Skoog 1962) medium containing 0.8% (w/v) agar with 2% (w/v) sucrose and growth regulators. The pH of the media was adjusted and maintained to 5.7. Growth regulators like 6-Benzylaminopurine -BAP (0.5 to 1 mg.L⁻¹) and Kinetin -KN (0.5 to 1.0 mg.L⁻¹) were experimented with MS medium at different combinations or alone for multiple shoot induction. The shoot proliferation in different combinations was recorded in the present study. The effect of Kinetin on *in vitro* shoot elongation was observed by inoculating the *in vitro* shoots on MS medium supplemented with various concentrations of KN (0.25, 0.5, 1.0, 1.5, 2.0 mg.L⁻¹). The *in vitro* regenerated shoots were transferred to full strength MS and half MS with Indole Butyric Acid-IBA (0.25, 0.5, 1.0 mg.L⁻¹) for rooting. For hardening, two to three weeks old rooted shoots were removed from the culture tubes, wash thoroughly and transferred to polycups containing soil and vermiculite in the ratio 1:2 and kept in mist chamber for acclimatization.

Statistical analysis

All experiments were performed with three replications, having 30 samples each. The effect of various treatments on selected growth parameters was measured quantitatively and statistically tested using analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences) version 11.0. The significance of the mean values of various treatments was assessed by Duncan's New Multiple Range Test (DMRT) at $p < 0.05$.

RESULTS

Table 1. Effect of BAP and Kinetin on shoot induction of *Pueraria tuberosa*.

Treatments	MS + Growth regulators		% explants showing shoot formation	Number of days for shoot induction
	BAP (mg.L ⁻¹)	KN (mg.L ⁻¹)		
T ₀	0.0	0.0	0.000 ^f	0.000 ^f
T ₁	1.0	0.5	74.00±1.05 ^b	9.82±0.10 ^a
T ₂	0.5	0.5	83.00±2.10 ^a	6.05±0.05 ^e
T ₃	0.5	1.0	48.00±3.16 ^c	9.35±0.05 ^b
T ₄	-	1.0	31.40±2.06 ^e	7.50±0.11 ^d
T ₅	1.0	-	40.00±0.05 ^d	8.14±0.05 ^c

Note: Level of significance was measured at $p < 0.05$. Column values with same superscript are not differing significantly ($P > 0.05$).

Table 2. Effect of BAP and Kinetin on shoot multiplication of *Pueraria tuberosa* after 40 days of culture.

Treatments	MS+ Growth regulators		Number of multiple shoots per explants
	BAP (mg.L ⁻¹)	KN (mg.L ⁻¹)	
T ₀	0.0	0.0	00.000 ^f
T ₁	1.0	0.5	09.85±0.47 ^b
T ₂	0.5	0.5	17.95±0.48 ^a
T ₃	0.5	1.0	08.84±0.21 ^c
T ₄	-	1.0	06.25±0.16 ^e
T ₅	1.0	-	08.36±0.20 ^d

Note: Level of significance was measured at $p < 0.05$. Column values with same superscript are not differing significantly ($P > 0.05$).

Morphological changes of the nodal explants were noticed within 7 days of inoculation. No shoots were developed in explants grown on the control medium. All the plant growth regulators used induced multiple shoots singly or in combination with variable response. Subcultures were done at every 15 days interval into fresh nutrient media with same nutrient composition (Table 1). MS medium supplemented with 0.5 mg.L⁻¹ BAP with 0.5 mg.L⁻¹ KN was found to be the most suitable medium for shoot induction from nodal explants of *Pueraria tuberosa*. 83 % of bud initiation was observed within 6 days of culture. Increasing the concentrations of growth regulators in culture medium resulted in callus formation at the basal part of the shoots. The same hormonal combination gave the maximum number of multiple shoots in 40 days of culture (Table 2; Figs. 1 &

2). Multiple shoot formation with the rate of 17.95 ± 0.48 were observed in concentrations of BAP (0.5 mg.L^{-1}) with KN (0.5 mg.L^{-1}) and it can be attributed to its synergistic effect of the combination. Shoot bud elongation occurred when the shoots were placed on MS medium supplemented with different concentrations of KN ($0.5, 1.0, 1.5, 2.0, 2.5 \text{ mg.L}^{-1}$). MS supplemented with 1.5 mg.L^{-1} KN was found to be the single best treatment to achieve maximum shoot length ($6.21 \pm 0.98 \text{ cm}$) and a number of leaves (5.94 ± 0.05) on cultures within 10 days. It is also evident from the results that the shoot length of *P. tuberosa* increases with increasing KN concentration up to 1.5 mg.L^{-1} (Table 3). In the present study, a further increase in KN concentration ($2.0, 2.5 \text{ mg.L}^{-1}$) had a negative effect on shoot length.

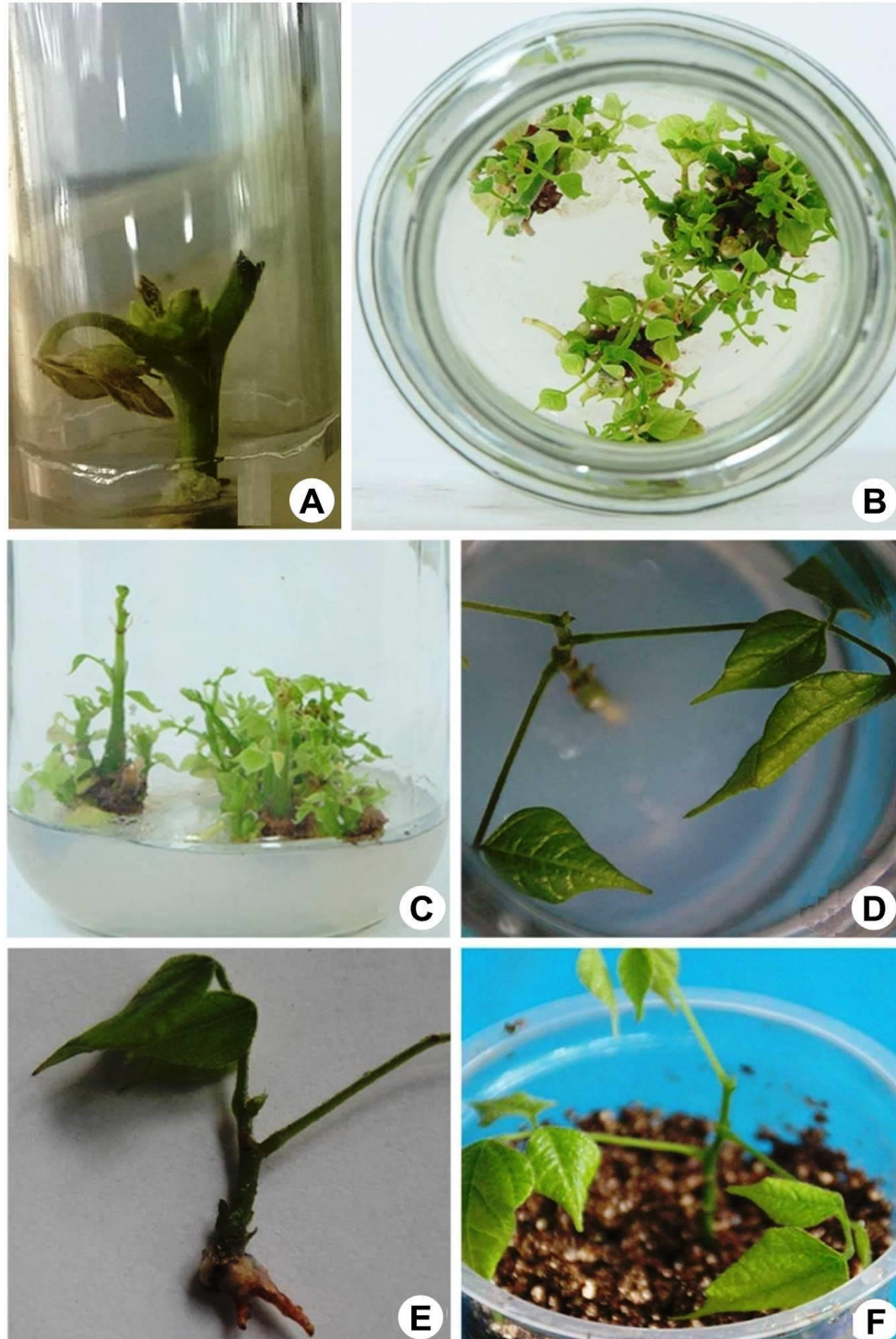


Figure 1. A, Shoot bud initiation of *Pueraria tuberosa* from nodal explants on MS medium supplemented with 0.5 mg.L^{-1} BAP and 0.5 mg.L^{-1} KN; B–C, Multiple shoot induction after 40 days of culture on 0.5 mg.L^{-1} BAP and 0.5 mg.L^{-1} KN; D, Shoot elongation on MS medium with 1.5 mg.L^{-1} KN; E, In vitro rooting on half strength MS with 0.5 mg.L^{-1} IBA; F, Acclimatization.

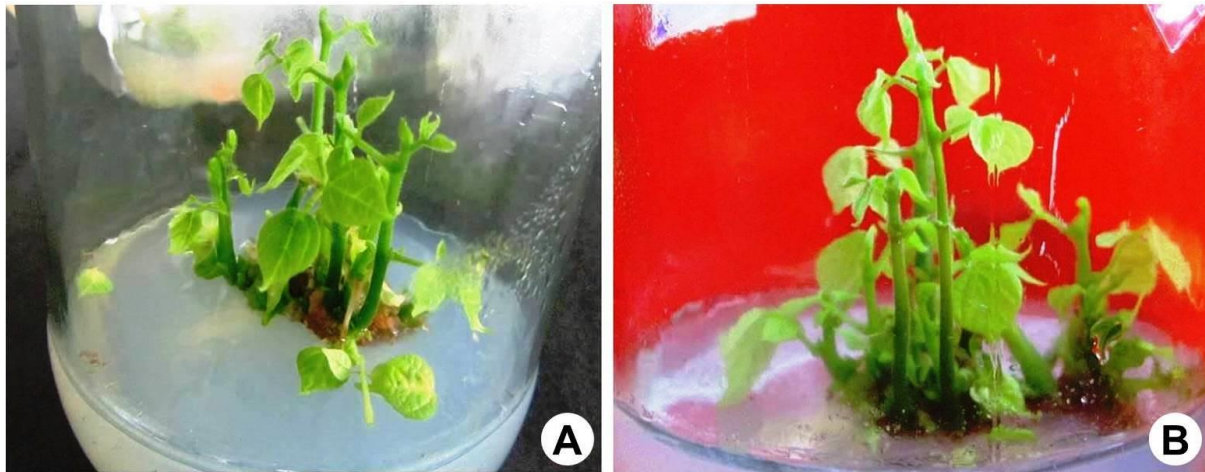


Figure 2. A, Multiple shoot induction after 40 days of culture in 0.1 mg.L⁻¹ KN; B, Multiple shoot induction after 40 days of culture in 0.1 mg.L⁻¹ BAP.

Table 3. Effect of KN on shoot elongation after 10 days of culture.

Treatments	MS+KN (mg.L ⁻¹)	Shoot length (cm)	Number of leaves
T ₀	0.0	0.000 ^f	0.000 ^f
T ₁	0.5	2.89±0.14 ^e	2.35±0.05 ^e
T ₂	1.0	3.38±0.22 ^d	3.35±0.10 ^c
T ₃	1.5	6.21±0.98 ^a	5.94±0.05 ^a
T ₄	2.0	4.76±0.61 ^b	4.05±0.05 ^b
T ₅	2.5	4.13±0.04 ^c	3.15±0.05 ^d

Note: Level of significance was measured at $p < 0.05$. Column values with same superscript are not differing significantly ($P > 0.05$).

To induce rooting, *in vitro* shoots were cultured on half strength MS media alone and in combination with various concentrations of growth regulator IBA. None of the *in vitro* shoots differentiated roots in full strength MS media without hormones. A number of roots, length of roots and number of days for root induction were observed and recorded. The differentiation of root buds varied with the growth regulator combination of the half MS (Table 4). A number of days for root induction differed significantly among the IBA concentrations in half strength MS. The number of roots and length of roots were significantly reduced on media with two levels of IBA (0.25, 1.0 mg.L⁻¹). Among the different concentrations of IBA studied half strength MS media with 0.5 mg.L⁻¹. IBA induced an optimum number of robust, healthy roots within 14 days. The micro shoots excised from *in vitro* cultures were successfully planted out to the soil in polypots with 80% survival. They were later planted in the field after about a month where they established well.

Table 4. Effect of Media and IBA on *in vitro* rooting.

Treatments	Media MS	IBA (mg.L ⁻¹)	Number of roots	Root length (cm)	Number of days for root induction
T ₀	Full	0.0	0.000 ^c	0.000 ^d	0.000 ^d
T ₁	Half	0.25	1.18±0.40 ^b	0.81±0.07 ^c	18.36±0.50 ^a
T ₂	Half	0.5	3.54±0.52 ^a	2.96±0.10 ^a	14.18±0.42 ^c
T ₃	Half	1.0	1.36±0.50 ^b	1.14±0.11 ^b	16.63±0.51 ^b

Note: Level of significance was measured at $p < 0.05$. Column values with same superscript are not differing significantly ($P > 0.05$).

DISCUSSION

After 6 weeks of culture, a maximum number of *in vitro* shoots (17.95±0.48) was obtained on MS medium containing 0.5 mg.L⁻¹ BAP with 0.5 mg.L⁻¹ KN which differed significantly from other concentrations and combinations of BAP and KN tested as well as in the control (Fig. 1). The cytokinins BAP and KN were effective in terms of shoot induction from nodal explants of *Stereospermum suaveolens* (G. Don) DC. (Trivedi & Joshi 2014) and *Costus speciosus* (Raghu *et al.* 2006). The combinations of 0.5 mg.L⁻¹ BA and 0.5 mg.L⁻¹ KN proved to be most effective for culture initiation with healthy shoots (Shubha *et al.* 2016). It was reported that *in vitro* cultures of *P. tuberosa* showed high shoot proliferation in KN supplemented media (Rathore & Shekhawat 2009). It has been reported that *in vitro* shoot induction and multiplication are largely based on media

composition containing cytokinins as major plant growth regulators (Afshin *et al.* 2011). Multiple shoot formation clumps having 6–7 shoots per explants have been observed at synergistic combinations of BAP and KN on *in vitro* cultures of *Achyranthes aspera* (Fazlima *et al.* 2008). The use of comparatively lower concentration of growth regulator in present protocol is an important factor worth mentioning, as it minimizes the risk of producing genetically altered individuals (Raghu *et al.* 2007). It is seen that treatment with KN alone significantly inhibited shoot production compared to treatments with BAP. It was reported that KN was less effective on multiple shoot induction of *Gentiana kurroo* as compared to BAP (Sharma *et al.* 1993). In several medicinal plant species, BAP enhances shoot multiplication of *in vitro* cultures (Lakshmi & Mythili 2003), which is in conformity with the result of our study.

Shoot length was enhanced in the presence of all tested concentrations of KN compared to control and there was a significant difference among the KN concentrations. The shoot length differed significantly among the KN concentrations. In the present protocol, shoot bud elongation was maximum when the multiple shoots were placed on MS medium supplemented with 1.5 mg.L⁻¹ KN. These results correspond to other reports where the best shoot length and a maximum number of nodes of *Matthiola incana* resulted in KN enriched media (Afshin *et al.* 2011). There are reports which state that KN promotes elongation of buds in *Vigna radiata* (L.) Wilczek (Chandra & Pal 1995). The length of shoots was observed to increase with increasing concentration of Kinetin (0.5–1.5 mg.L⁻¹). Similar finding was observed in *Stereospermum suaveolens* (Trivedi & Joshi 2014) when KN supplemented medium with higher concentrations form long healthy shoots with large leaves. It is evident from the study that micro shoots failed to exhibit high shoot elongation on media with two different levels of KN (2.0, 2.5 mg.L⁻¹).

In the present study *in vitro* shoots rooted in different concentrations of IBA in half MS. Among the different concentrations of IBA tested, 0.5 mg.L⁻¹ IBA significantly favoured maximum rooting, where the average root number increased to 3.54±0.52 with highest root induction response. These strong, short, pointed and healthy roots originated with an average length of 2.96±0.10 cm showed high survival rate during hardening. Half strength MS media with two levels of IBA (0.25, 0.1 mg.L⁻¹) developed slim and tender roots that were damaged during transfer. *In vitro* shoots of *P. tuberosa* rooted in IBA supplemented half strength MS media (Rathore & Shekhawat 2009). It has been reported that *in vitro* rooting in *Paulownia elongata* exhibited a maximum number of roots and 100% rooting on half strength MS medium supplemented with 0.5 mg.L⁻¹ IBA (Zayova *et al.* 2014). The number of roots and its length decreased with 1.0 mg.L⁻¹ IBA supplemented media. Earlier, Nayak & Kalidass (2016) reported similar observations in *Blepharispermum subsessile*.

CONCLUSION

This protocol for regeneration of *Pueraria tuberosa* through nodal explants is highly effective for clonal multiplication and conservation. *In vitro* regeneration holds tremendous potential to select, multiply and conserve medicinally important genotypes, which are a potential resource of bioactive compounds.

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