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In vitro multiplication and phytochemical analysis of *in vivo* and *in vitro* plant parts of *Pueraria tuberosa* (Roxb. Willd DC)

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ABSTRACT

An important medicinal plant, *Pueraria tuberosa* (Roxb. Willd DC), is a perennial woody climber in the Fabaceae family. The current study aims to develop callus from the leaves and nodes of *Pueraria tuberosa* as well as to optimize the *in vitro* regeneration method for *Pueraria tuberosa*. Murashige and Skoog's medium supplemented with Benzlyaminopurine (4.44 µM) and naphthalic acetic acid (2.68 µM) is more effective for leaf and node callus. The nodal explant was cultured in Murashige and Skoog medium supplemented with (BAP+NAA) (0.66+0.52) µM to achieve direct shooting from nodal segments. Shoot multiplication was significantly influenced by the type and concentration of plant growth regulators as well as by subculturing *in vitro* regenerated shoot lets on fresh media. Transferring *in vitro* shoots to rooting media containing 2.68 µM NAA and 0.49 µM indole butyric acid (IBA) resulted in the growth of healthy roots. An efficiency of 80% was achieved during the well-rooted plantlet's acclimatization. This study compares the phytochemical content of *P. tuberosa* plants that grew in the wild and those that were developed *in vitro*. *In vivo* and *in vitro* qualitative and quantitative phytochemical investigation revealed that they are a valuable source of secondary metabolites. In a conclusion, the present study will be helpful in the herbal formulations that use any *in vitro* parts of *P. tuberosa* without destroying plants in wild conditions. Additionally, the work will aid in the conservation of this rare medicinal plant.

Keywords: Callus formation, Micropropagation, Phytochemical analysis, *Pueraria tuberosa*

Abbreviations: 2,4-D – 2,4-Dichlorophenoxtacetic acid, BAP – Benzlyaminopurine, IBA – Indole butyric acid, KN – Kinetin, MS – Murashige and Skoog, NAA – Naphthaleneacetic acid, NPK- Nitrogen, phosphorous, potassium, µM – Micromolar

INTRODUCTION

In many indigenous communities, people have been using medicinal plants for a long time to treat various chronic conditions. Many of the people living in rural areas of the nation are dependent on medicinal plants, which were a significant component of traditional Indian medicine in the past (Deshmukh, 2017*). Pueraria tuberosa*, also known as "kudzu" and "Vidarikand," is a member of the Fabaceae family. *P. tuberosa* usually grows in shady, warm, and humid areas (Patel *et al*., 2021). The tuber is used by some Indian tribes as a complementary food and as a diabetic cure. Ayurvedic experts exploit the tuber to treat general weakness, reproductive disorders, and anti-aging (Pandey et al., 1998). The tuberous roots have galactagogue, stimulant, and emollient properties (Warrier *et al*., 1995). Several ayurvedic formulations like Nityananda rasa, Mahavisagarbha taila, Marma gutika, Shatavaryadi ghrita, Sarasvatarista, chyavanprasam (Anonymous, 1998), Goknura Vidari ghrita and macatmagupta sarpi, Asvagandharista, it is an important essential element (Venkata and Venkata, 2009). So, there is a significant market demand for the tuber part of this plant.

P. tuberosa tuber part contains puetuberosanol, tuberosin and a high amount of isoflavonoids and terpenes (Khan *et al.,* 1996). *P. tuberosa* has been reported to have several pharmacologically active parts, such as puerarin, daidzein, genistein, tuberosin, and lupinoside (Mazi *et al*., 2012). Antiallergic, antioxidant, hypothermic, hypotensive, anti-inflammatory, hepatoprotective, anti-aromatic, hypocholesterolemic, wound healing, and anti-diabetic properties are some of the properties of *P. tuberosa* contains. (Kintzios *et al.,* 2004, Mao and Gu, 2005, Zheng *et al.,* 2002). It is crucial to maintain a concerted effort to establish an effective propagation method to fulfill market demand. *Pueraria candollei* var. mirifica callus culture could produce a significant level of isoflavonoids such as genistein and daidzein (Sudarat and Sanha, 2006). The interest in isoflavonoids has grown significantly during the last few decades. Isoflavonoids played an essential and successful part in the treatment of cancer, postmenopausal symptoms, and cardiovascular illnesses (Dixon and Ferreira, 2002), (Nestel; 2004; Duncun *et al*., 2003 and Vitrac *et al*., 2004). Because of its nutritional and therapeutic value, *P. tuberosa* is regularly utilized, due to its limited germplasm basis. Furthermore, as this species reproduces by

tuber and seeds, various anthropogenic activities and non-scientific harvesting also threaten its existence. The rate of seed germination is relatively low. The most common multiplication method is vegetative propagation (via tuber), but it is insufficient to meet the needs of tribal people and the pharmaceutical industry. To increase the production of *P. tuberosa* on a commercial scale, it is necessary to develop a rapid propagation method (Rathore and Shekhawat, 2009). Additionally, *in vitro* plant cell culture has been recognized as a significant source of secondary metabolites from plants. *In vitro* technique also provides opportunities for genetically desired genotypes and proliferation. (Manisha *et al.,* 2012). Offsite conservation and micropropagation have benefited from plant tissue culture (Edson *et al.,* 1997; Arya *et al.,* 2003). For *P. lobata*, Thiem 2003 developed a micropropagation system; *P. candollei* Var. mirifica has been successfully micro-propagated, according to Thanokeo and Panichajakul's (2006) research. The synthesis of puerarin from hairy root cultures produced from *Phaseoloides* leaf explants transformed with *Agrobacterium rhizogenes* utilizing bioreactors was also reported by Kintzios *et al.,* (2004).

Thus, using nodal explants from *in vitro* seedlings, an effort was made to establish an effective and enhanced *in vitro* propagation technique for *P. tuberosa.* Compared to earlier studies on this plant, the shoot growth rate was high in this study. Although there are many reports available on the micropropagation of *P. tuberosa*, there is no report that compares the phytochemical profiles of plants of this species that were grown in the wild and those that were micro propagated *in vitro*. This is necessary to ensure that *in vitro* regenerated plants retain their medicinal and nutritional characteristics.

MATERIAL AND METHODS

Explant source and surface sterilization of explants:

In May 2021, mature pods were collected from the Junagadh Forest of Gujarat, India. Mechanical separation of well-grown seeds from pods was done. Running tap water was used to wash the seeds for 15 minutes. The seeds were soaked with Tween 20° (1%) for 5 minutes and then washed with autoclaved water twice. The seeds were immersed in 0.5% Bavistin and 0.2% streptomycin in sterile autoclaved water and then put on a magnetic stirrer for 30 min and washed with autoclaved water 2-4 times. After being surface sterilized for 3 minutes with continuous shaking in a laminar flow cabinet with freshly prepared 0.1% aqueous $Hgcl_2$, seeds were washed three times in autoclaved water to eliminate $Hgcl_2$ traces. In the last treatment, the seeds were rinsed in 8% Sodium hypochlorite for 5 min and then washed with sterile water and inoculated in the respective medium.

Medium composition, culture conditions and callus development:

Murashige and Skoog (Murashige and Skoog, 1962) medium (MS) were supplemented with BAP (0.29 M) and GA (1.44 M), 3% (w/v) sucrose (Hi-media) as a carbon source, and agar (0.8%) as a gelling agent for *in vitro* seed germination. In addition, 0.1 N NaOH or 0.1 N HCl were used to keep the media's pH at 5.7 pH (before the inclusion of the gelling agent). A 200 ml jar container was filled with the 20-30 ml medium, which was then autoclaved for 15 to 25 minutes at 121 °C and 15 psi pressure. The culture was incubated at 25°C, 55% relative humidity, a 16/8 h (light/dark) photoperiod, and an intensity of 1800–2000 lux of cool white fluorescent light.
Leaf and node samples were taken *in vitro* from germinated seedlings and used for callus development. For callus formation, concentrations of BAP (2.22 and 4.44) µM, NAA ((2.68 and 5.37) µM and 2,4-D ((2.26 and 4.52) µM were used, along with combinations of BAP + NAA (2.22 + 2.68, 2.22 + 5.37, 4.44 + 2.68, 4.44 + 5.37) µM and BAP + 2,4-D (2.22 + 2.26, 2.22 + 4.52, 4.44 + 2.26, 4.44 + 4.52) µM were used. Data has been collected, and the best combination of developed calluses was chosen and used for the phytochemical study.

In vitro Shoot formation:

A 2-3 cm long nodal explant was cut from an *in vitro* seedling for shoot formation. Nodal explants cultured in MS media supplemented with an ideal combination of BAP + NAA and BAP + KN. Three percent sucrose (w/v), 0.8% agar (w/v), and the hormone BAP (0.44, 0.66, 2.22 and 4.44) µM, NAA (0.53, 2.68) µM and KN (0.46, 2.32, 2.68 and 4.65) µM were added to the Murashige and Skoog medium. These cultures were maintained in the incubation room after inoculation. After three subculturing cycles, the process of shoot multiplication was completed. Every four weeks, the culture was sub cultured into a similar new media. These results were noted after three successive subcultures.

In vitro root formation:

Healthy elongated shoots with two to three nodes were removed from the cultures and transferred to full MS medium containing 3% sucrose and 0.8% (w/v) agar fortified with various concentrations of IBA with NAA (0.49+0.53, 0.49+2.68, 2.46+ 0.53, 2.46+ 2.68) µM and IBA (0.49, 0.98, 2.46, 4.92) µM individually. Rooted plantlets were gently rinsed under running tap water to remove agar residues after being taken from the culture

media. These plantlets were moved to plastic cups having disinfected cocopeat and sprinkled with ½ MS basal solution every five days for two weeks. These plants in pots were maintained in the humidity of a culture room by covering them with clear plastic cups. After 28 days, a mixture of thick wood ash, IBA (0.5%), and fungicide powder was applied to the root tip. The plantlets were put in clay pots with (Soil: NPK: cocopeat) maintained in a shaded net house for improved growth and development.

Experimental design and statistical analysis:
Each experiment had five replicates and was performed three times. The experiments were set up using a randomized block design (RBD). Ten explants were used in each trial for each treatment. The callus formation, callus growth frequency, number of days for callus induction, number of shoots, average shoot length, number of roots, and average root length were all observed. ANOVA was used to analyse the data, and Duncan's multiple range test (DMRT) was used to determine significant differences at the 0.05% probability level.

Phytochemical analysis:
For qualitative and quantitative analysis, *P. tuberosa* leaf, stem, tuber, and root were obtained from *in vivo* plants, and *in vitro* plant parts such as node callus, leaf callus, leaf, stem, and root were used.

Preparation of extract:
For qualitative analysis, extracts were prepared using aqueous, methanol, chloroform, and acetone solvents. First, fresh plant parts (*in vivo* and *in vitro*) were dried in a shaded area and crushed into a fine powder using a mechanical grinder. Next, 1 g of each plant part was mixed in 10 ml of water, methanol, chloroform and acetone, adequately covered with aluminium foil, labelled, and then kept in a shaker for 24 hours. After 24 hours of extraction, each sample was filtered using Whatman's filter paper no. 1. The filter sample was stored at 5 °C in the refrigerator. In the quantitative analysis above two types of extract methanol and acetone were used.

Qualitative phytochemical analysis:
The presence of alkaloids, phenol, flavonoids, steroids, tannin, glycosides, and saponin was determined via qualitative analysis from aqueous, Methanol, chloroform, and acetone extracts. Dragendroff test was used to determine the presence of alkaloids. The ferric chloride test was used to identify phenol and tannins. Lead acetate testing was used to detect flavonoids. Lead acetate testing was used to determine the presence of saponin. Salkowski test was used to identify steroids. By using the Borntrager test, glycosides were found to be present (Harbone, 1973).

Quantitative phytochemical analysis:
The samples were subjected to different analysis methods to quantify other phytochemicals such as total phenol, flavonoids, tannin, and natural steroids. Using the Folin-Ciocalteu reagent, total phenols were measured. Gallic acid (1 mg/ml) was used for the standard reading. The aluminium chloride technique was used to know the total flavonoid content. For standardization, 1 mg/ml of Quercetin was used. Estimation of total steroids using the Libermann-Burchard test. β-sitosterol (1mg/ml) was used for standard reading. For the Folin-Denis reagent-determined total tannin. Tannic acid (1mg/ml) was used as a reference (Obadoni and Ochuko, 2001).

RESULTS

The combined impact of BAP (0.29 µM) and GA (1.44 µM) in MS medium were found to have the highest percentage of seed germination *in vitro*. This percentage was ninety per cent in 10 days (Data was not shown).

Callus induction:
For the callus induction, various explants, such as nodes and leaves, were taken from the *in vitro* seedling. Then, the leaf and node explants were inoculated in an MS medium with different concentrations of BAP + NAA and BAP + 2,4-D. Under the effect of hormones, the explant showed a positive reaction. The leaf explant was inoculated directly into MS media treated with various concentrations of BAP (2.22, 4.44 µM), NAA (2.68, 4.65 µM), and 2,4-D (2.46, 4.92 µM). As a control, MS media without hormones was used. Callus development was very low in the control media. However, the most remarkable outcomes were obtained in the MS medium with a combination of BAP and NAA. The percent response decreased at a lower concentration of BAP 2.22 µM with 2.68 µM NAA (52 %). The MS medium treated with BAP (4.44 µM) and NAA (2.68 µM) resulted in the most significant formation of leaf callus. This hormone combination produced a callus induction rate of 90% within 11 days after being inoculated. In varied concentrations and combinations of BAP and 2,4-D, 76% callus formation was seen in 14 days in 2.22 µM BAP+ 5.37 µM NAA hormonal concentration. For node callus induction nodal

explants were used and various hormonal conditions were applied. The combination of BAP + NAA (4.44 µM + 2.68 µM) recorded the highest 87 % growth frequency in 12 days. Furthermore, there was a correlation between an increase in BAP and NAA concentration and a reduction in the growing frequency of callus induction. Compared to BAP and NAA hormone, BAP combined with 2,4-D at varying concentrations resulted in much less callus development (Table 1)(Figure-1(b-c)).

Table 1. Effect of various hormone concentrations and combinations on *P. tuberosa* callus induction

MS + Hormone	Concentration of hormone (µM)	Leaf callus		Nodal callus	
		Number of days for callus induction	**% Growth frequency**	**Number of days for callus induction**	**% Explant showing callus induction**
Control	0.0	71.8±3.69^{a}	19.2±1.35^{f}	62.8±2.41^{a}	23.8±1.52^{e}
BAP+NAA	2.22+2.68	17.2±0.86^{cd}	52.2±1.06^{d}	19.4±1.07^{bc}	55.8±2.92^{d}
	2.22+5.37	13.8±0.58^{de}	75.2±1.85^{b}	15.2±1.06^{ef}	72.4±1.56^{c}
	4.44+2.68	11±0.70^{e}	90±1.30^{a}	12.2±0.73^{f}	87.6±2.50^{a}
	4.44+5.37	21.4±0.50^{bc}	76.8±1.06^{b}	18.6±1,.20^{cde}	54.2±2.65^{d}
BAP+2,4-D	2.22+2.68	18±1.41^{bcd}	60.2±5.94^{c}	19±0.70^{bcd}	72.2±1.71^{c}
	2.22+5.37	14.2±0.8^{de}	76.2±3.11^{b}	13.4±1.07^{f}	78±2.21^{b}
	4.44+2.68	21.6±1.07^{b}	45.6±2.86^{b}	22.4±1.20^{b}	67.4±3.6^{c}
	4.44+5.37	19.8±1.28^{bc}	60.8±2.04^{c}	15.4±1.20^{def}	53.2±1.56^{d}

(Note: Treatments were found significant at 1% and 5% levelsl of significance. Superscripted different letters in Column indicate difference within treatments acording to Duncan's Multiple Range Test-DMRT).

***In vitro* shoot formation**

Table 2 presents the formation of *In vitro* shoots from seedling nodal explants in Ms media using varied concentrations of BAP +NAA and BAP + KIN. Almost all hormone concentrations tested shoot development and multiplication. However, very few shoots were observed in the explant grown on the control medium. When BAP + NAA (0.66 µM + 0.53 µM) was added to MS medium, the number of shoots per explant was the highest (11.4 ± 0.92) and the length of the most extended shoot was 10.1 ± 0.46 cm. This was recorded after 3 weeks of incubation (Table: 2)(Figure 1, d-f).

***In vitro* root formation**

When shoots were developed in MS media and treated with different concentrations of IBA and NAA or alone IBA at different concentrations, roots started growing. The highest 88% root formation was observed in 0.49 µM IBA + 2.68 µM NAA with the most increased root length of 8.8 ± 0.34 cm most significant number of roots per explant observed (Table:3)(Figure 1(g)). This plantlet showed better growth than that obtained with IBA with NAA. Only IBA (2.46 µM) in MS media observed 83.6± 2.06 % shoot formation with 6.52±0.21 root length. The proportion of NAA increases, and there is a noticeable reduction in root development.

Table 2. Effect of various hormone concentrations and combinations on *P. tuberosa* seedling nodal explant shoot development

WPM + Hormone	Concentration of hormone (µM)	Shooting		
		% Of shoot formation	**Number of shoots per explant**	**Shoot length (cm)**
Control	0.0+0.0	14.4±2.15^{f}	1.4±0.50^{f}	0.48±0.18^{f}
BAP+NAA	0.44+0.53	56.4±2.58^{e}	8.75±0.85^{cd}	4.66±0.37^{d}
	0.66+0.53	88.4±1.72^{a}	11.4±0.92^{a}	10.1±0.46^{a}
	2.22+2.68	80.2±2.26^{bc}	6.8±0.73^{cde}	8.04±0.54^{b}
	4.44+5.37	72.8±2.47^{cd}	4.6±0.50^{de}	4.18±0.39^{de}
BAP+KN	0.44+0.46	60.4±2.58^{e}	4.4±0.4^{e}	3.32±0.31^{e}
	0.44+2.32	70.2±3.27^{d}	8.6±0.87^{bc}	5.26±0.35^{cd}
	2.22+2.68	83±2.07^{ab}	9.8±0.58^{ab}	8.2±0.20^{b}
	4.44+4.65	53.2±2.85^{e}	8.8±0.37^{bc}	6.3±0.34^{c}

(Note: Treatmets were found signficant at 1% and 5% level of significance. Superscripted different letterns in Column indicate difference within tratments acording to Duncan's Multiple Range Test-DMRT).

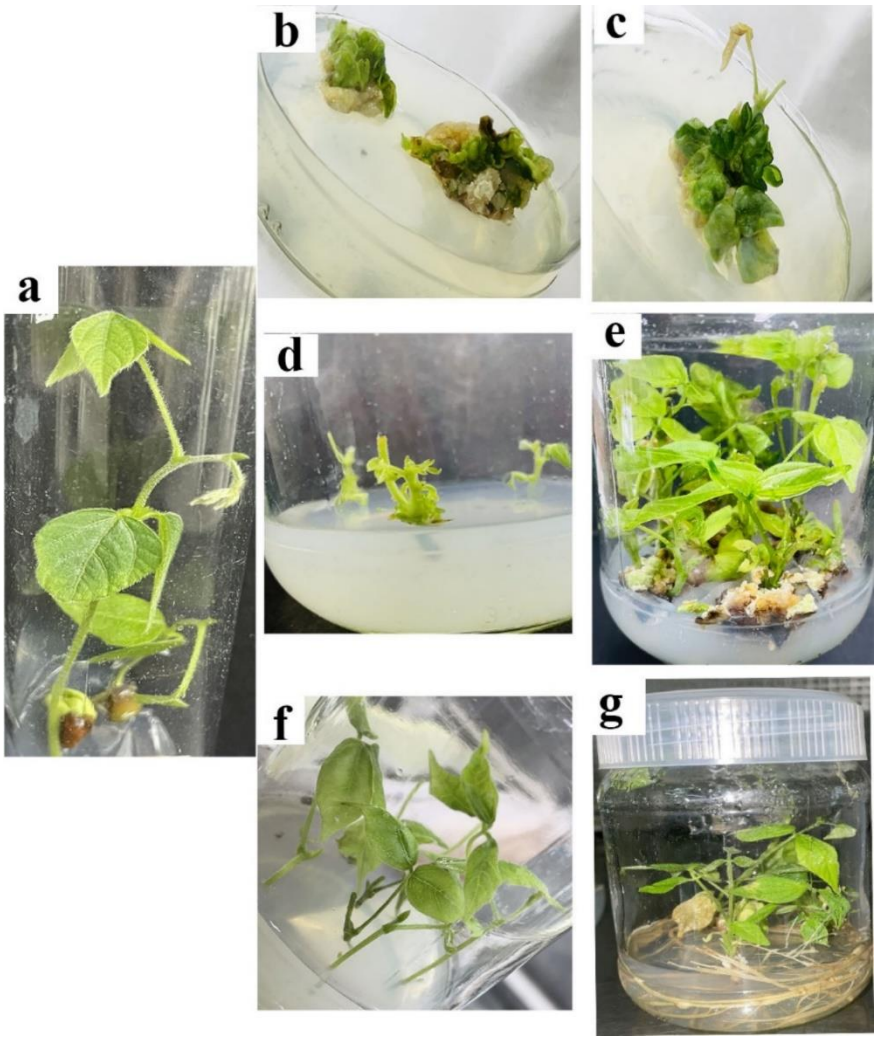


Figure 1. Callus formation and *in vitro* regeneration of *P. tuberosa.* a. *In vitro* seed germination b. Leaf callus c. Node callus d. Nodal explants e. Multiple shoot formation from nodal explants f. 5-6 cm long nodal explants for root formation g. Dense root formation from nodal explants**.**

Table 3. Effect of various hormone concentrations and combinations on *P. tuberosa in vitro* roots development

MS + Hormone	Hormone Concentration (µM)	Rooting		
		% Of root formation	**Number of roots per explant**	**root length (cm)**
Control	0.0+0.0	16.8±2.08^{f}	1.8±0.58^{f}	0.7±0.15^{e}
IBA+NAA	0.49+0.53	50.2±1.71^{d}	6.2±0.37^{bc}	5.34±0.37^{c}
	0.49+2.68	88±2.62^{a}	10.2±0.58^{a}	8.8±0.34^{a}
	2.46+0.53	64.8±3.51^{c}	5.2±0.58^{cd}	6.36±0.29^{b}
	2.46+2.68	47.2±2.61^{de}	3.6±0.50^{e}	5.54±0.38^{c}
IBA	0.49	42±3^{c}	4.6±0.67^{de}	4.04±0.24^{d}
	0.98	68.6±2.11^{c}	6.8±0.73^{b}	5.6±0.22^{c}
	2.46	83.6±2.06^{a}	7±0.70^{b}	6.52±0.21^{b}
	4.92	75.2±2.31^{b}	4.2±0.37^{dc}	3.6±0.35^{d}

(Note: Treatmets were found signficant at 1% and 5% level of significance. Superscripted different letterns in Column indicate difference within tratments acording to Duncan's Multiple Range Test-DMRT).

Qualitative phytochemical analysis in different plant samples

The qualitative phytochemical analysis was done in the 1) *In vivo* plant: - leaf, Stem, tuber and root and 2) *In vitro* plant: - leaf callus, node callus, leaf, Stem, and root. The results are presented in Table 4. The qualitative analysis showed the presence of different secondary metabolites, like alkaloids, phenol, flavonoids, steroids, tannin,

glycosides, terpenoids, and saponin in four extracts such as water, methanol, chloroform and acetone. In qualitative analysis, it was revealed that maximum phytochemicals were present in the methanolic extract. Very low phytochemicals were present in water extracts, so methanol and acetone were selected for further analysis. Flavonoids are recorded in maximum plant parts and saponin are found in less quality.

Table 4. Qualitative phytochemical analysis of in vivo and in vitro plant parts of *P. tuberosa* in different extracts

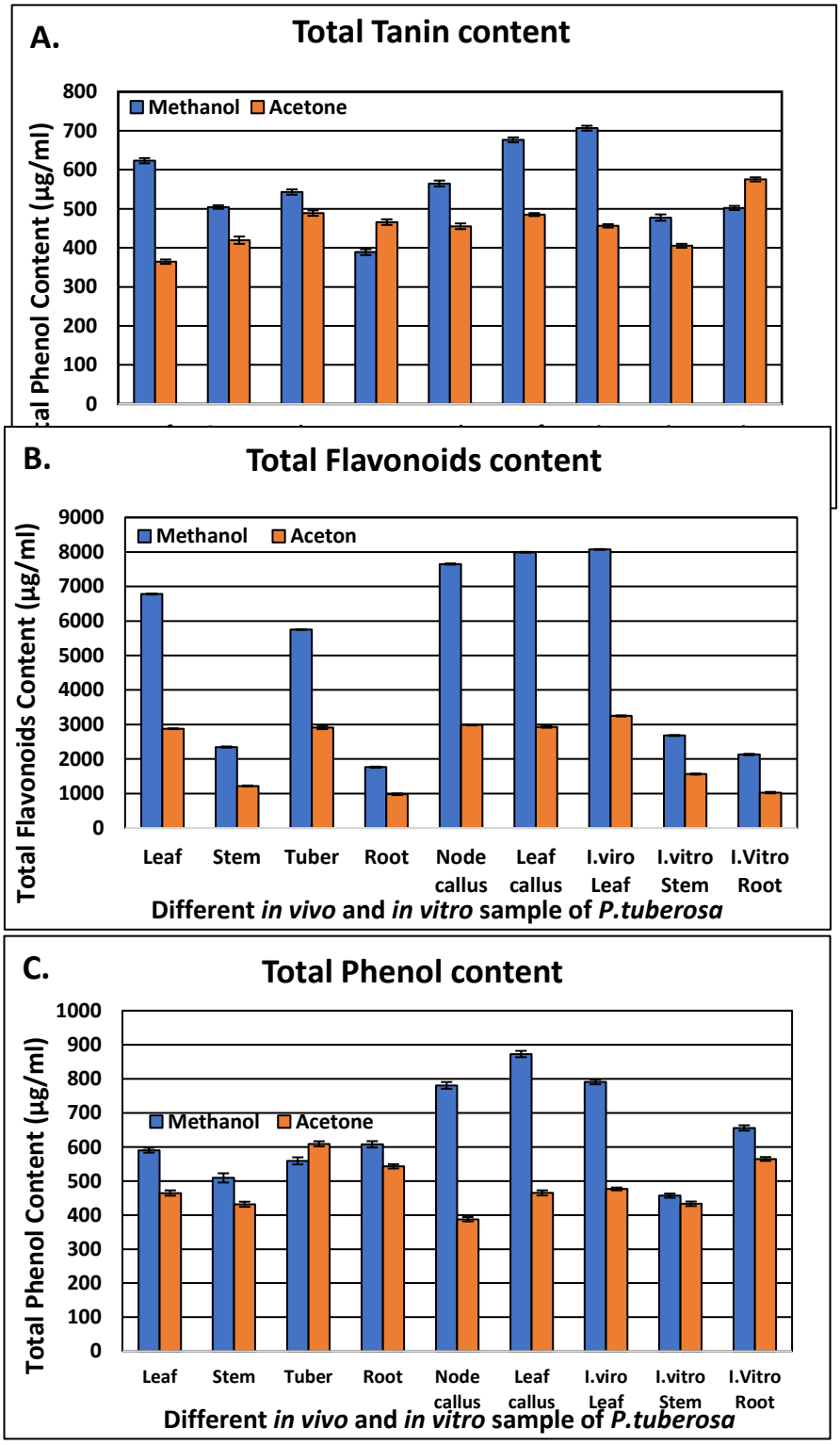
Plant parts name		Alkaloids	Phenol	Flavonoids	Steroids	Tannin	Glycosides	Terpenoids	Saponin
Leaf	**WE**			+		+			
	ME	+	+	+	+	+	+	+	+
	CE				+	+		+	
	AE	+	+	+	+		+	+	+
Stem	**WE**	+				+			
	ME	+	+	+	+	+	+	+	+
	CE	+			+				
	AE		+	+		+	+	+	+
Tuber	**WE**		+					+	
	ME	+	+	+	+	+	+	+	+
	CE	+	+				+		
	AE	+	+	+	+	+	+	+	+
Root	**WE**		+						+
	ME	+	+	+	+	+	+	+	
	CE		+		+		+		
	AE	+		+	+	+		+	
Leaf callus	**WE**		+						
	ME	+	+	+	+	+	+	+	+
	CE		+					+	
	AE	+		+	+	+	+	+	+
Node callus	**WE**		+			+			
	ME	+	+	+	+	+	+	+	+
	CE		+		+				+
	AE	+		+	+	+	+	+	
In vitro Leaf	**WE**								
	ME	+	+		+	+	+	+	+
	CE			+	+			+	
	AE	+	+	+	+	+	+	+	+
In vitro Stem	**W.E**						+		
	ME	+	+	+	+	+		+	+
	CE				+		+	+	
	AE	+	+	+	+	+	+	+	+
In vitro Root	**WE**								
	ME	+	+	+	+	+	+	+	+
	CE			+		+		+	
	AE	+	+	+	+	+	+	+	+

(Note: += present, -= Absent, WE- water extract, ME- methanol extract, CE- chloroform extract, AE- acetone extract)

Quantitative phytochemical analysis in different plant samples

Based on the qualitative phytochemical results, quantitative estimation of plant was carried out by standard method for major phytochemicals such as flavonoid, phenol, tannin and steroids. Two types of solvents were used for quantitative analysis – Methanol and Acetone. As shown in figure 2, the total tannin content maximum *in vivo* leaf (623.3 ± 6.64 µg/ml) followed by *in vitro* leaf callus (676.6 ± 6.38 µg/ml)and leaf (706.6 ± 6.33 µg/ml) of methanolic extract. Tannin was noted to be very low *in vivo* leaf (354 ± 5.60 µg/ml) followed by *in vitro* (398 ± 5.04 µg/ml) stem of acetone extract. Total phenol content was low in node callus (387 ± 6.35 µg/ml) and *in vitro* stem part (433 ± 6.65 µg/ml) in acetone. In the *in vivo* plant: leaf (590 ± 7.21 µg/ml) and root (607 ± 9.26

µg/ml) than in *in vitro*: leaf callus (872 ± 9.38 µg/ml), leaf (790 ± 6.93 µg/ml) and root (655 ± 7.83 µg/ml) Maximum phenol was reported part in methanolic extract. The flavonoid content was reported highest in *in vivo* leaf (6779.3 ± 12.78 µg/ml) and tuber (5751.6 ± 10.74 µg/ml) than in *in vitro* node callus (7650.6 ± 16.82 µg/ml), leaf callus (7992 ± 10.13 µg/ml) and leaf (8072.6 ± 11.09 µg/ml) parts in methanolic extract. Common flavonoids were noted in *in vivo* stem (1219.3 ± 13.73 µg/ml) and root (977.6 ± 22.99 µg/ml) than *in in vitro* Stem (1562.6 ± 16.59 µg/ml) and root (1026 ± 20.84 µg/ml) in acetone extract. The highest amount of total steroid was found in the *in vivo* plant; leaf (1452.3 ± 9.70 µg/ml) and tuber (1551 ± 6.38 µg/ml) than *in vitro* plant leaf (1543 ± 6.35 µg/ml) in methanolic extract. Total steroid found in low amounts in acetone extract in *in vivo* stem (244.33 ± 7.51 µg/ml) and *in vitro* stem (432.6 ± 4.35 µg/ml) (Figure 2).



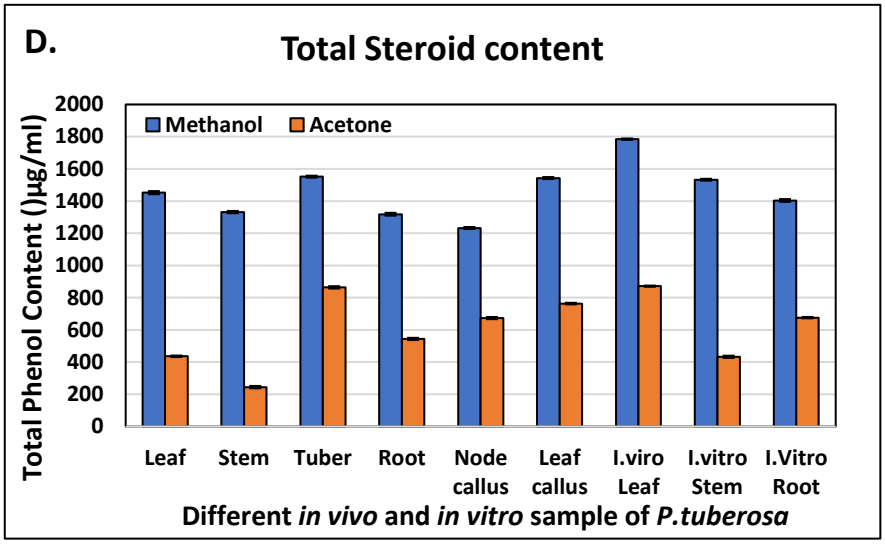


Figure 2. Quantitative analysis of important secondary metabolites from *in vivo* and *in vitro* parts of *P. tuberosa* A. Total tannin content B. Total flavonoid content C. Total phenol content D. Total steroid content

DISCUSSION

P. tuberosa, also known as Indian Kudzu is a plant considered to have great medicinal value in the Indian traditional medical system in ayurveda. This plant is referred to Vidari, in the Indian ayurvedic pharmacopoeia (Jain and Choudhary 2016). Reports indicate that *P. tuberosa* tuber is mainly used in traditional and ethnomedical systems for medicinal purposes. Verma *et al.,* (2012) reported that the tuber contains cardioprotective, emollient, laxative, galactagogues aphrodisiac, diuretic, allergenic, cooling, rejuvenating, and expectorant properties, according to Ayurveda. It is essential to select, propagate, and characterize vital medicinal plants due to the increasing demand for plant-based medications for commercial application. Furthermore, the development and genetic enhancement of desired genotypes are achieved by *in vitro* methods. *In vitro* propagation cultures have been discovered as a significant source of secondary metabolites from plants (Manisha *et al*., 2012). Different parts of a plant have other numbers of secondary metabolites. Qualitative analysis and quantitative research have revealed which areas of a plant contain the most significant number of secondary metabolites. Table 1 shows the results of callus formation from *in vitro*-grown leaf and seedling nodal explants of *P. tuberosa*. In tissue culture, the proper balance of auxin and cytokinin is an important and essential part of regulating cell division (Bindu *et al.,* 2018). Plant hormones play a vital role in callus formation and micropropagation. Auxin and cytokinin concentrations are essential in the growth of calluses in various plant species. A high ratio of auxin and cytokinin increases root and shoot growth, while an average amount of auxin and cytokinin helps to grow calluses (Skoog and Miller, 1957). In this study, different auxin and cytokinin concentrations influenced how calluses were established from leaf and node explants. MS media treated with 4.44 µM BAP and 2.68 µM NAA in 11 days had the highest 90 percent of leaf callus growth. BAP + NAA combine effect on the formation of callus in *Alstroemeria* cv. Fuego is consistent with the findings similar to this study (Seyyed *et al.,* 2013). Various auxin and cytokinin concentrations stimulate callus formation (Patel *et al.,* 2021). In *P. lobata*, leaf and stem callus formation and growth were greatest in medium containing 25 µM benzyl amino purine (Matkowski 2004). Reddy *et al.,* (2011) reported that leaf explant developed callus in half-strength MS media treated with BAP and 2,4-D. According to previous research, a 2,4-D + NAA (2.0 + 0.5 mg/l) was the most excellent induction of callus formed from leaf explants (Manokesh *et al*., 2014). Leaf explants of *P. tuberosa* grew best on MS media treated with benzyl aminopurine 6.6 µM and 2.69 µM naphthalene acetic acid (Bindu *et al*., 2018). After two weeks of culture, *Tectona grandis* formed a compact callus on MS medium containing BAP + NAA (1.5 + 0.5 mg of L-1) (Egodawatta *et al.,* 2014).

Maximum growth was reported in 4.44 µM BAP with 2.68 µM NAA in MS medium. It was discovered to have an 88 percent growth frequency in 12 days when analyzing the callus development from the nodal explant of *P. tuberosa*. Nodal explants of *P. tuberosa* generated a green nodular compact callus in MS media treated with 0.53 µM NAA + 8.88 µM BAP (Sadguna and Mustafa, 2014). In the same way, adding 8.88 BAP µM + 9.3 µM kinetin in MS medium formed a green compact callus from the nodal explant. (Gowda *et al.,* 1988). In soyabean, similar data have been achieved. So, not important what kind of callus is made from an explant, it can be used for regeneration and somatic embryogenesis, and it can also be used to get secondary metabolites that can be

used in medicine. MS medium treated with 11.1 µM BAP and 2.68 µM NAA was the best medium for leaf explants. This was because it had a high fresh weight, a high percentage of callus, and helped *Ombrychis stiva* form embryogenic stages (Mohajer *et al*., 2012). In our studies, two different kinds of callus, could be helpful for future micropropagation research.

For the development of shoots from the nodal explant, various combinations of BAP + NAA are applied. In MS media supplemented with 0.66 µM BAP and 0.53 µM BAP, the highest shoot formation frequency was 88 percent, the number of shoots per explant of 11.4, and a maximum shoot length of 10 cm was reported (Table:2). In MS media supplemented with 2.22 µM + 2.72 µM (BAP + KN), 83 percent shoot development was seen with ten shoots per explant at a length of ten centimetres. The data indicate that NAA was significantly more effective than KIN for shoot growth. Several *in vitro* experiments have found that BAP is the most effective cytokinin. BAP is prominent among cytokinin due to its stability and easy absorption. Media containing BAP showed more significant potential for shoot regeneration than those from the media supplemented with other cytokinins (KIN and IDZ) in many different plant species, including *Eclipta alba* (Ray and Bhattacharya, 2008), *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012), *Ceropegia evansii* (Chavan *et al.,* 2015), *Bacopa monnieri* (Jain *et al*., 2014) similar result noticed. Shoot induction and differentiation are influenced by the relative concentration of cytokinin and auxins in the growing media. For the induction and multiplication of shoots in *D. bulbifera*, a high concentration of cytokinin combined with a low quantity of auxin produced favourable results (Bhat *et al.,* 2022). Nodal explants of *Costus speciousus* (Raghu *et al*., 2006) and *Streospermum suaveolens* (G. Don) DC (Trivedi and Joshi, 2014), the cytokinins BAP and KN significantly promoted shoot formation. In earlier research, Kadota *et al*., (2003) reported that at a high growth regulator level-sensitive in the proliferation stage. When BAP with NAA, it significantly affected shooting in the culture media. In *Rosa hybrida* were noted similar result (Alibad *et al.,* 2019). For *in vitro* root formation, regenerated shoots were inoculated in an MS medium containing IBA + NAA and IBA alone. Out of all the different concentrations of IBA and IBA with NAA that were tested, the one with the most roots were 0.49 µM + 2.68 µM (IBA + NAA), where the average number of roots was 10.2 ± 0.58 and the highest percentage of roots formed was 88 ± 2.62. Average root length of 8.8 ± 0.34 cm at formation, these pointed, strong, and healthy roots had a reasonable survival rate during hardening. Half MS medium with 2.46 µM IBA gave good results in *P. tuberosa*. This same way of result was obtained Bindu *et al.,* in 2017. IBA with NAA better result compares to IBA alone for root formation. In *Nopalxocia ackermannii* (Deng *et al.,* 2018) and *Abutilon ranadei* (Survase *et al.,* 2016) the same type of result was obtained in this important medicinal plant.

Plants produce two types of metabolites; 1) carbohydrates, lipids, and proteins etc., are primary metabolites that are involved in various metabolic processes, 2) alkaloids, flavonoids, saponin, glycosides, tannins, terpenoids etc. these are secondary metabolites they are not involved in metabolite and considered the product of primary metabolite (Pal, 2007). The primary source of food additives, medicines, insecticides, fragrances and herbicides come from these secondary metabolites (Okwu, 2005; Ramawat and Dass, 2009; Ramu and Mohan, 2012). *In vitro* plants had higher levels of flavonoids, while undifferentiated tissues had lower flavonoid concentrations than organized tissues. Such variations in the concentrations of secondary metabolites between entire plants and cell or tissue cultures have been observed frequently (Patel *et al.,*1979), (Pramanik *et al.,* 1995). Qualitative analysis was performed for various extracts of *P. tuberosa* in both the *in vivo* plant and *in vitro* plant parts. The maximum phytochemical present in methanolic extract. Both *in vivo* plant and *in vitro* plant parts present all phytochemicals like phenol, flavonoids, glycosides, alkaloids, saponin, steroids, tannin and terpenoids. Flavonoids present in both *in vivo* plant and *in vitro* plant parts. These analyses show the existence of several bioactive secondary metabolites, which may be the source of their therapeutic properties. These secondary metabolites have an essential role in the biological activities of medicinal plants, including their ability to act as antidiabetic, anti-inflammatory, and antibacterial agents. *R. sativus* contains tannins, saponins, flavonoids, anthraquinones, carbohydrates, steroids, phytosterol, alkaloids, amino acids, terpenoids, cardiac glycosides, and chalcones, according to earlier *in vitro* phytochemical research (Janjua *et al.,* 2013). Singh in 2013 reported in their research secondary metabolite such as tannins, saponins, phenolic, alkaloids, steroids and flavonoids compounds found in *cucurbita maxima* fruits. Flavonoids from plants act as antioxidants both in the *in vivo* plant and *in vitro* plant (Soorbrattee *et al*., 2005; Geetha *et al*., 2003; Shimoi *et al.*, 1996). In Pteris species, both quantitative and qualitative phytochemical analysis was recorded in Gracelin *et al.,* research. They noted the plant have most found flavonoids followed by phenolic and alkaloids. The extract of selected fern had low amount of saponins and tannins (Gracelin *et al.*, 2013).

Analysis of phytochemicals is a highly helpful process in medicinal plants, and that might be applied to the discovery and development of new drugs. Researchers have found that the type of solvent used in the extraction process affects how well phytochemical components are obtained. Solvents like Methanol and acetone were used in this study. On the basis of qualitative analysis of phytochemicals, quantitative analysis was also done on tannins, phenols, flavonoids, and steroids, which are the main phytochemicals. Highest amount of

tannins content was found in *in vitro* leaf in methanol extract where highest number of phenols was found in *in vitro* leaf methanolic extract while highest flavonoids content was examined in *in vitro* leaf. Steroids were found to be in highest in *in vitro* leaf. All phytochemical maximum found in methanolic extract. It has more solubility for bioactive components of *P. tuberosa* than other solvents. Due to the variety and availability of phytochemicals, this plant could be used to make medicine (Yamaguchi *et al.*, 1998). Previous research found qualitative and quantitative phytochemicals in selected plants (Khan *et al.,* 2011). This study found that the phytochemical components of the extracts were affected by the type of solvent used (Nayak and Singhari; 2003). Quantitative estimation showed that the *in vitro* plant samples accumulated higher amounts of tannins, phenols, flavonoids and steroid content than samples of wild-growing plants.

In vitro plant samples were shown to have a much higher number of phenols, tannins, steroids, and flavonoid content when compared to samples of the *in vivo* plant. This was determined using quantitative analysis. *In vitro* plants like In *Thymus lotocephalus* (Ncube *et al.*, 2011) and *Tulbaghia violacea* (Costa *et al*., 2012) have higher amounts of secondary metabolites recorded in few research studies. The findings showed that *in vitro* plants have higher phenol content in their leaf, leaf callus, and node callus samples than compare *in vivo* plants. Some plants, like *Sussurea involucrate* (Guo and Singh, 2007) and *Habenaria edgeworthii* (Giri *et al*., 2007), had the same results. The phenolic content of *in vitro* plants was higher than that of *in vivo* plants. The highest-growing *P. major* calli had the lowest flavonoid concentrations. Many protocols for making secondary metabolites in a factory have two steps; The first involves growing tissues or cells in a growth medium and then subculturing them in a production medium. (Kurian and Josekumar; 2017 and Sarmah *et al*., 2017).

CONCLUSION

The method of micropropagation can be applied as a new solution to increase the production of secondary metabolites and plantlets on a large scale. According to the most current data, the synergetic and positive effects of BAP and NAA on *P. tuberosa* cultures enhanced callus development and shoot multiplication. With the help of plant growth regulators and *in vitro* conditions optimized for callus formation, shooting, and rooting in *P. tuberosa*, is a more efficient and less expensive method for the multiplication of this plant. All *in vitro* regenerated shoots were effectively rooted via rhizogenesis, which might reduce time and lower the cost of producing plantlets. It could be an effective strategy for cultivating this seasonal, vulnerable, and important plant species. A phytochemical study also showed that the protocol could be used to increase the production of bioactive compounds. The current findings suggest that the study may help to identify and standardize active compounds for the production of new drug formulations for future research.

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