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Phytochemical Diversity And Micropropagation of Paris Polyphylla Rhizomes From Northeast India

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Abstract: *Identification of medicinal plant for elite genotypes requires assessment of phytochemical diversity across different populations. Such study is fundamental for further scale of plant resources as well as subsequent drug development for treatment of human ailments. The present study was taken up to assess the total steroidal saponins diversity in Paris polyphylla across the Northeast region of India. From the study, it was found that P. polyphylla populations from Khonoma showed the highest total saponins content, recording an average of 32.06mg/g DW in comparison with all the populations under study. The findings of the study were taken up for micropropagation of the Khonoma populations for mass propagation of rhizomes of this high valued plant. Efficiency of two cytokinins with different sucrose concentrations on minirhizome induction was studied, and it was found that 0.5mg/l BAP+6% sucrose and 1.0mg/l 2iP+6% gave the best response giving 88.6% and 89.2% response with 1.27±0.02g fresh weight and 1.36±0.10g fresh weight minrhizome respectively.*

Keywords: *Cytokinins, diversity, genotypes, minirhizomes, saponins*

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I. Introduction

Discovery of new and novel drugs from plants for treatment of various human ailments, require the screening of plant extracts, and thus will provide continued access to the vast plant biodiversity of the Earth[1]. Carrying out phytochemical analysis of medicinal plants from different climatic zones/geographical zones can help for scale up of the plant species on a commercial scale to increase its pharmaceutical marketing values[2].Paris polyphylla Sm. (family Trilliaceae) is an important medicinal herb with proven therapeutic potential. Its rhizome is known to produce steroidal saponins that have immense medicinal applications. The information on utilization of this plant in India and Nepal is limited to the traditional folk-healers where it is mainly used in the treatment of common ailments like fever, as an expectorant, digestive stomachic, antihelmintic, antispasmodic, treatment of cuts and wounds and of poisonous snake and insect bites[3-7]. It is an extensively researched plant in China; it is used in Traditional Chinese Medicine (TCM) as haemostatic, parotisis and snake bite, analgesic, treatment of fractures, anti-microbial and anti-inflammatory agent. Also, it is widely documented for the presence of secondary metabolite in the form of steroidal saponins and its proven anti-cancer properties[8-22]. Owing to its high medicinal applications, the plant suffers tremendous pressure of over-collection from the wild.In addition, unscientific and unregulated exploitationas well as lack of significant conservation strategies have driven the plant to vulnerable state[23].

Secondary metabolites biosynthesis varies among plants, even in different organs of the same plant, and their biosynthesis depends on genetic factors as well as the environmental factors in which they grow[24]. Estimating the amount of variability of a desired trait in genotypes/accessions and the influence of a particular trait on other traits contributing to the yield of plant is of prime importance in bringing about improvement in both quantitative and qualitative traits[25].For bringing about improvement in quality control of plant phytochemicals for maximum drug efficacy and safety, it is fundamental to screen medicinal plants for the presence of the active constituent of choice[26]. Since, there exists variability in the active constituents of same plants at various developmental stages growing under different environmental conditions and, quantification of the phytochemicals is fundamental for correct identification of zonal population for genotypes with superior secondary metabolite yield.

Phytochemical screening and diversity assessment is thus empirical for zonal identification of populations which will ultimately supplement in downstream processing of the phytochemicals and subsequent drug development.The present studywas taken up to assess the phytochemical diversity of P. polyphylla germplasm collected from across the Northeast region of India.Micropropagation of the rhizome through tissue culture for mass propagation of the elite genotypes was undertakento ultimately negate the pressure of extensive

collection of this high-valued medicinal plant from the wild. This approach may be helpful in formulating strategies for conservation of P. polyphylla, a highly valuable medicinal plant of the region.

II. Materials And Methods

2.1 Survey and plant collection

Survey was made across the Northeast regions of India. Four states viz., Nagaland, Manipur, Meghalaya and Arunachal Pradesh were considered in the study. The details of the populations undertaken in the study are given in **Table 1**.

2.2. Steroidal saponins estimation

2.2.1.Plant materials

Eight-ten plant samples from each population were randomly collected and maintained in the net house of Plant Biotechnology Laboratory, Dept. of Botany, North-Eastern Hill University, Shillong, Meghalaya. To maintain uniformity in the experimental setup, 6 samples from each population having matured rhizomes measuring up to 5-8cm in length and 1-2cm girth size were only considered for the study.

2.2.2. Extraction and steroidal saponins determination

The method of extraction was followed using the protocol as described by Shivei et al. (2015)[27]. Harvested matured rhizomes were individually washed under running tap water to remove the mud and dirt adhering on them. Final rinsing of the washed rhizomes was carried out in distilled water. The washed rhizomes were blot dried, removal of dead scales and necrotic portions was carried out using scalpel blade. These clean and dried rhizomes were sliced into 0.5-1mm sections and oven dried at 50ºC until constant dry weight (DW) was achieved. Dried rhizomes sections were then ground into fine powder using mortar and pestle. For extraction of steroidal saponins, 1g of sample powder was loaded into Soxhlet apparatus containing 120ml of 90% aqueous ethanol solution (v/v) and kept for refluxing for about 3h. After which, the solvent was completely evaporated at 50ºC and the left over slurry was re-dissolved in 5ml methanol by vortexing. The solution was subjected to centrifugation at 10,000rpm for 10min and the resulting supernatant was membrane-filtered using Himedia membrane filter of 0.22µm size. The final filtrate was then used as the test solution.

2.2.3. Determination of steroidal saponins using HPLC

Reverse phase HPLC (RP-HPLC) was used for qualitative steroidal saponins profiling and quantification. The HPLC analysis was carried out using Agilent Technologies 1260 Infinity LC system equipped with a diode array detector (DAD). The HPLC separation was carried out in analytical reverse C_{18} column, (250x4.6mm, 5µm) eluted using gradient elution containing mobile phase acetonitrile (A) and water (B). The ratio of A: B was applied in gradient elution regime: 45:55 (A/B) to 55:45 in 20min, 90:10 in 5min. A 5min pure acetonitrile wash was applied after each run. Flow rate was kept at 1ml/min, sample size was set at 20µl and the detection was set at 203nm. The chromatographic peaks of the samples were confirmed by comparing their retention time with those of the three standard saponins viz., polyphyllin I, polyphyllin II and polyphyllin VII and also by spiking samples with standard steroidal saponins.

For preparation of standard calibration curve, all the three standard polyphyllins were prepared in various concentrations 0.1-1.0mg/l in methanol. A 20µl volume of each standard was injected in the HPLC for obtaining the standard curve plot of peak area with a run time of 25min. To check linearity of the method developed, each standard saponin was analysed at six different concentrations ranging from 0.1-1.0mg/l. The calibration curve was constructed by plotting the peak area (y) against concentration in µg/ml of standard solutions (x). The standard equation obtained from the curve was used for quantification of the steroidal saponins in the unknown test samples. The final analyzed steroidal saponin content was expressed as mg steroidal saponins/g dry weight. All solvents (HPLC grade) were obtained from Himedia, India and the authentic saponin standards were procured from SanghaiYaji Biological Technology Co., Ltd. (Sanghai, China)[27].

2.5. Micropropagation of P. polyphylla

2.5.1. Collection, sterilization and explant preparation

Rhizomes of the genotypes with the highest saponins content (as already reported in Chapter 3) were harvested from the plants growing in the glass house of Plant Biotechnology Laboratory, North-Eastern Hill University, Shillong, India. Healthy and actively growing rhizomes were collected during the months of April-May. These were washed thoroughly first under running tap water, treated with 1% bavistin for 15min, washed thoroughly with fungicide and then rinsed with sterile distilled water several times. The rhizome explants were given two step surface sterilization procedure; first treatment with 15% sodium hypochlorite (NaClO) for 15min with 2-3 drops of Tween-20, and second with 0.2% mercuric chloride ($HgCl_2$) (w/v) for 10min. The explants were rinsed several times with sterile distilled water after each sterilization treatment. Subsequently, the

explants were blot dried, outer scales were removed and 0.5x1mm sections, roughly weighing about 0.15g were inoculated on the nutrient medium.

2.5.2. Nutrient requirements and culture conditions

Rhizome sections were inoculated on ½ strength MS medium (half-strength macro- and micro-nutrients; Murashige and Skoog 1962) with varying concentrations of sucrose (3-9% w/v) and solidified with 0.2% clerigel (w/v) (Himedia, India). The culture medium was supplemented with various concentrations (0.25, 0.5, 1.0, 1.5 and 2.0mg/l) of Benzyladenineaminopurine (BAP) and 2-Isopentyl (2iP) individually. The intrinsic combinatory effect of 0.5mg/l BAP with 2iP in the medium was also evaluated. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C at 1.05kg/cm^2 pressure for 15min. All the cultures were initially incubated in dark inside the culture room at controlled temperature of 25±2ºC. The percentage response of the explants and increase in fresh weight of explants were recorded after 6 months of culture. No subculturing was carried out until then. After the record of percentage response, subculture of the induced rhizomes was carried out on ½ MS medium in cytokinin-free medium followed by regular subculture after every 3 months intervals. Number of sprouted shoots from the induced rhizomes was recorded after 5 months, which were individually detached from the mother explant and inoculated on the same fresh medium. The culture vessels with the shoots were kept under culture conditions of 14h photoperiod and photosynthetic photon flux of 60µmolm^{-2}s^{-1} provided by cool-white fluorescent lamps. Well-developed shoots with rhizomes after one and half years in culture were subjected to acclimatization.

2.5.3. Hardening of regenerated plantlets

Plantlets with well developed rhizomes were taken from culture vessel and washed under running tap water to remove all traces of adhering agar. The clean plantlets were then transferred to thermocol cups with holes to provide aeration plus proper drainage and the compost mixture of soil: peatmoss: cow manure in the ratio of 2:1:1 was added. The cups with the plantlets were initially covered with polythene bags having holes pierced on it, to maintain adequate humidity. The polythene bags were removed after a month of acclimatization transfer. All the plantlets were kept in green house providing required sunlight, and watering of the plantlets was monitored. Survival percentage of the transferred plantlets was recorded after 3 months of transfer to green house conditions.

2.5.4. Data analysis

For data analysis, ten replicates per treatment for all the experiments and the whole exercise were repeated thrice. All statistical analysis was done by Analysis of variance (ANOVA) at 5% significance level and the means were compared by using Tukey's LSD test (PC version Origin 8.0 NORTHAMPTON, MA, USA).

III. Results

3.1. Steroidal saponins estimation

The steroidal saponin contents in all the collected samples were analyzed by RP-HPLC and the standard saponinspolyphyllin I, plyphyllin II and polyphyllin VII (Fig. 1: a-c) were used to construct a calibrated graph by plotting peak area versus amount of the respective steroidal saponins. The representative HPLC chromatograms from each population obtained are shown in Fig. 2: a-d. The amounts of compounds present in the crude samples were calculated by using the equation generated from the curve by external standard method. The three standard steroidal saponinspolyphyllin I, polyphyllin II and polyphyllin VII showed peak at retention times of 11.67min, 11.07min and 5.90min respectively. The methanolic extracts of the samples across all the population analyzed showed retention time corresponding to all the three standard steroidal saponins confirming the presence of the three saponins in the crude extracts. The total steroidal saponins content recorded was the sum of individual saponins i.e., polyphyllin I, polyphyllin II and polyphyllin VII in each sample. In the present study, 54 genotypes representing 9 populations were assessed for total saponin content using HPLC. It was found that, polyphyllin II content was highest among the three saponins quantified followed by polyphyllin VII and polyphyllin I. This trend was observed in all the samples under study. Variability in the saponins content across all the samples was observed in the study undertaken. The total saponins content varied from 13.93mg/g DW – 39.75mg/g DW (**Table 2**). The highest total steroidal saponin content was recorded in KH5 under Khonoma population having total steroidal saponins content of 39.75mg/g DW rhizome, with 3.04mg/g DW of polyphyllin I, 28.92mg/g DW of polyphyllin II and 7.79mg/g DW of polyphyllin VII. On taking average of all the samples in each population, the rhizome samples from Khonoma population showed the highest total steroidal saponins (32.06mg/g DW), followed by Ukhrul population (30.76mg/g DW) and Tuensang population (30.72mg/g DW). The least total steroidal saponins content was observed in samples fromWestKhasi Hills population (20.03mg/g DW) (**Table 2**).

3.2. Micropropagation of rhizomes of P.polyphylla

3.2.1. Effect of BAP and sucrose concentration on rhizome induction from rhizome sections

The response of the rhizome sections cultured on ½ MS medium supplemented with various concentrations of sucrose and BAP is given in **Table 3,** Fig. 3: a-d. No response was observed in explants in the absence of cytokinin in the medium, whereas in the medium supplemented with BAP there was significant increase in fresh weight of induced MR and number of shoots. The concentration of sucrose as well as that of BAP in the medium had differential influence on the growth and development of the explants in culture. The optimal response of the explants (88.6%) was obtained in medium containing 6% sucrose and 0.5mg/l BAP with MR fresh weight of 1.27±0.02g, followed by 87.2% in medium containing 3% sucrose and 0.5mg/l BAP wherein MR fresh weight of 1.12±0.03g was recorded. The induced MRs on subsequent subculture on to the basal medium (½ MS with 3% sucrose without cytokinins) gave rise to 5.1±0.4 and 4.8±0.7 shoot buds per explant, respectively. It can be mentioned here, that the subculture of the induced MR on the same medium showed an increase in size, but on being subcultured in basal medium small shoot protuberances started to appear after 3-4 months. The highest percentage response and MR fresh weight in medium containing sucrose at both 3% and 6% concentration, was observed in the medium incorporated with 0.5mg/l BAP, showing that it is the optimal concentration for explant responsiveness. Further increase in BAP concentration in the medium had a negative impact on the growth of the MR. It was also observed in the study that the increase in sucrose concentration to 9% had a drastic effect on the growth of the explant resulting in a significant decrease in the overall parameters under study.

3.2.2. Effect of 2iP and sucrose concentration on rhizome induction from rhizome sections

Similarly, experiment with 2iP incorporated in the medium to study its effect on the growth and development of rhizome explants was carried out. From the experiment conducted, it was observed that in medium containing 6% sucrose with 1.0mg/l 2iP highest percentage response of 89.2% resulted with MR fresh weight of 1.36±0.10g (**Table 4**, Fig. 4: a-d). With 3% sucrose in medium supplemented with 1.0mg/l 2iP, 85.30% percentage response and MR fresh weight of 1.07±0.05g were recorded. These MRs on being further subcultured in basal medium resulted in 4.6±0.3 shoot buds per explant and 5.3±0.3 shoot buds per explant. It was observed that the optimal concentration of the 2iP in the medium for the explant responsiveness was 1.0mg/l. The present study showed the higher sucrose concentration in the medium to be detrimental for the growth and development of the MR; a similar observation with the incorporation of BAP was found.

IV. Discussion

The commercial importance of phytochemical analysis in medicinal plants for preliminary identification of potential for new and novel drugs for the treatment of human ailments drives great interests among the pharmaceutical industries. Secondary metabolites are plant compounds which serve protection, competition and species interactions but not necessarily survival. These compounds have generated potential as raw material for drugs that can cure human ailments. Studies on phytochemical diversity will provide a helpful insight into the identification of zonal populations as chemotypes of a particular plant species vary significantly among populations. In the backdrop of immense therapeutic value of P.polyphylla and meagre data on its phytochemical diversity, the information on its variationserves as an important factor for selection of divergent genotypes for crosses and effective conservation and management of its germplasm resources.

From our study, it was observed that the total steroidal saponins content was found to be highest in Khonoma population followed by Ukhrul population. Therefore, Khonoma populations can be considered for plant improvement programmes and may be subjected to mass propagation technologies to feed consumer demand. An important mention can be made here that the biological activity of the rhizomes may be due to the synergistic effect of all the three known saponins and not because of a single compound. The concentration and composition of secondary metabolites varies in nature amongst the members of same species which is evidenced by the present study. The observed variation in the steroidal saponin content in the rhizomes of the samples under study could be attributed to a number of factors. Earlier reports have suggested the influencing property of environmental factors such as geography, climate, soil type, growing season, sun exposure, grazing stress, seasonal changes, etc. on the phytochemical composition of plants. All of these factors ultimately affect the total phytochemical property of the plant[28-36].Plants grow in populations in extreme topographical variations and under various climatic zones and temperature conditions therefore variations within and between populations are not uncommon. Earlier, much of the variation in phenotype was believed to be influenced by environmental factors. Many botanists reasoned that distinct intra-specific variations of plants were merely due to phenotypic plasticity brought about by habitat modifications and adaptation to environment. However, Turesson (1922)[37] have shown the genetic aspect of such variation stating that significant hereditable variation occurs amongst members of same species under certain conditions where such variations are longer plastic in nature because they were consistently transferred to generations after generations irrespective of the cultivation

conditions.Natural populations of the plant species may have superior and inferior chemotypes based on the content of active phytochemicals, brought about by either intrinsic (genetic) or extrinsic (environmental) factors or the interaction between the two[38-39]. The observed results in our study might be speculated to be due to the interaction of both the genetic and environmental factors. Of all the important factors contributing to the functionality of the medicinal plant, genotype is particularly important for determining the quantity of phytochemicals, and consequently affects the overall plant quality.Plant phytochemical diversity to assist in breeding programmes for improvement of breeding lines as well superior chemotypes identification for commercialization have been carried out in Andrographis paniculata[25], tomato[40].

Micropropagation of the rhizomes for mass propagation was carried out using half-strength MS medium, as it was earlier reported by Raomaiet al. (2015)[27] that MS full strength was unfavourable for the growth and development of tTCL of P. polyphylla, due to its high nutrient content. Coherent report on efficiency of ½ MS medium on MR formation was made by Pence and Soukup (1993)[41] on Trillium erectumand T. grandiflorum. The responsiveness of the explants in culturecan be attributed to the type of organ used as explants. Since rhizomes are basically modified stem which contains higher density of vascular tissues, it may have a contributing factor to its responsiveness. Similar observation was reported inLycastearomatica[42]wherebasal section gave better response in shoot regeneration than the apical portions. However such response can vary depending on the plant as documented inL. longiflorumin which tip portions gave high shoot regeneration than the basal portions[43].

Cytokinins incorporation in media for shoot organogenesis which can be modified to generate tubers, bulbils, rhizomes and corms are well documented. Cytokinins types and concentrations used in the study had a positive influence on the rhizome induction and shoot development on rhizome sections used in the study. Both the cytokinins under study proved to be effective in MR induction where BAP and 2iP used singly in medium gave highest response at 0.5mg/l and 1.0mg/l respectively. Fresh weight of the induced MR was found to be slightly higher in the 2iP treatment than the BAP treated MR. Similar report given by Rahimaet al. (2013)[44] where 2iP was found to be the most effective cytokinin in multiple shoot regeneration in Rhodendronindicum. Shahzadet al. (2013)[45] also reported the efficacy of various cytokinins and found that cytokinin BAP was better in multiple shoot induction than 2iP treatment. BAP is the most widely and most commonly used effective cytokinin for shoot induction in numerous plants. Tuberization and rhizome induction using BAP have been reported by many researchers: Cucurma aromatica[46], Armoracia rusticana[47],Colocasia esculenta[48],Bambusa bambos[49]and Crocus sativus[50]. The effectiveness of BAP in inducing multiple shoots have been carried out in multiple number of medicinal plants[51-58]. Possibility of feedback inhibition by cytokinins at higher concentrations may be the reason for decrease in percentage response. Similar findings were also given by Kapoor and Rao (2007)[49].

Contrary to several reports, inhibitory nature of BAP in in vitro MR induction in Cucurma sp. and Dioscorea sp.,is also reported which shows that BAP effectcan vary from plant to plant[59-60]. Generally, cytokinins have been proven to promote cell division and involve in the development of storage organs such as tubers[61]. Increase accumulation of starch in growing tubers by cytokinin incorporation has been shown by Sarkaret al. (2006)[62]. Efficacy of cytokinin supplementation in tuberization and development of underground storage organs have been reported by Sharma and Singh (1995)[63], Suriet al. (1999)[64], Ghoshet al. (2007)[65].

Influence of sucrose concentration was also studied in the present study and it was found that sucrose has a profound effect on the MR induction in the excised rhizome. Coherent reports on high sucrose concentration in medium to enhance microtuber formation have been shown in many Dioscorea species[59,60,66]. Nayak (2000) have also reported that rhizome induction in Zingiberoffinalewas optimal with $60gl^{-1}$ sucrose addition. However, contradictory report on inhibitory effect of high sucrose augmentation in medium for tuberization in vitro was documented by Ovonoet al. (2007)[67] in Dioscoreacayanensis-Dioscorea rotunda complex and in D. fordii by Yan et al. (2011)[68]. As rhizome serves mainly to store starch and fibres availability of sucrose in increased amount may play a role in their development and formation of larger rhizomes[69]. Formation of storage or perennating organs by high sucrose incorporation has been shown[65,70-73]. Presence of sugar in the medium was responsible of a large part of the osmotic strength. Jo et al. (2009)[74] suggested that sucrose may be essential as an osmoticum, as an energy source and at higher concentration it may have a significant role as a signal formation of microtuber. Massive accumulation of starch with a change in the pathway of sucrose metabolism is a characteristic of tuberization and tuber growth[75]. During these phases of the tuber life cycles, the tuber is clearly a sink for carbohydrates and subsequent optimal response in higher sucrose concentration[76]. Studies on carbohydrate metabolism during tuber formation show that key enzymes (sucrose synthase, AGPase, and starch synthases) involved in starch biosynthesis indeed increase in activity[77]. Moreover, Geigenberger (2003)[78] suggested that sucrose synthase and AGPase enzymes are subjected to activation in response to sucrose and oxygen availability. Importance of sucrose and reduction in development of storage organs of the plant when carbohydrate availability is low has already been reported[79]. Significant inhibitory effects of lower (0-5%) and higher (11%) concentrations of sucrose on microrhizome production was reported in

Cucurma longa[46,60,80, 81].Shirgurkaret al. (2001)[60] also stated that a lower concentration of sucrose decreased the size and number of rhizomes or even completely prevented the induction of any MRs.

Figures And Tables

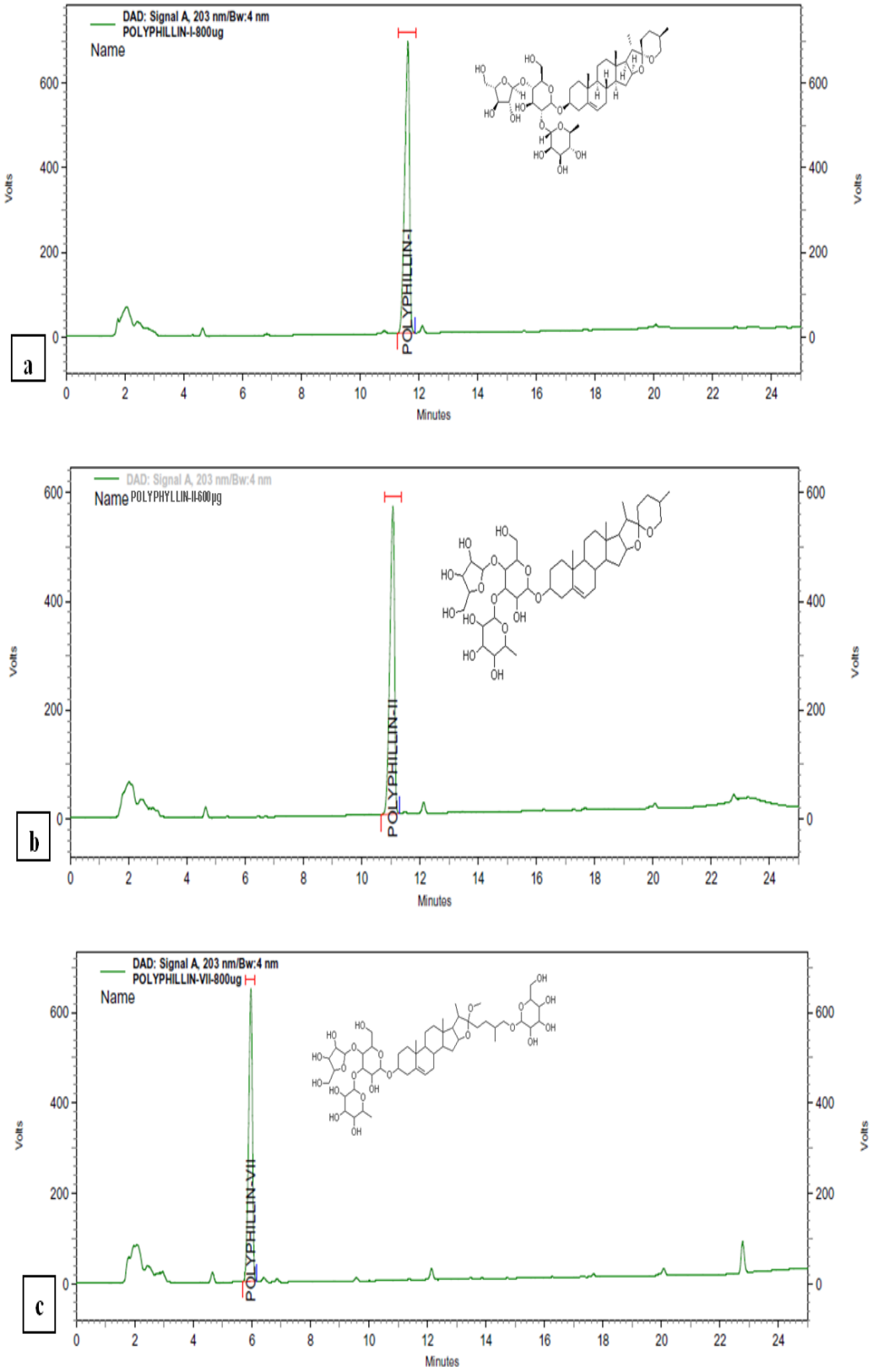

Fig. 1: Standard chromatograms. (A) Polyphyllin I, (B) Polyphyllin II, (C) Polyphyllin VII.

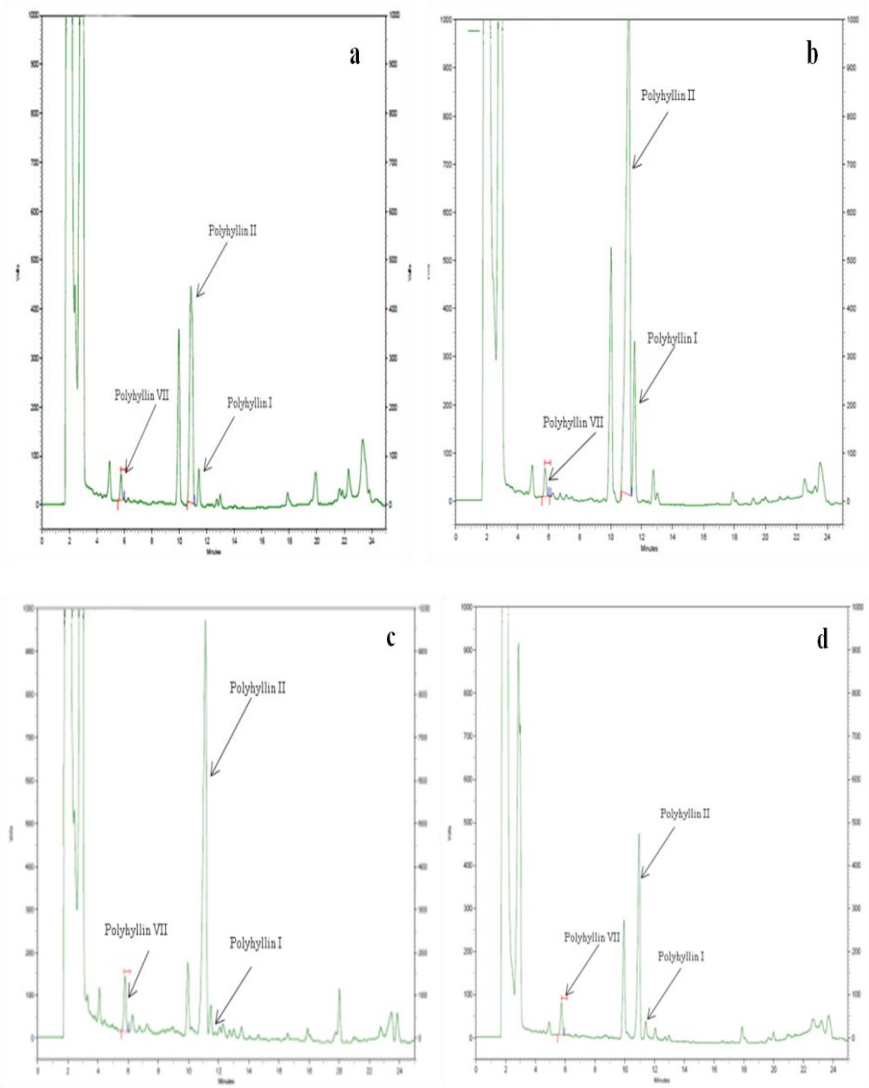

Fig. 2: population representative chromatograms. (A) Chromatogram of Nagaland population, (B) hromatogram of Manipur population, (C) Chromatogram of Meghalaya population, (D) Chromatogram of Arunachal population.

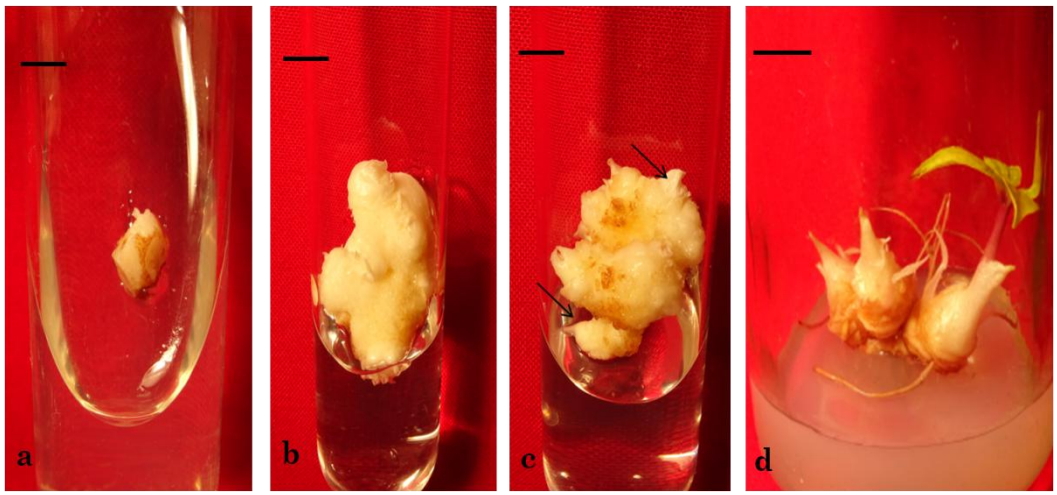

Fig. 3: Minirhizome induction from mature rhizome section using BAP+sucrose. (a) rhizome section, (b) growth of rhizome, (c) emergence of shoot buds, (d) shoot buds. *Arrow indicates shoot bud. Bar: a, b, c =1mm, d=1cm

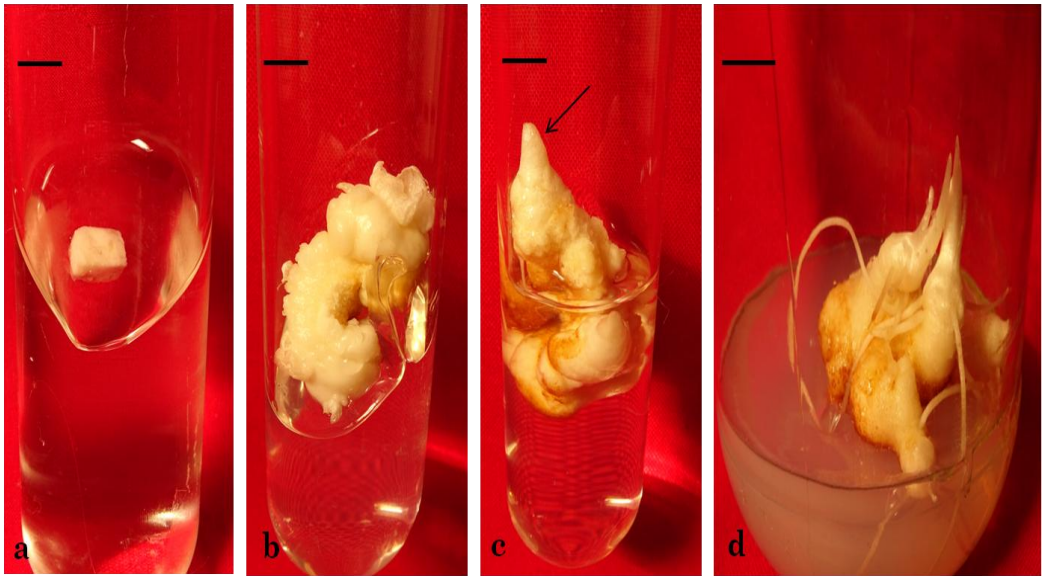

Fig. 4: Minihizome induction from mature rhizome sectionusing 2iP +sucrose (a) rhizome section, (b) growth of rhizome, (c) emergence of shoot buds, (d) shoot buds. *Arrow indicates shoot bud. Bar: a, b, c =1mm, d=1cm.

Fig. 5: (a) Minihizome derived regenerated plantlet showing well developed shoot and rhizome with roots, (b) Hardened plantlets (1 year old), (c) 3 months old hardened plants, (d) 2 year old plants. Bar: a=1cm, b=2.5 cm

State	Districts	No. of plants	Collection name	Latitude (N)	Longitude (E)	Altitude (m)
Nagaland	Tuensang	8	TN1 to TN8	26°13'20''	94°47'50''	1732
	Kohima	9	KH1 to KH9	25°39'29''	94°01'47''	1572
	Mokokchung	10	MK1 to MK10	26°15'52''	94°24'36''	1463
	Wokha	8	WK1 to WK8	26°06'52''	94°15'42''	1373
Manipur	Ukhrul	8	UK1 to UK8	24°48'50''	94°21'49''	1475
	Senapati	10	SE1 to SE10	25°28'57''	94°07'48''	1657
Meghalaya	East Khasi Hills	9	EH1 to EH9	25°31'47''	91°48'47''	1769
	West Khasi Hills	8	WH1 to WH8	25°33'41''	91°38'12''	1681
Arunachal Pradesh	Dibang valley	9	AR1 to AR9	28°47'51''	95°55'12''	1538

Table 1: Details of populations sampled across Northeast India

Table 2: Steroidal saponins estimation from rhizomes collected from different parts of Northeast India

Place	Collection name	Polyhyllin I (mg/g DW)	Polyphyllin II (mg/g DW)	Polyphylllin VII (mg/g DW)	Total steroidal saponins (mg/g DW)	Average
Tuensang	TN1	4.12	25.36	4.31	33.79	30.72
	TN2	4.31	14.76	6.08	25.15	
	TN3	4.60	23.27	8.70	36.57	
	TN4	2.24	11.65	9.10	22.99	
	TN5	3.43	17.56	7.45	28.44	
	TN6	2.86	27.81	6.73	37.40	
Khonoma	KH1	4.36	25.70	5.37	35.43	32.06
	KH2	4.86	19.66	5.16	29.68	
	KH3	2.05	16.13	5.54	23.72	
	KH4	5.50	22.97	3.58	32.05	
	KH5	**3.04**	**28.92**	**7.79**	**39.75**	
	KH6	4.71	21.44	5.62	31.77	
Mokokchung	MK1	5.73	22.14	2.40	30.27	23.81
	MK2	4.03	11.38	4.56	19.97	
	MK3	2.12	10.85	4.52	17.49	
	MK4	3.04	17.84	7.46	28.34	
	MK5	1.70	12.77	7.22	21.69	
	MK6	2.76	16.52	5.84	25.12	
Wokha	WK1	3.24	14.03	2.91	20.18	22.92
	WK2	3.51	15.06	3.16	21.73	
	WK3	2.33	16.69	2.88	21.90	
	WK4	1.98	21.41	2.98	26.37	
	WK5	4.32	18.09	3.74	26.15	
	WK6	2.86	16.04	2.34	21.24	
Ukhrul	UK1	4.18	19.76	2.27	26.21	30.76
	UK2	3.92	24.65	3.75	32.32	
	UK3	6.82	27.93	1.97	36.72	
	UK4	4.65	24.31	1.34	30.30	
	UK5	2.97	26.81	1.82	31.60	
	UK6	3.91	21.93	1.59	27.43	
Senapati	SE1	3.43	17.64	1.11	28.62	22.77
	SE2	1.86	18.21	2.16	22.23	
	SE3	2.20	11.28	1.97	15.45	
	SE4	3.50	21.08	4.04	22.18	
	SE5	3.07	13.82	2.74	19.63	
	SE6	4.02	21.39	3.14	28.55	
East Khasi Hills	EH1	2.18	17.63	4.36	24.17	27.91
	EH2	1.09	14.59	4.77	20.45	
	EH3	1.98	18.22	5.54	25.74	
	EH4	1.39	26.27	4.45	32.11	
	EH5	2.57	29.54	3.92	36.03	
	EH6	1.74	22.02	5.25	29.01	
West Khasi Hills	WH1	1.26	20.93	2.81	25.00	20.03
	WH2	1.41	10.49	2.03	13.93	
	WH3	2.81	14.21	4.02	21.04	
	WH4	1.93	14.83	3.04	19.80	
	WH5	1.91	13.32	3.55	18.78	
	WH6	3.12	14.11	4.42	21.65	
	AR1	5.85	21.26	3.81	30.92	
	AR2	5.25	14.32	1.08	20.65	

Arunachal Pradesh	AR3	3.65	15.06	1.34	20.05	23.00
	AR4	5.10	16.93	2.58	24.61	
	AR5	3.81	14.79	3.01	21.61	
	AR6	3.54	16.12	1.55	21.16	

Table 3: Effect of BAP and sucrose incorporated in MS medium on the growth and development of rhizome explants

Sucrose concn.	**BAP(mg/l)**	**Response %*#**	**FW (g)*#**	**No. of shoot buds**#**
Control	0.00	0.00	0.00	0.00
3%	0.25	84.7^{b}	0.96±0.05^{ab}	3.6±0.3^{abc}
	0.50	87.2^{ab}	1.12±0.03^{ab}	4.8±0.7^{ab}
	1.00	85.3^{b}	0.95±0.06^{ab}	4.4±0.3^{ab}
	1.50	81.8^{c}	0.92±0.04^{ab}	3.8±0.2^{abc}
	2.00	77.5^{d}	0.89±0.03^{bc}	3.0±0.5^{bc}
6%	0.25	83.9^{b}	0.98±0.06^{ab}	4.2±0.5^{ab}
	0.50	88.6^{a}	1.27±0.02^{a}	5.1±0.4^{a}
	1.00	85.4^{b}	0.96±0.08^{ab}	4.7±0.3^{ab}
	1.50	82.7^{bc}	0.93±0.06^{ab}	4.2±0.2^{ab}
	2.00	79.8^{c}	0.86±0.10^{c}	3.7±0.5^{abc}
9%	0.25	58.7^{e}	0.45±0.04^{d}	2.8±0.5^{bc}
	0.50	53.4^{e}	0.32±0.08^{de}	2.3±0.4^{abc}
	1.00	48.3^{ef}	0.28±0.05^{ef}	2.1±0.8^{bcd}
	1.50	44.9^{f}	0.23±0.03^{f}	2.0±0.6^{cde}
	2.00	38.6^{f}	0.20±0.04^{f}	1.8±0.6^{e}

Table 4: Effect of 2iP and sucrose incorporated in MS medium on the growth and development of rhizome explants

Sucrose concn.	**2iP (mg/l)**	**Response %*#**	**FW (g)*#**	**No. of shoot buds**#**
Control	0.00	0.00	0.00	0.00
3%	0.25	80.6^{abc}	0.94±0.06^{bc}	3.5±0.05^{b}
	0.50	84.2^{abc}	0.95±0.08^{abc}	3.9±0.4^{ab}
	1.00	85.3^{abc}	1.07±0.05^{ab}	4.6±0.3^{ab}
	1.50	83.3^{abc}	0.98±0.05^{abc}	4.0±0.3^{ab}
	2.00	79.6^{bc}	0.92±0.03^{bc}	3.6±0.6^{b}
6%	0.25	83.5^{abc}	1.02±0.07^{ab}	4.1±0.4^{ab}
	0.50	86.2^{ab}	0.98±0.06^{abc}	4.7±0.2^{ab}
	1.00	89.2^{a}	1.36±0.10^{a}	5.3±0.3^{a}
	1.50	84.8^{abc}	0.90±0.04^{bc}	4.6±0.5^{ab}
	2.00	80.4^{ab}	0.85±0.02^{de}	4.0±0.4^{ab}
9%	0.25	42.9^{d}	0.30±0.03^{de}	2.1±0.5^{bc}
	0.50	48.1^{de}	0.38±0.03^{d}	2.5±0.6^{cd}
	1.00	38.5^{ef}	0.32±0.07^{de}	2.0±0.5^{cde}
	1.50	32.6^{f}	0.25±0.08^{ef}	1.8±0.3^{de}
	2.00	30.5^{f}	0.20±0.10^{ef}	1.6±0.4^{de}

*Data recorded after 6 months; **Data recorded after 5 months of transfer to PGR free medium. #Different letters within a column indicate significant differences at P = 0.05 by Tukey HSD test

V. Conclusion

The present study showed the presence of variability in the steroidal saponins content in P. polyphylla collected from different geographical regions. The preliminary identification and the present findings can be taken up for further analyses to understand the biochemical pathway governing the production of the desired phytochemical. In addition, application of cutting edge technologies like next generation sequencing and trancsriptome analysis will immensely assist in genotype identification and identification of the key genes or enzymes responsible for the steroidal saponins production. These studies can be considered for future prospects.In addition, the micropropagation protocol described here can be applied for scale up for production of plants within limited time and space. This will definitely help in bringing about a balance in the supply demand statistics and ultimately aid in conservation and management of plant resources in its natural populations.

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