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ULTRASTRUCTURE, CALLUS INDUCTION AND MICROPROPAGATION OF *PHYLA NODIFLORA* (L.) GREENE

DALILAH ABU BAKAR[1,2*], RUBASHINY VEERAMOHAN[3], ABDUL BAKRUDEEN ALI AHMED[4], NOORMA WATI HARON[1] AND ROSNA MAT-TAHA[1]

[1] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Advanced Biotechnology and Breeding Centre, Malaysian Palm Oil Board, 43000 Kajang, Selangor, Malaysia
[3]Institute of Systems Biology (INBIOSIS), Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[4]Department of Biochemistry, Centre for Research and Development (CRD), PRIST Deemed University, Vallam, India
[*]Corresponding author's email: dalilah.ab@mpob.gov.my

Abstract

The present paper reports the ultrastructure and a protocol for the efficient *in vitro* callus induction and micropropagation on *Phyla nodiflora* (L.) Greene. Foliar micromorphology study of *Phyla nodiflora* was investigated by JEOL-7500F Field Emission Scanning Electron Microscope (FESEM). Diacytic stomata are abundant on both the adaxial and abaxial surfaces. Trichomes are simple unicellular and densely distributed. Callus induction and shoot regenerations were achieved through culturing leaf and stem explants after disinfecting with 70% ethanol 3 minutes, 0.1% $HgCl_2$ for 3 minutes and 20% sodium hypochlorite for 2 minutes on Murashige and Skoog (MS) medium (pH5.8) supplemented with single and combinations of auxins and cytokinins, which are naphthaleneacetic acid (NAA), 2,4-dicholorophenoxyacetic acid (2,4D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and benzyladenine (BA), kinetin (KN). Maximum 92.5% callus response on single plant growth regulators (PGRs) is 2.5 mg/L NAA (118.6 mg/L) with light green friable callus, 90.0% response from 2.0 mg/L BA (42.7 mg/L) with green compact callus and 89.2% response from combination 3.0 mg/L NAA: 2.0 mg/L KN (165.0 mg/L) with green compact and friable callus and 2.5 mg/L IBA: 1.0 mg/L BA (139.6 mg/L) with green compact callus. Highest shoot regenerations response was from MS media without plant growth regulators (90%) while shoot regenerations from MS media supplemented with combination IAA and BA gave highest multiple shoots with 3 shoots per explants. Mass propagation of callus and plantlets protocol of *Phyla nodiflora* can assist for future metabolic research.

Key words: *Phyla nodiflora*, Verbenaceae, *In vitro*, Micropropagation, Micromorphology, FESEM.

Introduction

Phyla nodiflora (L.) Greene (also known as *Lippia nodiflora* (L.) Mihex), is a small creeping perennial herb which belongs to the family Verbenaceae and the distribution is reported worldwide, found close to rivers and it has pan- tropical native distribution including human- mediated dispersal (Mako *et al*., 2006; Siddiqui *et al*., 2007; Terblanche *et al*., 1996; Gross *et al.*, 2017). It has shown to possess high medicinal value involving diuretic potential, antidiabetic (Shukla *et al*., 2009; Balamurugan *et al*., 2011), anti-hepatotoxic (Sudha *et al*., 2013), and antimelogenesis (Ko *et al*., 2014). Ravikanth *et al*., (2002) has verified the presence of two significant bioactive compounds, halleridone and hallerone, which are anti-cancer, anti-tumor, anti-malarial and antifungal. Shukla *et al*., (2009), reported the presence of carbohydrates, flavonoids, phenolics, sterols and essential oil in *Phyla nodiflora*.

Ahmed *et al.,* (2011a) discovered embryogenic callus induced using MS media supplemented with 2, 4-D and ascorbic acid. However, previous studies of tissue culture on callus induction on media supplemented with PGRs reported the browning of callus by Ahmed *et al*., (2011b), brown friable callus was observed in the callus induction of *P. nodiflora.* Gupta *et al*., (2001) recorded a dark-brown calli after two to three weeks of initiation on *Lippia alba* (Verbenaceae) while Jibina Bai *et al*., (2014) reported *Lippia alba* to induce brown friable callus and profuse callusing after 9 days of inoculation.

Morphologically *Phyla canescens* was found to have almost similar characteristics to *P. nodiflora,* however it can be distinguished by its canescent stems and leaves, and blunt short teeth on its leaf tips (Munir, 1993). Taxonomic status of both species must be interpreted in the current order of taxonomic classification as there may be an overlap in the morphology and habitat of these two species (Leigh & Walton, 2004). Mashile & Tshisikhame (2017) mentioned the importance of separation and linkage in higher level of taxonomic hierarchy provided through leaf epidermal features in some cases, and its properties could be associated in functional aspects of leaf venation. Furthermore, additional taxonomic data based on its foliar micromorphology can improve the identification of these two species.

Based on the research discussed above, there are lack of taxonomic reports on *P. nodiflora* in Malaysia. Therefore, this study aims to carry out taxonomic study particularly on the micromorphology to add more taxonomic information to the species. Also, identification of compounds through screening of metabolites could greatly improve pharmaceutical industry. To conduct this study, induction of callus and plantlet on *P. nodiflora* was investigated under sterile conditions. Hence, the objectives of this study are: (i) to study the micromorphology of *P. nodiflora* particularly the stomata and trichomes, (ii) to develop callus from leaf and stem explants of *P. nodiflora,* (iii) to optimize the callus growth using biomass in *P. nodiflora*, and (iv) to mass propagate *P. nodiflora* from tissue culture to produce whole new plants.

Materials and Methods

Plant materials and identification: Healthy, young leaf and stem explants of *P. nodiflora* were collected from Taman Rimba Ampang, Selangor, Malaysia (Latitudes 3° 8' 57", longitudes 101° 47' 28"), and maintained at the Institute of Biological Sciences (IBS) Garden, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Herbarium specimen was also prepared (KLU47924) and kept at the University of Malaya herbarium (KLU). The plants were maintained in 10 pots at Institute Biological Sciences Garden, Faculty of Science, University of Malaya for tissue culture work.

Ultrastructure study of *Phyla nodiflora:* Fresh leaves of *P. nodiflora*, 4-6 mm in length, were fixed in 8% glutaraldehyde with Sorensen's Phosphate Buffer solution (1:1) for one hour and rinsed using distilled water: Sorensen's Phosphate Buffer solution (1:1). Samples were soaked in 4% osmium: distilled water (1:3) for 14 hours in low temperature (5°C) and for one hour in room temperature inside the fume chamber. *P. nodiflora* leaves were dehydrated for 15 minutes in every three rinse of ascending series of ethanol (20–100%). This was followed by dehydration of samples in three times degradation of 100% ethanol: 100% acetone for 20 minutes with ratio of 3:1, 1:1 and 1:3 and four times degrade in 100% acetone for 20 minutes. Then, samples were placed for critical point drying with liquid carbon dioxide (CO_2) (Autosampler 810 critical point dryer) and sputter-coating with gold-palladium (LEICA EM SCD005 sputter coater). Observation was done on both adaxial and abaxial surfaces using a JEOL- 7500F Field Emission Scanning Electron Microscope. Structures such as stomata, trichomes and oil gland were observed and analyzed.

Sterilization method: Leaf and stem explants of *P. nodiflora* were thoroughly washed in running tap water for 30 minutes, then rinsed with sterile distilled water added with 2% (v/v) Teepol (Labchem, Malaysia) for 10 minutes. Explants were further washed with 70% ethanol for three minutes, followed by 20% sodium hypochlorite (Clorox, Malaysia) for three minutes and lastly washed with 0.1% mercury (II) chloride ($HgCl_2$) (Sigma-Aldrich, Germany) for two minutes. Explants were washed three times with sterile distilled water prior to inoculation.

Callus induction of *Phyla nodiflora:* *P. nodiflora* was inoculated in aseptic conditions to obtain callus induction. The leaf and stem explants were inoculated in MS media (Murashige & Skoog, 1962) supplemented with PGRs such as auxin, naphthaleneacetic acid (NAA), 2, 4- dicholorophenoxyacetic acid (2, 4-D), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA), also cytokinin of benzyladenine (BA) and kinetin (KN) which will be tested in many concentrations (0.5-4.0 mg/L). The leaf and stem explants were faced the media abaxially after injuring the surface using scalpel by slightly cutting it to five to six cuts. Maintenance of cultures at 25±2°C, 16/8-hour (light/dark) photoperiod and low light intensity. Optimization and evaluation of callus were done qualitatively and quantitatively based on their nature and biomass (Ahmed *et al.,* 2011b). Treatments of various concentrations of auxin, cytokinin and combination of auxin and cytokinin were observed based on their fresh and dry weights in seven days (one week) interval thus, harvest of callus at 7, 14, 21 and 28 days after inoculation. At every selected duration, three replicates of callus were separated carefully from media using forcep and scalpel, fresh and dry weight were recorded accordingly.

***Phyla nodiflora* shoot regenerations:** *P. nodiflora* was inoculated in aseptic conditions to regenerate the shoots. The leaf and stem explants were inoculated vertically in MS media (Murashige & Skoog, 1962) supplemented with PGRs such as auxin, NAA, 2, 4-D, IBA and IAA, also BA and KN of cytokinin with its combinations which will be tested in many concentration (1.0-3.0 mg/L). The regenerated shoots were subcultured to the fresh MS media supplemented with similar PGRs for further shoot development. Maintenance of cultures at 25±2°C, 16/8-hour (light/dark) photoperiod and low light intensity. Number and length of shoots were recorded and tabulated.

Root induction and acclimatization: The minimum length of developed shoots were about 3.0 cm, the shoots were excised and transferred into rooting media (MS media + 1.0- 3.0 mg/L IAA and IBA). Observation for the numbers of roots as per shoot was recorded and tabulated after 20 days of inoculation. Complete plantlets were successfully obtained. For acclimatization, the roots were washed in running tap water to remove the media. The plantlets were transplanted in Plantaflor® (Young Plant Substrate, 50% white peat + 50% black peat + 1.0 kg NPK fertilizer, including trace elements) soil, that was placed in small plastic pots enclosed with a transparent and punctured plastic. After four weeks, the plantlets were introduced into greenhouse and transferred into the field.

Data analysis

Analysis of variance (ANOVA) was performed to test if there were significant differences in callus induction, plant regeneration and root induction among the samples tested on MS media supplemented with single and combination PGRs, using Minitab statistical computer software v.17 (Minitab Inc., Pennsylvania, USA). Comparison procedures assuming equal variances were analyzed using Tukey's Honest Significant Difference (HSD) Tukey's test at a confidence level of 95% ($p \leq 0.05$).

Results and Discussion

Ultrastructure of *Phyla nodiflora:* The ultrastructure

of the leaf surface of *P. nodiflora* is shown in Fig. 1(A-J). *P. nodiflora* showed epidermal cells with convex shape and covered with cuticular folds. According to Prüm *et al.*, (2012), epicuticular wax crystals or cuticular foldings that were observed on the plant surface may influence the insect attachment based on the shape of cell, similar to traction forces on surfaces of convex cells. *P. nodiflora* leaves are amphistomatous where the stomata are present on adaxial and abaxial sides of leaf surfaces and diacytic stomata were observed abundance on both surfaces (Figs. 1A and 1B). The amphistomatous characteristics might be an adaptive feature of the plant to the environmental conditions of tropical and subtropical countries. The function of having abundant distribution of trichomes on the surface of the leaves is as a mechanism of defense against foraging insects and airborne fungal propagules (Pendota, *et al*., 2008) and to prevent water loss by trapping air over the stomata especially on the abaxial surface (Naidoo *et al.*, 2009). The leaf of *P. nodiflora* possess simple, unicellular trichomes which were densely distributed but more on the abaxial compared to the adaxial surface (Figs. 1E and 1F). There were also more trichomes on the midrib of abaxial surface compared to the edge of the leaf. Trichomes development on the epidermis usually results from a degree of extension and consequent divisions of the epidermal cells and their derivatives (Ashafa *et al.*, 2008). The correlation between density of trichomes towards insect resistance were positively expressed in diverse plant species (Valverde *et al.*, 2001). The trichomes (Fig. 1G) also, varied in lengths (175.4µm, 222.6µm and 376.7µm, not shown in figures) and its centre was connected to the epidermis (Fig. 1H). Shukla *et al*., (2009) extracted essential oil from *P. nodiflora*, therefore oil gland was spotted throughout the leaf surfaces (Fig. 1I). Both adaxial and abaxial surfaces showed different oil gland characteristics where the glands observed on the adaxial surface had smooth exterior (Fig. 1I), while shrivel exterior with an opening was observed on abaxial surface (Fig. 1J).

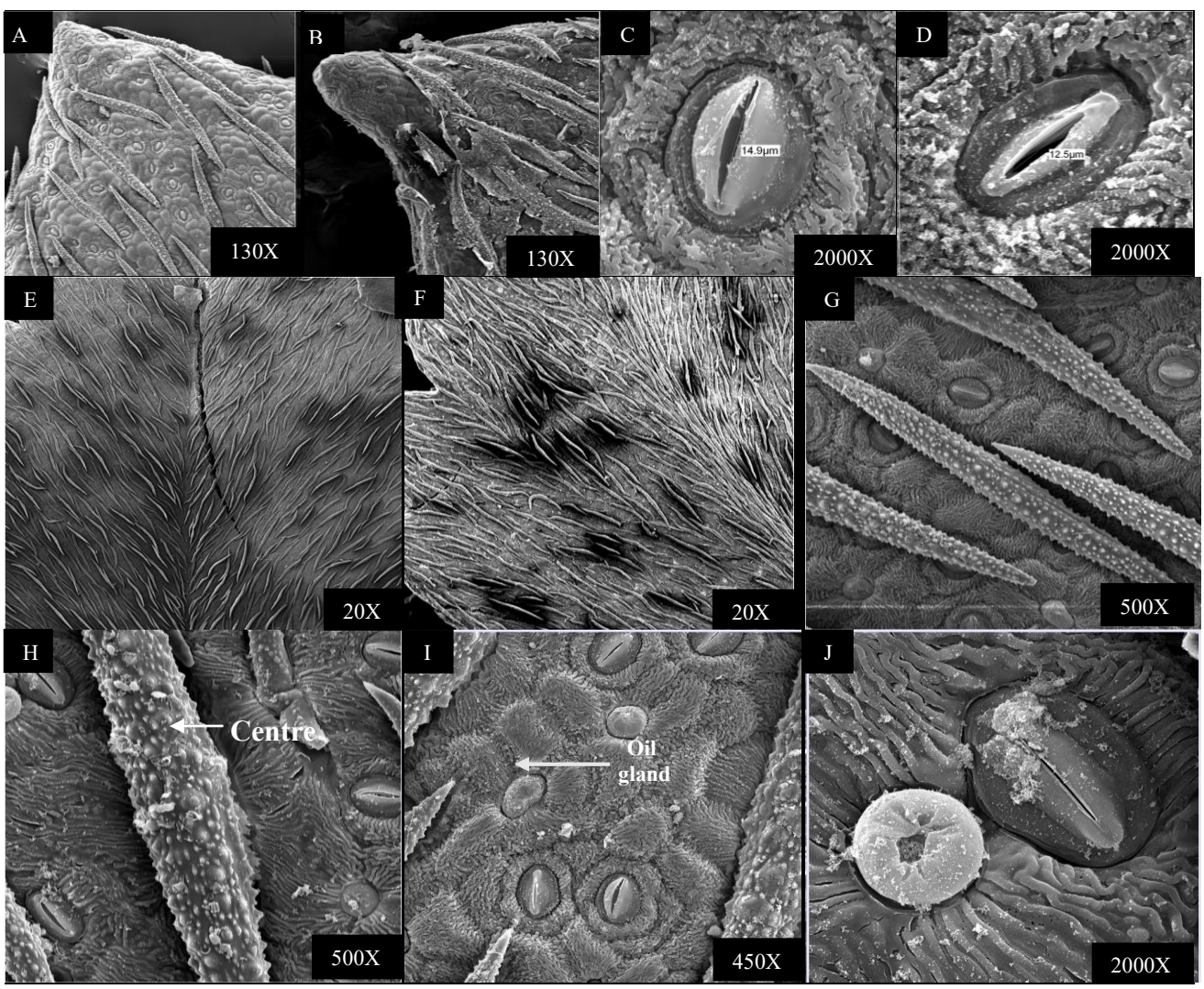

Fig. 1. Stomata distribution in *P. nodiflora* (A) Adaxial surface (B) Abaxial surface (C, D) Stomata on adaxial and abaxial surfaces, respectively. Simple, unicellular trichomes (E) Adaxial surface (F) Abaxial surface (G) Simple, unicellular trichomes and stomata (H) Trichomes showing the middle part attached to the epidermal surface. The oil gland on epidermis (I) Oil gland structure on adaxial surface (J) Oil gland on abaxial surface.

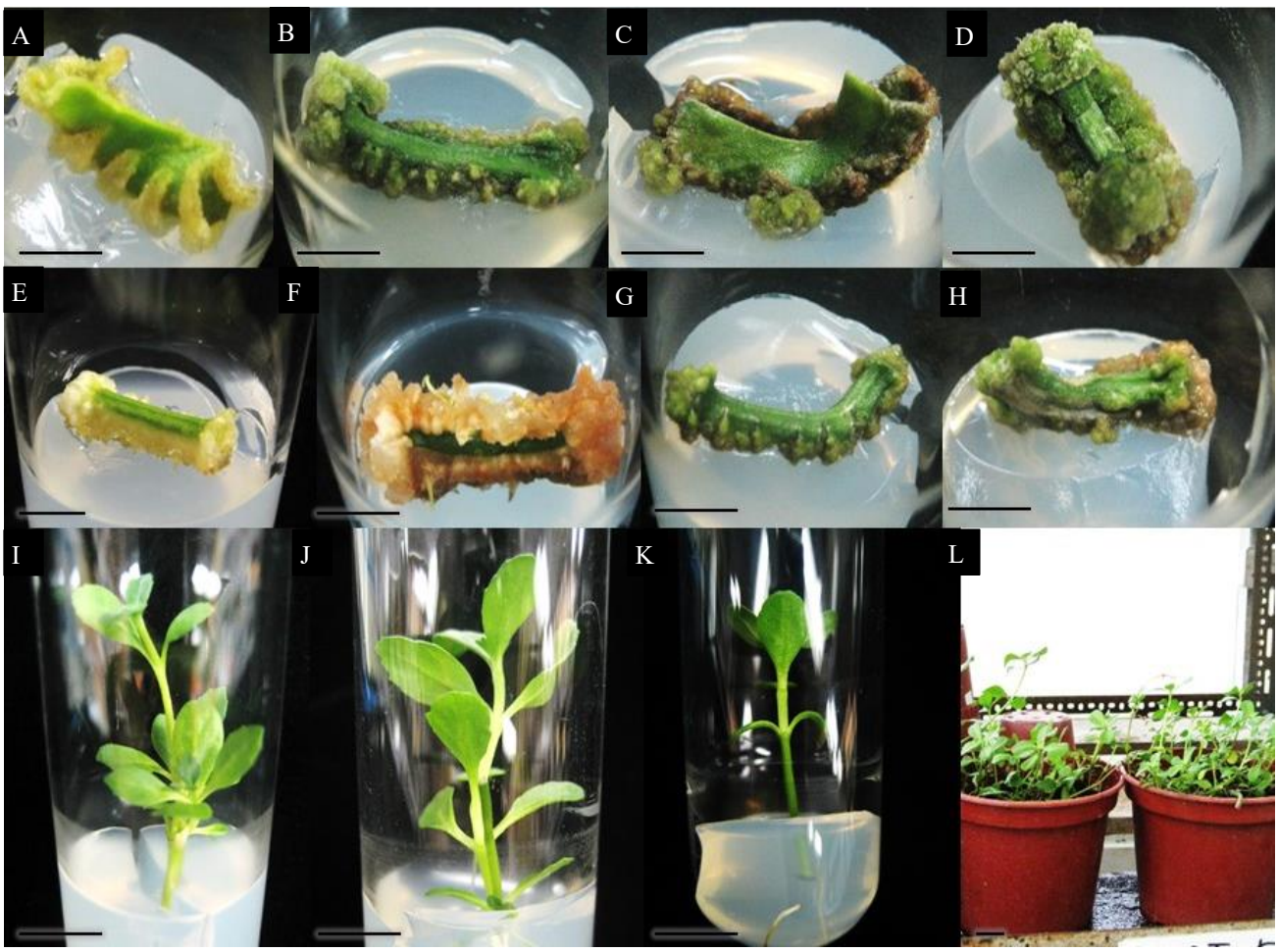

Fig. 2. The nature of callus obtained induced from *P. nodiflora* on MS media supplemented with different PGRs (A) Light green compact callus - 2.0 mg/L NAA (B) Green compact callus - 3.0 mg/L IAA (C, D) Leaf and stem of 2.5 mg/L IBA+1.0 mg/L BA, respectively. (E, F) Browning of callus on single PGRs in MS media supplemented with 3.0 mg/L NAA on the first week and 3rd week respectively (G, H) Browning of callus on single PGRs in MS media supplemented with 2.0 mg/L IAA+ 2.0 mg/L KN on the 2nd week and 5th week respectively (I) Shoot regeneration of stem on control (J) Shoot regeneration of stem on MS media supplemented with 2.0 mg/L KN (K) Roots formation on regenerated shoots of *P. nodiflora* (L) *P. nodiflora* plant has propagate and increased in number after 30 days acclimatization. Bar represent 1 cm for (A-K) and 2 cm for (L).

Callus induction of *Phyla nodiflora:* *P. nodiflora* plant was tested with different auxin and cytokinin concentrations and combinations. Saleh Alansi *et al*., (2018) reported the importance of balanced auxin and cytokinin supplemented in media resulted in induction of callus. In the study, the obtained calluses were mostly green and compact, followed by light green compact callus and some were white colored. The nature of callus varied on the media supplemented with PGRs, NAA and 2,4-D while others were mainly green compact callus (Fig. 2 A-H). A variety of callus can be seen on single PGRs, NAA and 2,4-D (Fig. 2A-B) while other than these two PGRs, IAA, IBA, BA, KN, and their combinations all developed green compact callus. The texture of the callus was rough and hard as could be seen in Fig. 2C and D. Callus, can mostly be classified into two categories which are friable and compact. The cells of compact callus are densely aggregated and hard, whereas the cells of friable callus are loosely associated with each other and the calluses are soft and can be easily broken apart. The friability of a species' callus can sometimes be improved by manipulating the media components or by repetitive subculture (Slater, 2003). Gürel *et al*., (2001) mentioned that regardless of the media containing auxins, with the presence of single high BA concentrations (1.0 mg/L) or in combination with auxins produced green and compact callus for various beets explants. In this study, starting from combinations IAA and IBA for auxin, BA and KN for cytokinin, to any combination of auxin and cytokinin, exhibits green compact callus. However, previous studies suggested that there are organogenic potential depending on structure of callus. Large cells of white and friable callus may generate roots and shoots (Konwar & Coutts, 1990; Saunders & Daub, 1984; Shimamoto *et al*., 1993), while small cells of green and compact callus do not possess organogenic capacity (Ritchie *et al*., 1989; Tetu *et al*., 1987).

Based on Table 1, the two explants (leaf and stem), both showed high similarity percentage of overall response, but data recorded that stem explants obtained higher percentage of induction. Similar to Ahmed *et al*.,

(2011a), stem explants were able to obtain higher frequency of callus induction compared to shoot tips explants by 92% to 83% respectively. The highest callus response was recorded from PGRs, IBA in leaf explants and BA in stem explants (84.6% and 88.3% respectively). There was no induction of callus in MS media without PGRs (control). The highest dry weight of callus was recorded on MS media supplemented with 3.0 mg/L NAA + 2.0 mg/L KN (165.0 mg/L), followed by 2.5 mg/L IBA + 1.0 mg/L BA (139.6 mg/L). There was large gap of dry weight between single and combination PGRs. Therefore, mass production of callus is suitable to perform using MS media supplemented with 3.0 mg/L NAA + 2.0 mg/L KN for further phytochemical study.

Oxidation of polyphenols and formation of quinones results in browning of explant's tissue and tissue culture media are highly reactive and toxic to the plant tissues. The inhibitory effect may be due to the bonding of phenols with protein and their subsequent oxidations to the quinones (Jain, 2000; Raj Bhansali & Singh, 1982). In *P. nodiflora* callus, browning effect can occur as fast as the second week to some friable callus, Fig. 2E and F shows the third week of callus induction on MS media with supplemented 2.0 mg/L NAA. However, minor browning of callus occurred on green compact callus of MS media supplemented with 2.0 mg/L IAA + 2.0 mg/L KN only at third week. Previous research was done to minimize browning effect such as pre-soaking explants in ascorbic acid and citric acid prior to the sterilization method (Murashige, 1974). In recent studies of Naz & Khatoon (2014), overcame the problem of callus browning in *Achyranthes aspera*, the callus induction was inoculated in MS media supplemented with 2.0 mg/L 2,4-D and 0.2% charcoal and also higher sucrose level (>3%). The callus browning still occurred and reduction in callus fresh weight was recorded. In the present study, the browning effect that had occurred during the callus induction on *P. nodiflora* was as fast as second week onwards for single PGRs and third week onwards for combination PGRs. Stem explants could be seen on MS media supplemented with 2.0 mg/L NAA on week one (Fig. 2E), the nature of the callus was light green and friable. However, approaching second to third week, the callus started to turn brown (Fig. 2F). The result of auxin and cytokinin combination was investigated to enhance biomass but additionally, was found to decrease the browning effect that was occurring throughout the experiments on single PGRs especially on NAA and 2,4-D. At the declining stage of callus growth curve, the callus induced on 2.0 mg/L NAA had started to decline significantly at week four while callus from 2.0 mg/L IAA + 2.0 mg/L KN declined slowly from week five onwards when it was not subcultured. This investigation could further support the mass production of callus to be supplemented using combination PGRs.

Table 1. *Phyla nodiflora* callus nature and explants percentage of induction (%) for individual and combination plant growth regulators (PGRs).

Plant growth regulators (PGRs)	Nature	Percentage of induction (%)	
		Leaf	Stem
Individual			
NAA	Light green friable callus Light green compact callus Whitish greenish compact callus	84.4 ± 1.56[a]	86.4 ± 0.4[a]
2,4-D	Light green friable callus Light green compact callus Whitish greenish compact callus	84.4 ± 0.54[a]	87.1 ± 0.47[a]
IAA	Green compact callus	83.9 ± 0.84[a]	86.6 ± 0.84[a]
IBA		84.6 ± 0.97[a]	85.4 ± 0.91[a]
BA		83.4 ± 0.83[a]	88.3 ± 0.83[a]
KN		81.7 ± 0.49[a]	84.7 ± 0.27[a]
Combination			
NAA + BA	Green compact callus	78.4 ± 1.15[a]	86.3 ± 1.32[a]
NAA + KN		83.1 ± 1.12[a]	86.1 ± 1.39[a]
2,4-D + BA		80.2 ± 1.53[a]	83.9 ± 1.74[a]
2,4-D + KN		80.7 ± 1.60[a]	82.8 ± 1.42[a]
IAA + BA		79.3 ± 0.60[a]	86.8 ± 1.21[a]
IAA + KN		81.8 ± 0.76[a]	85.7 ± 1.21[a]
IBA + BA		80.8 ± 1.63[a]	87.5 ± 0.58[a]
IBA + KN		78.9 ± 0.19[a]	84.7 ± 1.51[a]

Means ± SE indicated with the same letter were not significantly different (HSD Tukey's test: $p<0.05$)

Table 2. Shoot regeneration of stem explants of *Phyla nodiflora*.

Plant growth regulators (PGRs)						Response (%)	No. of shoots	Length of shoots (cm)
NAA	2,4D	IAA	IBA	BA	KN			
-	-	-	-	-	-	90 ± 2.89^{a}	1.0 ± 0.00^{d}	6.4 ± 0.45^{a}
√						18 ± 3.63^{ef}	1.2 ± 0.13^{cd}	0.5 ± 0.08^{e}
	√					8 ± 3.00^{f}	1.1 ± 0.10^{d}	0.4 ± 0.04^{e}
		√				38 ± 4.33^{cde}	1.7 ± 0.20^{abcd}	2.9 ± 0.26^{d}
			√			33 ± 4.33^{def}	1.6 ± 0.15^{abcd}	3.3 ± 0.15^{cd}
				1		63 ± 6.82^{abc}	2.0 ± 0.20^{abcd}	2.6 ± 0.22^{d}
				2		73 ± 4.64^{ab}	2.3 ± 0.38^{abc}	3.3 ± 0.35^{cd}
				3		73 ± 6.29^{ab}	1.9 ± 0.26^{abcd}	4.6 ± 0.19^{b}
					1	60 ± 1.44^{bcd}	1.4 ± 0.15^{bcd}	4.3 ± 0.19^{bc}
					2	62 ± 0.83^{abc}	1.8 ± 0.19^{abcd}	2.8 ± 0.33^{d}
					3	68 ± 3.00^{ab}	1.9 ± 0.26^{abcd}	2.9 ± 0.35^{d}
		√		√		78 ± 0.83^{ab}	2.7 ± 0.14^{a}	3.5 ± 0.13^{bcd}
		√			√	71 ± 4.41^{ab}	2.0 ± 0.24^{abcd}	3.7 ± 0.15^{bcd}
			√	√		79 ± 2.20^{ab}	2.5 ± 0.29^{ab}	3.6 ± 0.32^{bcd}
			√		√	76 ± 2.20^{ab}	2.1 ± 0.26^{abcd}	3.3 ± 0.11^{cd}

Means ± SE indicated with the same letter were not significantly different (HSD Tukey's test: $p<0.05$)

***Phyla nodiflora* shoot regenerations:** Fig. 2I and J showed shoot regeneration on stem explants that was inoculated vertically on MS media supplemented with PGRs and a control. Every stem explants were ensured with the presence of node. MS media without PGRs (MSO) was used as control and explants that were inoculated on them showed successful regeneration (Figs. 2I and J) with the highest percentage (90%) compared to all the other treatments (Table 2), contrasting from Saleh Alansi *et al.*, (2018) and Muhammad Imtiaz *et al.*, (2019) that were not able to obtain any response from MS media that do not contain any PGRs. In spite of this, explants that were inoculated on MS media supplemented with auxin (NAA and 2,4-D) had induced more callus than the initiating shoot. Successfully regenerated plant was stunted at the induction of callus and resulted in short length shoots on the plant. PGRs IAA and IBA on the other hand, gave positive response of plant regenerations including a few roots along with minimal shoots and possessed high plantlet length. Cytokinins, BA and KN were more responsive than the auxins from each of the concentrations tested; more than one over four responded, unlike 2,4-D, where minimal response from one batch was about one over eight. Explants inoculated in MSO had given high response to shoots length where it might be suitable for *in vitro* shoot regeneration. However, explants inoculated in 2.0 mg/L KN gave the highest shoot number with five shoots but it was not consistent where average of 1.8 shoots was recorded in Table 2. In average, the PGR combination IAA + BA gave highest shoot number of 2.7 with mostly three to five shoots per explant with a minimum of one shoot per explant. Shoot regeneration with a combination of PGRs was successful, however, the length of the shoots was only about 3 to 5 cm long. This might be due to the interruption of callus proliferation. Callus induction and proliferation could inhibit or slow down the growth of the regenerated shoots. Nevertheless, shoots generated from callus induction explants proved that favorable condition of the plant might be able to regenerate if the parts of the nodes were intact. There was no shoot regeneration from leaf explants, while only callus was initiated on the explants.

Ahmed *et al*., (2005) reported that the axillary nodal explants used inoculated in MS media supplemented with cytokinins promoted shoot buds initiation and BA (2.5 mg/L) was proven to be most efficient with 6 cm length and high number of shoots with mean of 12.5 shoots per node. In this study, *P. nodiflora* nodes gave good response too with a PGR combination of 2.5 mg/L BA + 0.5 mg/L IBA with mean 8.7 shoots per node (not shown). As a comparison to this study, two to three out of ten regenerated shoots had one responsive shoot while the highest number of shoots obtained was five. This however, may result in low mean number in total as shown on Table 2. Priya & Ravindhran (2011) also successfully micropropagated *P. nodiflora* and found that the most efficient media was MS media supplemented with 3.0 mg/L BA (mean 10.50 shoots per node) and shoot multiplication induced better with PGRs BA than KN which produced less shoots.

Root induction and acclimatization: Regenerated plantlets that were inoculated in MSO were able to induce roots aside from shoot regeneration. Shoots regenerated from single and combinations of auxin and cytokinin exhibited different lengths (3-8 cm); they were excised and transferred to MS media supplemented with 2,4-D, IAA, IBA and NAA using different concentrations (1.0, 2.0 and 3.0 mg/L) as root induction was observed and recorded. Table 3 showed that MS media supplemented with 3.0 mg/L IAA had most responsive number of roots (mean 4.0±0.33) with a maximum of eight roots per plantlet and the distribution of all three concentrations were similar. No roots were observed on MS media supplemented with 2,4-D and NAA (concentration 1.0, 2.0 and 3.0 mg/L respectively for all PGRs tested) but

callus formed at the end of the plantlet of the excised surface. Roots of *P. nodiflora* were present on most of its nodes with at least one long root or two short roots (Fig. 2K) and can go up to 5 to 8 roots per node, however, an established root in *in vivo* plants possess a cluster of roots in interval of several nodes. Roots observed on regenerated plantlets inoculated in MSO were mostly with one to two long roots. The development of few roots however, was adequate for the plantlet to survive in soil. *P. nodiflora* was mentioned as a creeping plant (Sharma & Singh, 2013) where developing roots on its nodes is essential to provide them good nutrient intake to its elongating stems and leaves. A series of experiments with several hardening process and acclimatization had been successful with 3 cm plantlets. Growth development and size of *P. nodiflora* depends on the fertility of the soil. It can appear to be ground covering (short length) or creeping herbs with 7 to 10 cm height.

Table 3. Observation of root induction on *Phyla nodiflora* regenerated shoot.

Plant growth regulators (PGRs) concentration (mg/L)	Number of roots
IAA	
1.0	3.1 ± 0.18^{b}
2.0	3.3 ± 0.15^{ab}
3.0	4.0 ± 0.33^{a}
IBA	
1.0	3.0 ± 0.21^{b}
2.0	3.3 ± 0.21^{ab}
3.0	3.7 ± 0.26^{ab}

Values are mean of 10 replicates per treatment and repeated thrice. Means ± SE indicated with the same letter were not significantly different (HSD Tukey's test: $p<0.05$)

Microenvironment can be defined as an environment, supplied with media containing sugars and nutrients within culture vessels assisted with low light intensity, aseptic conditions and sufficient humidity to allow heterotropic growth. Exposure of unique microenvironment to *In vitro* plantlets provide them minimum stress and optimal conditions for plant proliferation and development. This can promote survival of the plantlets in the natural environmental conditions, introduced in the greenhouse then further developed in the field (Hazarika, 2003). So, acclimatization would be an important process in tissue culture to maintain the condition of the plantlets. Complete *P. nodiflora* plantlets that were obtained from root induction and control cultures, had gone through hardening process in culture room (25±2°C, 16/8-hour). Then, acclimatization was done for successfully regenerated plantlets through the microenvironment to IBS greenhouse and garden where 90% of the plantlets showed positive response. *P. nodiflora* elongated and propagated in the small pot and then transferred into a longer square pot (24" x 6") on the 30th day of acclimatization (Fig. 2L) then to the 6x6 feet garden plot. They were watered and maintained regularly for further phytochemical study.

Conclusion

P. nodiflora was known for its ornamental value and also as a medicinal plant. The ultrastructural study on the leaf of *P. nodiflora* showed the presence of diacytic stomata, simple unicellular trichomes and oil glands on both abaxial and adaxial leaf. The trichomes exhibited a simple unicellular structure with two ends connected to the epidermal tissue on the centre of the trichome. The induction of callus was successful on most MS media supplemented with PGRs especially auxin and combinations of auxin and cytokinins. The highest biomass and best nature obtained for callus in MS media supplemented with single PGRs were 2.5 mg/L NAA (118.6 mg/L) with light green friable and 3.0 mg/L NAA + 2.0 mg/L KN (165.0 mg/L) with green compact and friable callus for combination PGRs. Induction of callus on combination PGRs treatments reduced browning effect on callus and increased dry weight. Plant regeneration of *P. nodiflora* was successfully generated in both control (MS media without PGRs) and MS media supplemented with PGRs. However, high response of plant regeneration was found in control (90%) in comparison with the PGRs treatments. The successfully regenerated plant was able to generate roots in MS media supplemented with IAA and IBA PGRs, highest on 3.0 mg/L IAA with 5 roots per plantlet. The protocol of callus induction and plant regeneration had improvised to elongate culture duration by reducing browning effect without frequent sub culturing, thus saving cost and manpower. The mass propagation of *P. nodiflora* may assist in phytochemical and pharmacology aspect of study as the medicinal value of *P. nodiflora* offers many potential health benefits.

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