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Improvement of Regeneration of *Pelargonium radula* via Somatic Embryogenesis

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

In vitro stem segments of *Pelargonium radula* cultured for callusing then differentiated into somatic embryos and subsequently regenerated plantlets. Initiation of callus was observed in culture medium containing low concentrations of the plant growth regulators, 2,4-dichlorophenoxyacetic acid (2,4-D) and/or α-naphthalene acetic acid (NAA). At 0.2 mg/L 2,4-D and 0.2 mg/L NAA was showing the highest rate (92%) of callus induction. The callus showed no sign of browning after sub-cultured. Sub-culturing the callus onto medium with 0.2 mg/L 2,4-D showed the highest in proliferation rate resulted 13.45g weight of callus. The presence of agar at 6 g/L and 0.5 mg/L Gibberellic acid (GA3) improved the regeneration of the somatic embryos, which produced maximum number of plantlets (15 plantlets). Agar with concentration of 9 to12 g/L increased the incidence of browning. The former medium was more successful in plantlet regeneration when the selected embryos were subsequently transferred to regeneration medium with 3 g/L agar, 0.5 mg/L GA3 and 0.5 mg/L Benzylaminopurine (BAP).

Keywords: Pelargonium radula; regeneration; callus induction and plant growth regulator.

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1. INTRODUCTION

Pelargonium radula is a species in the *Pelargonium* genus and member of the *Geraniaceae* family. In Malaysia it is called as Jeremin and our ancestors call it as "pokok halau nyamuk". This plant can be used as a biopestidal plant or was used as mosquitoes repellent and will contribute the greener world. *Pelargonium* essential oil extract contains cintronellol and esters such as i-methnone, geranyl formate, citronellyl formate, eugenol and geraniol. These extract usually used as therapy in diabetes treatment and also for insect repellent formulation. According to widely accepted finding, these components found to have potential in insect repellence activity. There are also reports that the ethanolic plant extracts possess antimicrobial activity which against *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Bacillus subtilis*, *Escherichia coli* and *Serratia marcescens* [1]. Geraniol essential oil is used to treat many diseases such as dysentery, haemorrhoids, inflammation, heavy menstrual flow and cancer [2,3]. The essential oil of the plant is extensively used in fragrance, flavor and pharmaceutical industries and in aromatherapy.

Plant secondary metabolites are the sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals [4]. The production of secondary metabolites are influenced by environmental and physiological conditions, especially those that interfere with the stable production of the metabolite [5]. Accumulation of metabolites in the plants subjected to stresses including various elicitors, signal molecules or plant stress physiology. The production of secondary metabolite often low (less than 1% dry weight) and mostly depends on the physiological and developmental stage of the plant [6]. Plant cell and tissue cultures show a great promise in production of useful secondary metabolites on demand. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, especially in the production of bioactive plant metabolites through tissue culture technology. *In vitro* production of secondary metabolite in plant cell cultures or bioreactor has been reported in various medicinal plants. Plant cell and tissue cultures can be established under sterile conditions that initiated from explants, such as plant leaves, roots, meristems and stem for multiplication and extraction of secondary metabolites [7]. There is no report on somatic embryogenesis for *Pelargonium radula* in Malaysia and it is limited in world wide. In relation to studies on the cultivation of *Pelargonium radula* in Malaysia, the mass number of uniform plantlets through somatic embryogenesis was needed and tissue culture was employed. The objective of this study was to improve the in vitro regeneration system of *Pelargonium radula* via somatic embryogenesis.

2. MATERIALS AND METHODS

2.1 Plant Material and Callus Induction

The sterilized in vitro plant of *Pelargonium radula* was used in this study using method as described by Zuraida et al. [8]. For the callus induction, the petiole of the in vitro plants (Fig. 1a) were cut into lengths of 1–2 cm. Then the explants were cultured on MS medium [9] containing different concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2, 0.5, 1.0, 3.0, and 5.0 mg/L) and α-Naphthalene acetic Acid (NAA) (0.2, 0.5, 1.0, 3.0, and 5.0 mg/L) that supplemented with 3% sucrose and 3 g/L phytogel. Each growth regulator used on its own or in combination with one another as listed in Table 1. The cultures were incubated in a plant growth room at a temperature of 25ºC±1 and with 16 h photoperiod by cool-white fluorescent lamps (1000–2000 umol m^{-2} s^{-1}). Callus induction success was expressed as percentage of callus induction. The callus was sub-cultured three times at monthly intervals onto fresh media. The percentage of browning of the callus three month was recorded.

2.2 Proliferation and Regeneration

For the callus proliferation, the callus that was initiated from the treatments with 0.2 mg/l 2,4-D + 0.2mg/L NAA was used in the study. The callus was transferred onto fresh media with different concentrations of 2,4-D (0.2, 0.5, 1.0, 3.0, and 5.0 mg/L) and NAA (0.2, 0.5, 1.0, 3.0, and 5.0 mg/L). The each growth regulator used on its own or in combination with one another as listed in Table 2. After a month the weight of callus was recorded. Any further changes in the appearance and morphology of the callus were also noted. In a separate experiment, a friable callus that maintained on proliferation medium containing 0.2 mg/L 2,4-D (the media giving optimal for proliferation) were used to establish an appropriate medium for embryogenesis and shoot regeneration. Small pieces of calli (approximately 200 mg) were transferred to culture media containing different plant growth regulator (Indole-3-butyric acid; IBA, Kinetin;

Kin, NAA, GA3, Indole Acetic Acid; IAA,) at concentration of 0.5 mg/L and BAP at concentration of 0.1, 0.2, 0.3 and 0.5 mg/L. The agar was added into the treatment medium with different concentrations of 3.0, 6.0, 9.0 and 12.0 g/L. After 45 days of treatment, the fresh weight of proliferated callus obtained were recorded. All the callus were continued sub-culture on the same fresh treatment medium for 45 days before data on percentage of somatic embryos and browning incidence were recorded. The obtained somatic embryos were then transferred onto regeneration medium containing 3 g/L agar supplemented with 0.5 mg/L BAP+0.5 mg/L GA3. The number of plantlets regenerated were recorded after 4 weeks of culture. Regenerated shoots were then transferred to rooting MS medium containing 0.2 mg/L IAA for root induction. The plantlets were individually transplanted in soil contained in polybags and kept under controlled conditions in a net house with 75% shading. The survival rate of the plantlets was observed.

2.3 Statistical Analysis

The data (10x 3 replicates per treatment) were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using SPSS version 11.0 software. Significance of differences between means was tested by Duncan's Multiple Range Test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 Callus Induction and Proliferation

In this study, the frequency of callus induction varied at different concentration of 2, 4-D and NAA as shown in Table 1. Callus induction frequency occurred in approximately 40–90% of explants, depending on plant growth regulator type and concentration used. Results showed that all the media tested were suitable for callus induction using petiole explants. The highest number of callus forming was obtained in media containing 0.2 mg/L 2,4-D + 0.2 mg/L NAA, where the percentage of callus forming were 92% after a month in culture (Fig. 1b). Treatment of 0.5 mg/L 2,4-D + 0.5 mg/L NAA also give higher in the percentage of callus forming at 90%. The combination of 2,4-D and NAA was the most effective in inducing callus with no browning appear. When compared to both plant growth regulator, the callus showed favorable characteristics on the medium supplemented with 2,4-D. Application of 2,4-D alone in the culture medium produced good callus with less of browning. Treatments of 2,4-D at concentrations of 0.2-1.0 mg/L showed no browning at all. The number of callus forming browning was increased as the concentration of 2,4-D increases (3-5 mg/L). More browning was seen in treatment with NAA alone at all concentrations tested after sub-culture, which give 40-70% of browning.

These results are also supported by Zuraida et al. [10] who achieved callus induction on *Ruta graveolens* using culture medium containing 0.2 mg/L 2,4-D. They initiated callus on medium with 0.2 mg/L NAA and followed by sub-cultured on medium containing 0.2 mg/L 2,4-D give the optimum callus production with absence of browning. In addition, cultures that maintained on 2,4-D at 0.2 mg/L produced the best result for proliferation (95%). In contrast, according to Yasmin et al. [11], used of lower concentrations of 2,4-D showed no callus formation in mature wheat embryos. Turhan & Baser [12] who worked on *Triticum Aestivum* found that callus growth was higher with treatment of 4 mg/L 2,4-D in combination with 1 mg/L NAA. Shah et al. [13] reported that excellent callus was induced in wheat in treatment of 3.5 mg/L 2,4-D. Similar result was reported by Tahir et al. [14], where higher percentage of embryonic callus was observed at 3.0 mg/L 2,4-D in sugarcane. Higher concentration of 2,4-D ranged 4-5 mg/L found that callusing was the optimum in selected elite wheat with up to 82% [15]. In our result, high concentrations of 2, 4-D, there was a reduction in callus percentage of callus induction. Callusing and regeneration frequencies were higher with 2.0 or 4.0 mg/l 2,4-D for 10 or 15 days. Expose longer time in 2–4 mg/l 2,4-D in the medium beyond the initiation phase resulted detrimental to plant regeneration [16].

For callus proliferation, callus initiated in growth medium containing 0.2 mg/L 2,4-D + 0.2 mg/L NAA were sub-cultured on to different media with varying concentrations of 2,4-D (0.2-5.0 mg/L), NAA (0.2-5.0 mg/L) singly or combined as listed in Table 2. Generally, callus that was sub-cultured on growth media with lower concentration of 2,4-D (0.2-1.0 mg/L) showed higher proliferation give ranged 9-13 gram of fresh weight compared to other treatments, which treatment of 0.2 mg/L 2,4-D showed the highest (13.45g). On the other hand, there was no browning observed when the callus was sub-cultured onto these medium. These medium was

optimum treatment in terms of high callus production and absence of browning. However, browning of the callus occurred when 2,4-D (3-5 mg/L) were increased which only ranged 2.0-5.0 gram of fresh weight recorded. Callus initially cultivated on medium with 0.2 mg/L2,4-D+02.mg/L NAA and then sub-cultured on same medium also showed higher in fresh weight (8.91 gram). For callus proliferation.

Table 1. Effect of combination of 2,4-D with NAA on percentage of callus induction that initiated from petiole of *Pelargonium radula* and browning appear after two times sub-culture

2,4-D (mg/L)	NAA (mg/L)	Callus induction (%)	Percentage of browning after sub-culture
0	0	0	0
0.2	0	75±15	0
0.5	0	80±10	0
1.0	0	77±4	0
3.0	0	76±13	15±4
5.0	0	46±5	35±5
0	0.2	42±8	40±9
0	0.5	50±2	50±3
0	1.0	65±6	50±2
0	3.0	45±3	50±11
0	5.0	40±15	70±4
0.2	0.2	92±11	0
	0.5	85±21	0
	1.0	76±6	10±1
0.5	0.2	67±7	0
	0.5	90±9	0
	1.0	75±15	0
1.0	0.2	67±7	0
	0.5	58±14	0
	1.0	60±12	0

The best response, with no browning, was found with MS medium containing 2,4-D 0.2 mg/1. Similarly, according to Mungole et al. [17], response of callus (90%) and nature of callus in *Physalis minima* that obtained on 0.4 mg/l 2, 4-D proved the best response with greenish yellowish and very soft. Furthermore, Brown [18] reported that callus formation and average weight of calluses were obtained from young leaves cultured on a medium containing 0.5 mg/l 2,4-D in the light condition showed the greater performance. It also observed that the response of callusing reduced when the level of 2, 4-D further increased in the MS medium [19]. According to Al Abed et al. [20] increasing in levels of 2, 4-D decreased maize callus response and resulted in browning of callus. In our result, application of 2,4-D alone give better result on proliferation of callus. This is in agreement with observations by Kunta et al. [19] callus response of Indian Maize Inbreds showed maximum frequency of callusing at 80% in auxin 2, 4-D alone. In contrast with our result, reported by Mousavi et al. [21] found that 1.5 mg/L NAA produced significantly higher callus in *lisianthus*. They also supported that callusing was not response in all treatment of 2, 4-D. Wernicke and Mikovitis [22] stated that auxin was able to promote the growth of callus however with high concentrations of 2, 4-D could inhibit the callusing and it had effect as the herbicide.

Table 2. Effect of 2,4-D and NAA on callus proliferation from callus *Pelargonium radula* after two time sub- culture that initiated from 0.2 mg/L 2,4-D+0.2 mg/L NAA

2,4-D (mg/L)	NAA (mg/L)	Weight of callus	Callus morphology
0	0	0	-
0.2	0	13.45±2	Transparent, yellow
0.5	0	9.75±1	Transparent, yellow
1.0	0	9.01±2	Transparent, yellow
3.0	0	5.67±2	yellow
5.0	0	2.14±1	Dark Yellow
0	0.2	3.66±1	Dark yellow, brown
0	0.5	3.93±0.5	Dark yellow, brown
0	1.0	4.54±0.7	Dark yellow, brown
0	3.0	3.98±0.9	Dark yellow, brown
0	5.0	1.21±0.5	Dark yellow, brown
0.2	0.2	8.91±2	Transparent, yellow
	0.5	6.78±2	Pale yellow
	1.0	5.21±0.2	Light brown
0.5	0.2	5.36±0.6	Pale yellow
	0.5	5.22±0.4	Light brown
	1.0	3.56±0.9	Light brown
1.0	0.2	2.78±0.2	Light brown
	0.5	2.98±0.2	Light brown
	1.0	2.31±0.2	Light brown

3.2 Somatic Embryos and Regeneration

The proliferated callus that initiated from treatment of 0.2 mg/L 2,4-D+0.2 mg/L NAA were maintained on 0.2 mg/L 2,4-D for a month (Fig. 1c) then was used for regeneration treatment. The callus was cultured onto medium containing different type of plant growth regulator. The medium also supplemented with different concentrations of agar as a solidifying agent in order to examine their effect on the regeneration (Fig. 1d-i). In our results, the gelling agent showed a major factor contributing to

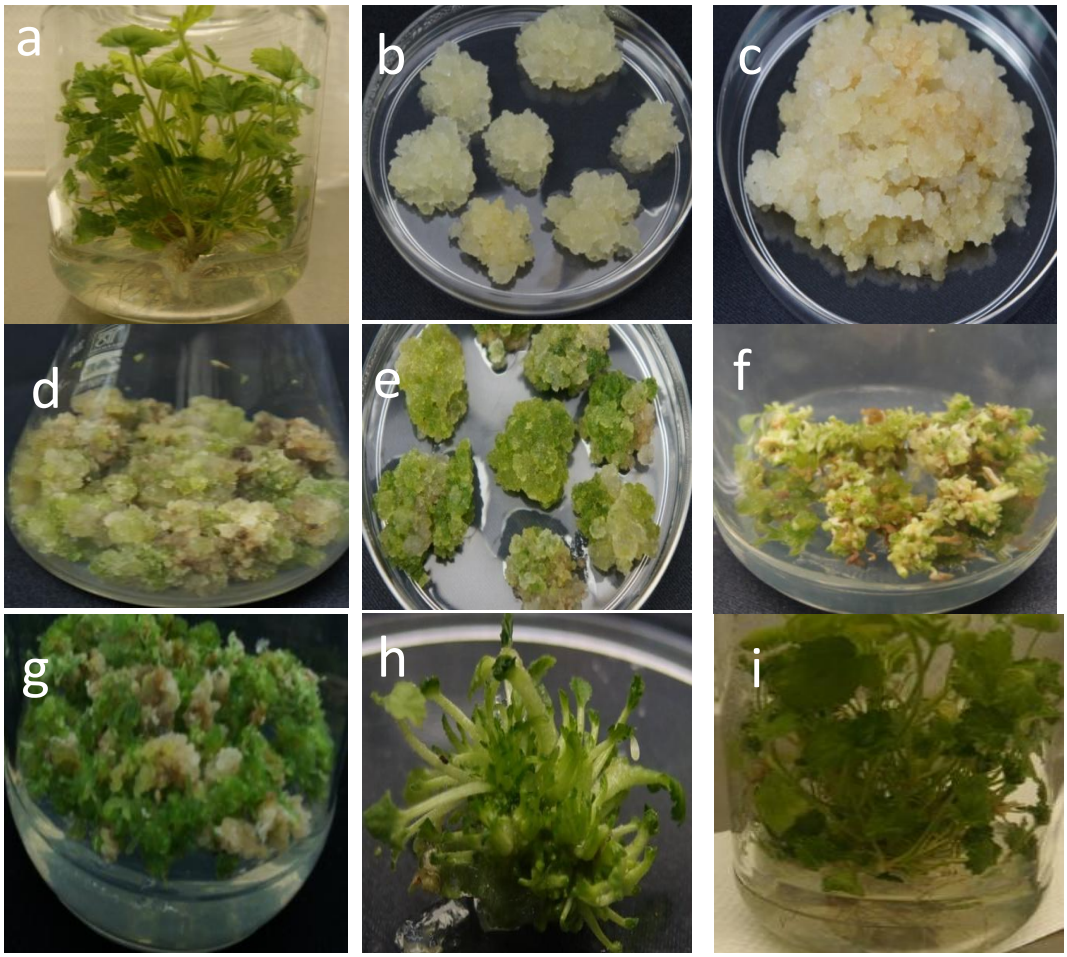

Fig. 1. Callus induction and plant regeneration in *Pelargonium radula* (a) Planting material used for callus induction, (b) Callus induction initiated on medium containing 0.2 mg/L NAA+0.2 mg/L 2,4-D, (c) Callus used for regeneration treatments (d-g) Development somatic embryogenesis (h) Regenerated plantlets (i) Complete plantlets with roots

regeneration success. This finding is in agreement with Huang et al. [23], stated that gelling agents is major element that affects the responses in in vitro culture. Generally, callus growth on media containing 6 g/L agar with plant growth regulator (IBA, Kin, GA3 and BAP) showed high in number of regenerated plantlets (Table 3). Among treatment involving 6 g/L agar with 0.5mg/L GA3 or 0.5 mg/L Kin given the higher number of regenerated plantlets, 15 plantlets and 12 plantlets, respectively. Increasing the agar concentrations to range 9-12 g/L incidence of browning increased. At these ranged of agar, only treatment of 9 g/L agar with 0.5 mg/L GA3 and 12 g/L agar with BAP (0.2 mg/L and 0.3 mg/L) showed response on regeneration. The highest fresh weight of somatic embryos and callus was observed in treatment with 3 g/L agar, but no regenerated plantlets produced. This treatment allowed rapid proliferation and most of the embryos or callus turned watery and brown.

Findings of the present study are in agreement with Zuraida et al. [10] for *Ruta graveolens*. They reported that medium containing 9 mg/L agar with 0.5 mg/L Kin give the highest in somatic embryos production rate up to 90% and low incidences of browning. Zuraida et al. [10] also reported treatment of 6 g/L agar+0.5 mg/L BAP produced higher number of regenerated plantlets. Abdoli et al. [24] in their study on sunflower observed that medium with 6 g/L agar given the highest in frequency of shoot development. Agar concentration is one of the factor influence the regeneration of shoot.

Table 3. Effect of agar concentration and plant growth regulators on somatic embryos, browning and regeneration

Agar (gram/L)	Plant growth regulator	Fresh weight of somatic embryos and callus (grams)	Green somatic embryos appear (%)	Browning incidence (%)	Number of regenerated plantlets
	0.5 IBA	12.6	45±5	55	0
	0.5 Kin	9.45	60±7	40	0
	0.5 NAA	9.92	40±5	60	0
3	0.5 GA3	11.33	25±2	75	0
	0.5 IAA	5.24	50±5	50	0
	0.5 BAP	12.12	50±4	50	0
	0.3 BAP	11.34	70±10	30	0
	0.2 BAP	8.62	70±11	30	0
	0.1 BAP	8.78	60±4	40	0
	0.5 IBA	1.2	60±2	40	7±1
6	0.5 Kin	2.08	50±2	50	12±3
	0.5 NAA	4.56	0	100	0
	0.5 GA3	2.72	40±4	60	15±1
	0.5 IAA	2.04	0	100	0
	0.5 BAP	2.48	40±7	60	7±1
	0.3 BAP	3.33	40±7	60	5±1
	0.2 BAP	3.15	40±9	60	4±1
	0.1 BAP	5.15	40±1	80	0
9	0.5 IBA	1.30	75±2	25	0
	0.5 Kin	2.87	30±3	70	0
	0.5 NAA	0.45	20±1	80	0
	0.5 GA3	1.34	10±1	80	3±
	0.5 IAA	1.31	0	100	0
	0.5 BAP	0.59	0	100	0
	0.3 BAP	1.09	0	100	0
	0.2 BAP	1.11	0	100	0
	0.1 BAP	1.04	5±1	95	0
12	0.5 IBA	0.50	0	100	0
	0.5 Kin	0.78	0	100	0
	0.5 NAA	0.61	0	100	0
	0.5 GA3	0.43	0	100	0
	0.5 IAA	1.25	20±1	70	0
	0.5 BAP	0.61	10±1	90	2±0.5
	0.3 BAP	0.32	5±1	95	2±0.7
	0.2 BAP	0.23	0	100	0
	0.1 BAP	0.31	0	100	0

A reduced amount of agars in the medium increased the water up by plantlets as seen in treatment 3 g/L agar which most of the cultures were watery. Furthermore, Abdoli et al. [24] also reported that increased in agar concentration to 8g/L significantly reduced the percentage of hyperhydric shoots. Hyperhydricity can be controlled through various ways included reducing cytokinin levels [25] and increasing the agar concentration [26]. Soft consistency gels allow tissue cultures to extract more water in medium [27]. In the finding, GA3 is the most important plant growth regulator to promote the plant regeneration. The maximum number of plantlet produced is when being pre-treated with 0.5 mg/L GA3. This supported finding by Sultana et al. [28], shoots elongation was found to be most excellent in medium supplemented with GA3. Reiterated by Sathyanarayana et al. [29] who stated that medium containing 2.89 mM GA3 producing maximum elongation of *Mucuna prureins*. In contras Ihsan et al. [30] found that GA3 at concentration 0.5-1 mg/L, less number multiplication shoot but shoot tended to elongate rather than to multiply.

Regenerated plantlets were transferred onto MS media containing 0.2 mg/L IAA for further rooting. The in vitro plantlets with roots through successive phases of acclimatization for two weeks before transplanted to polybags. They were maintained at about 70% relative humidity

in the greenhouse with 75% shading. After six weeks, a survival rate 90% was achieved.

4. CONCLUSION

In conclusion, we have developed a method for indirect plant regeneration from in vitro explants of *Pelargonium radula.* It was reported that culture medium containing 0.2 mg/L 2,4-D and 0.2 mg/L NAA followed transferred to medium containing 0.2 mg/L 2,4-D is optimal for callus induction and proliferation of *Pelargonium radula.* Combination of 0.5 mg/L GA3 and 6 g/L gelrite agar is suitable for somatic embryo production as a pre-regeneration medium. Subsequently treated somatic embryos transferred to regeneration medium with 3 g/L agar, 0.5 mg/L GA3 and 0.5 mg/L BAP found higher in multiplication shoot and appropriate for the regeneration. The indirect regeneration system developed for *Pelargonium radula* provided a step towards the application of such methodology, for this medicinal plant. Moreover, callus culture offer tools for genetic transformation through genetic engineering which much more rapid than conventional breeding

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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