academicJournals

Vol. 6(1), pp. 1-7, July 2014
DOI: 10.5897/JDBTE2014.0069
Article Number: 4F434B746465
ISSN 2141-2251 © 2014 Academic Journals

http://www.academicjournals.org/JDBTE

Journal of Developmental Biology and Tissue Engineering

Full Length Research paper

A study on the formation and development of *Panax bipinnatifidus* Seem. adventitious root

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Received 30 June, 2014; Accepted 11 July, 2014

This study aimed to generate adventitious roots from root and stem-derived callus of *Panax bipinnatifidus* Seem. Callus formation was observed best (83.3%) in MS media supplemented with 3.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L indolebutyric acid (IBA). Callus generated adventitious roots in MS media supplemented with 2.0 mg/L IBA at high frequency (66.7%). The optimal culture condition for the development of adventitious roots was liquid MS media supplemented with 40 mg/L sucrose and maintained at pH 6.0.

Key words: Adventitious root, stem, basic MS media.

INTRODUCTION

Panax bipinnatifidus SEEM. (Araliaceae) is a hygrophilous and shade-enduring plant, preferring cool and wet climate conditions with an average temperature of about 12-15°C. In nature, this plant is relatively rare and mostly found in the high mountainous region of Hoang Lien Son in the northwest of Vietnam. The root of *P. bipinnatifidus* has been used as a valuable tonic to increase mental and physical performance, improve thinking and memory, reduce cancer risk, and lower blood sugar in diabetics in the Vietnamese traditional medicine (Tung et al., 2011). Nowadays, bioactive compounds in *P. bipinnatifidus* Seem. are characterized, including compounds in oleanolic triterpenoid saponin group: Bifinoside A-C (1-3), narcissiflorine methyl ester (4), chikusetsu saponin IVa (5), pseudoginsenoside RP1 methyl ester (6), stipuleanoside R1 (**7**), pseudoginsenoside RT1 methyl ester (8), momordinIIe (9) and stipuleanoside R2 methyl ester (10) (Tung et al*.,* 2011). *P. bipinnatifidus* roots contain saponins triterpens (chikusetsu saponin IV, zingibrosid R1, ginseninosid Ro, Rb, Rd, Re, Rg1, Rg2,...), and phytosterol, reducing sugars, oils, uronic acids and fatty acids. Extracts from *P. bipinnatifidus* has metabolic activity (Dua et al., 1989), anti-inflammatory activity (Matsuda et al., 1991), anti-oxidation (Wen-Ta Chiu, 1999), cardiovascular benefits (Chen et al., 2008; Yang et al., 2005).

Adventitious roots are non-transgenic, proliferating through plant growth regulators supplemented in the media. Adventitious roots were rapidly proliferated and accumulated high concentrations of secondary metabolites (Hahn et al., 2003; Yu et al., 2005). This method could be used to increase the accumulation of secondary

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Figure 1. Stem segment.

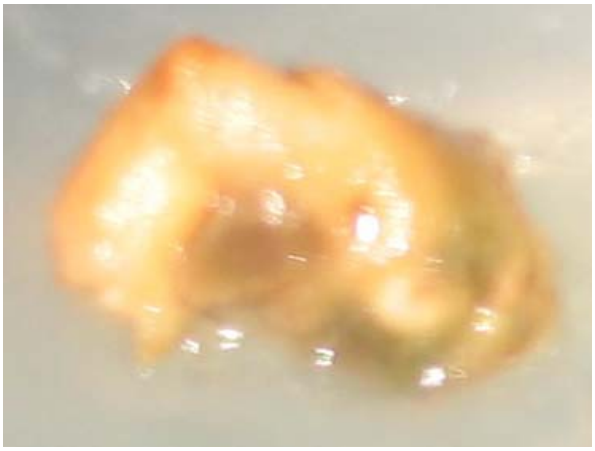

Figure 2. Root segment.

metabolites in plants. In this research, we have generated adventitious roots from callus of *P. bipinnatifidus* Seem. and investigated effects culture conditions on development of adventitious roots.

MATERIALS AND METHODS

Induction of adventitious roots

The roots and stems of *P. bipinnatifidus* were collected in Sapa, Laocai, Vietnam and were taxonomically identified by botanist Ngo Van Trai (Institute of Medicinal Materials, Hanoi, Vietnam). Explants were washed under running water and soap, then washed again with sterilized distilled water. Explants were then treated with Hypochlorite Na 50% (v/v) solution for 25 min and washed with sterilized distilled water. Explants were then soaked in cefotaxim (250 ppm) for 24 h, and washed with sterilized distilled water. Explants were cut into pieces and cultured on MS media (Murashige and Skoog, 1962). Explants were cut into 0.1-0.3 cm length and cultured on MS medium suplemented with 30 g/L sucrose, 8 g/L agar, pH 5.8-5.9 and 2,4-dichlorophenoxyacetic acid (2,4-D) (2 and 3 mg/L), indolebutyric acid (IBA) (0.1, 0.3, 0.5 mg/L), NAA (0.1, 0.3 and 0.5 mg/L). The callus induction frequency and percentage of callus were recorded after 8 weeks of culture. Callus were isolated and subcultured on MS media supplemented with 30 g/l sucrose, 8 g/l agar and IBA (0.5, 1.0, 1.5, 2.5 mg/L), NAA (0.5, 1, 1.5, 2, 3 mg/L), with pH adjusted to 5.8. Observations were made after 8 weeks of culture by percentage of explants producing adventitious roots. Cultures were maintained at 25±2°C in the dark.

Effects of culture conditions on the adventitious root

Adventitious roots were harvested from callus cultures and cut into 1 g and was cultured on different media including MS, Chu (N6) Medium and Gamborg's B5; supplemented with 30 g/l sucrose, 8 g/l agar, pH 5.8-5.9 and 2.0 mg/L IBA. Adventitious roots were culture selected media at three status: liquid (0 g/l agar, shaking at 80 rpm, semi-solid (4 g/L agar) and solid (8 g/L agar). Fresh weight and dry weight of cultured roots were recorded after four to eight weeks culture. Cultures were maintained at 25± 2°C in the dark.

Effects of pH, sugar content and mineral proportions on the adventitious root

Adventitious roots was cultured on media with different pH (5.5; 6.0; 6.5; 7.0 and 7.5), sucrose content (20, 30, 40, 50, 60 and 70 g/l), mineral proportions (25, 50, 75 and 100% when compared with the basic media). Fresh weight and dry weight of cultured roots were recorded after 4-8 weeks culture. The experiments were carried out in dark condition, at 25± 2°C, shaking at 80 rpm for liquid media.

Statistics

Experiments were repeated three times. Experimental data were processed by Duncan test ($p<0.05$, for experiment ≥ 6 treatments) or t test (for experiment with less than 6 treatments) by SAS program (ver. 6.12).

RESULTS AND DISCUSSION

Callus induction

After sterilization, explants were cut into small segments and cultured on MS media without plant growth regulators. The percentage of successfully sterilized explants was 65.2% after 14 days of culture (Figures 1 and 2).

Sterilized *P. bipinnatifidus* explants were cut into 0.1-0.3 cm segments and cultured on media for callus induction. Results are shown in Table 1, Figures 3 and 4.

Results from ANOVA table show that there is no significant difference between the effects of IBA and NAA on the callus induction efficiency ($P>0.001$); there was a significant difference of the D treatment (2,4-D) ($F=127.6$ với $p<0,001$), indicating that 2,4 D played a main role in callus induction capacity of *P. bipinnatifidus*, as in *Panax ginseng* roots (Thành and Yoeup, 2008).

Results indicate that the treatment C4 (2,4-D 3.0 and IBA 0.1 mg/l) induced the highest number of 83.3%.

Adventitious root induction from callus

The results showed (Figure 5 and Table 2), RI_4 and RI5 adventitious rooting sample rates are the same, RI_5 treatment had higher adventitious rooting average number

Table 1. Effects of plant growth regulators on callus induction.

Treatment	2,4-D (mg/L)	IBA (mg/L)	NAA (mg/L)	Callus induction (%)
C_1	2.0	0.1	0.0	22.3^{e}
C_2	2.0	0.3	0.0	33.3^{e}
C_3	2.0	0.5	0.0	33.3$_{e}$
C_4	3.0	0.1	0.0	83.3^{a}
C_5	3.0	0.3	0.0	64.0^{bc}
C_6	3.0	0.5	0.0	55.3^{bc}
C_7	2.0	0.0	0.1	30.3^{e}
C_8	2.0	0.0	0.3	36.3 ^{de}
C_9	2.0	0.0	0.5	49.7^{cd}
C_{10}	3.0	0.0	0.1	69.0^{b}
C_{11}	3.0	0.0	0.3	62.7^{bc}
C_{12}	3.0	0.0	0.5	55.0^{bc}

Source	DF	Type III SS	Mean Square	F Value	Pr> F
D	1	8464.000000	8464.000000	127.60	<.0001
IBA	2	216.777778	108.388889	1.63	0.2161
D*IBA	2	1258.111111	629.055556	9.48	0.0009
NAA	2	30.333333	15.166667	0.23	0.7973
D*NAA	2	852.111111	426.055556	6.42	0.0058

The average score with different letters are significantly different at p = 0.01 level. Letters a, b, c, d, e and f in the same column represent the differences among treatments by Duncan test.

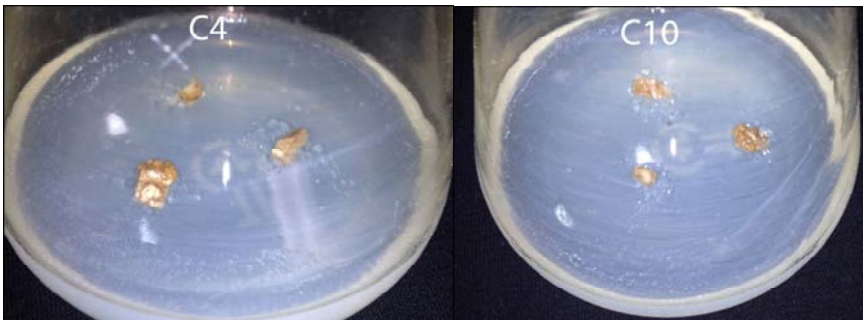

Figure 3. Callus induced on C_4 and C_{10} medium.

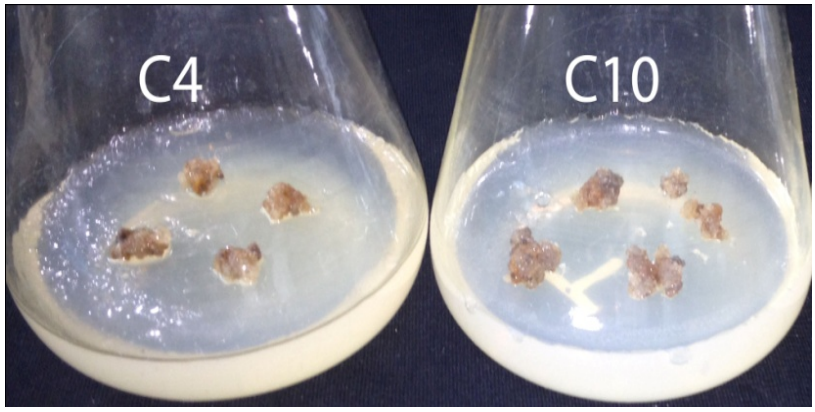

Figure 4. Callus induced on C_4 and C_{10} medium after 12 weeks culture.

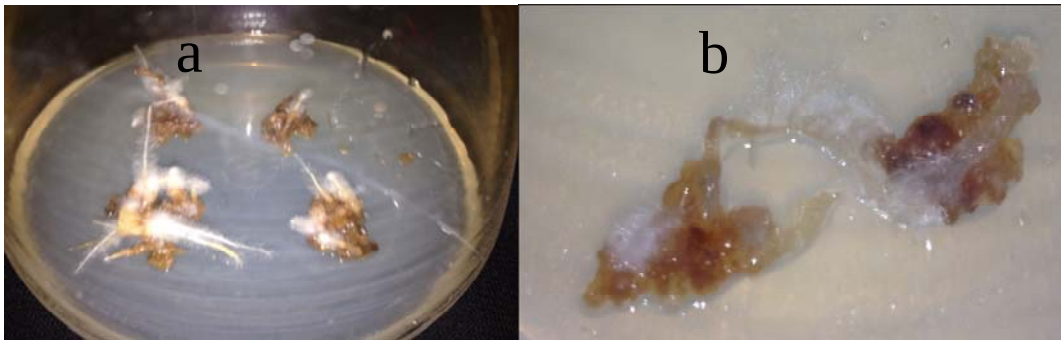

Figure 5. *P. bipinnatifidus*callus-induced adventitious roots after 12 weeks of culture.

Table 2. Effects of plant growth regulators on callus induction.

Treatment	NAA (mg/l)	IBA (mg/l)	Roots that induced callus (%)	Number of roots/explant	Root length (cm)
RN_1	0.5	0.0	17.9^{d}	1.23^{f}	0.47^{d}
RN_2	1.0	0.0	22.2^{dc}	2.47^{ef}	0.800^{bcd}
RN_3	1.5	0.0	36.1^{bcd}	3.80^{cde}	1.23^{ab}
RN_4	2.0	0.0	44.5^{abc}	3.87^{cde}	1.00^{abc}
RN_5	3.0	0.0	58.3^{ab}	4.87^{bc}	1.10^{abc}
RI_1	0.0	0.5	15.1^{d}	1.23^{f}	0.70^{bcd}
RI_2	0.0	1.0	27.8^{cd}	3.10^{de}	0.77^{bcd}
RI_3	0.0	1.5	47.2^{abc}	4.37^{bcd}	1.07^{abc}
RI_4	0.0	2.0	66.7^{a}	5.67^{b}	1.47^{a}
RI_5	0.0	3.0	66.8^{a}	7.33^{a}	1.03^{abc}

The average score with different letters are significantly different at p = 0.01 level. Letters a, b, c, d, e and f in the same column represent the differences among treatments by Duncan test.

Table 3. Effects of different media on adventitious root development.

Media	MS	N6	B5
Average fresh weight (g/75 ml)	1.99^{a}	1.70^{b}	1.83^{ab}
Average dry weight (g/75 ml)	0.206^{a}	0.182^{b}	0.190^{ab}

The average score with different letters are significantly different at p = 0.01 level. Letters a, b, c, d, e and f in the same column represent the differences among treatments by t Tests (LSD).

than RI_4, treatment RI_4 had adventitious rooting longer than RI_5. However, the actual culture, RI_4 created adventitious biomass better than RI_5. Therefore, we choose RI_4 to breeding adventitious biomass of *P. bipinnatifidus.* Callus are group of cells been localized, so the addition of growth regulators to stimulate callus form model differentiation depend on functional of growth regulator. Auxin (NAA and IBA) is the group of stimulants that starts rooting in cuttings and root development in tissue culture (Correa and Fett-Neto, 2004; Fletcher et al., 1965).

The result is simikar to that of Kim et al. (2002), the formation and development roots of *P. giseng* C.A. Meyer had optimum efficiency when environmental IBA (24.6 mM) or NAA (9.8 mM) are added. The ability to stimulate formation of adventitious rooting from callus of *P. giseng* have shown that IBA is suitable for the formation and growth of adventitious rooting, number of roots uncertainty also formed on each additional IBA environment is more when compared with NAA (Thanh and Paek, 2008).

Effects of different media on *P. bipinnatifidus* on adventitious root development

Adventitious root was cultured on three different media (MS, N6, B5) supplemented with 2 mg/L IBA. Fresh weight and dry weight of root cultures were recorded after 6 weeks culture (Table 3 and Figure 6).

The experiments indicated that MS media gave the best results for adventitious root development. Similar results were obtained when culturing *Astragalus membranaceus* adventitious roots *(*Thwe et al., 2012), *Vernonia amygdalina* (Khalafalla et al., 2009) and *Bupleurum falcatum* (Kusakari et al., 2000). According to

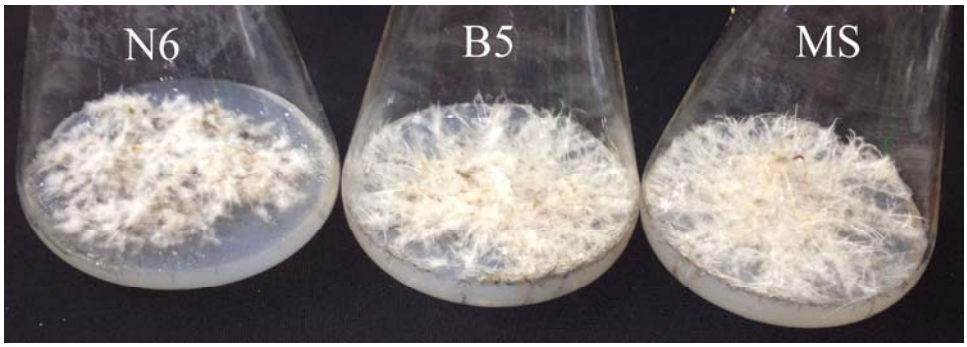

Figure 6. The adventitious root development of *P. Bipinnatifidus* on different media.

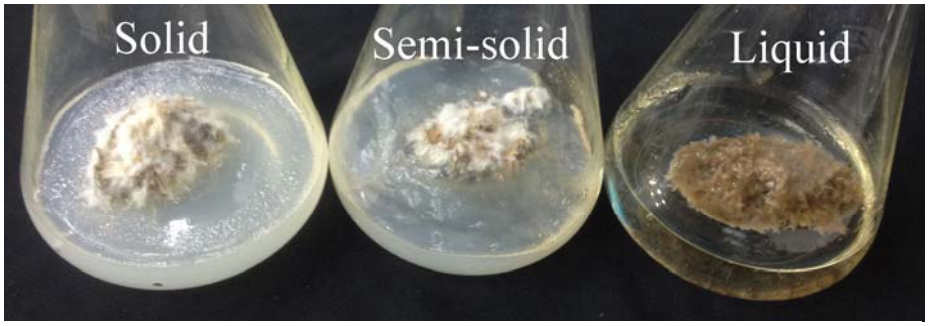

Figure 7. Effects of the physical states of media on *P. Bipinnatifidus* adventitious root development.

Table 4. Effects of physical states of media on *P. bipinnatifidus* adventitious root development.

Physical states of media	Solid	Semi-solid	Liquid
Fresh weight (g/75 ml)	1.90^{b}	1.75^{b}	2.13^{a}
Dry weight (g/75 ml)	0.190^{ab}	0.176^{b}	0.201^{a}

The average score with different letters are significantly different at p = 0.01 level. z: Letters a, b, c, d, e and f in the same column represent the differences among treatments by t-Tests (LSD).

Table 5. Effects of pH.sucrose on *P. bipinnatifidus* adventitious root development.

pH	5.5	6.0	6.5	7.0	7.5
Fresh weight(g/75 ml)	1.917^{c}	2.237^{a}	2.117^{ab}	2.047^{bc}	1.950^{bc}
Dry weight(g/75 ml)	0.193^{b}	0.231^{a}	0.206^{ab}	0.198^{b}	0.192^{b}

The average score with different letters are significantly different at p = 0.01 level. Letters a, b, c, d, e and f in the same column represents the differences among treatments by t tests (LSD).

Amzallag et al. (1992), the mineral content affected root development through the metabolism of plant growth regulators.

Effects of the physiscal states of the media on *P. bipinnatifidus* adventitious root development

Adventitious root was cultured on MS media at different physical states: solid (8 g/L agar), semi-solid (4 g/L agar) and liquid (0 g/L agar; shaked at 80 rpm), supplemented with 2 mg/L IBA. Fresh weight and dry weight of cultured roots were recorded after six weeks of culture (Table 4 and Figure 7).

Results show there was an effect from selected media on the adventitious root development of *P. bipinnatifidus,* particularly adventitious roots cultured on media without agar performed better as compared to those cultured on media supplemented with agar according to its fresh and dry weight (Table 4). The good contact of roots in the liquid medium could be the reason.

Effects of pH on *P. bipinnatifidus* adventitious root development

Adventitious roots (1 g/75 ml media) were cultured on liquid MS with pH 5.5, 6.0, 6.5, 7.0 or 7.5, supplemened with 2 mg/l IBA, in dark condition, 25±2°C, shaked at 80 rpm. Fresh weight and dry weight were recorded after 6 weeks of culture (Table 5 and Figure 8).

Results from Table 5 indicate that *P. bipinnatifidus* adventitious root developed best at pH 6.0. pH affected

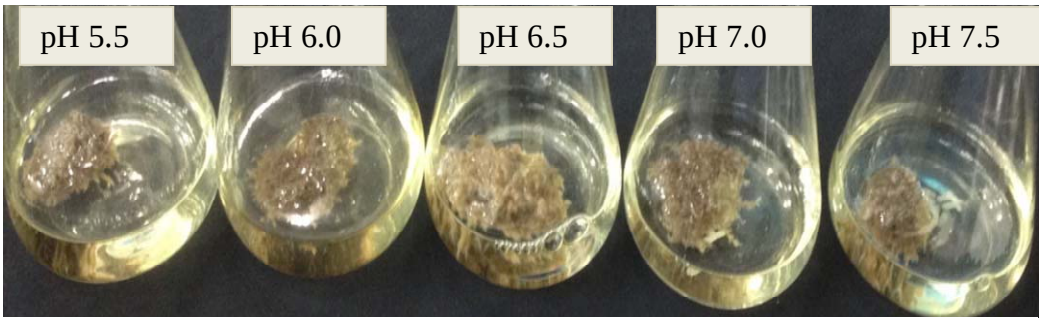

Figure 8. Effects of pH on the development of *P. bipinnatifidus* adventitious roots.

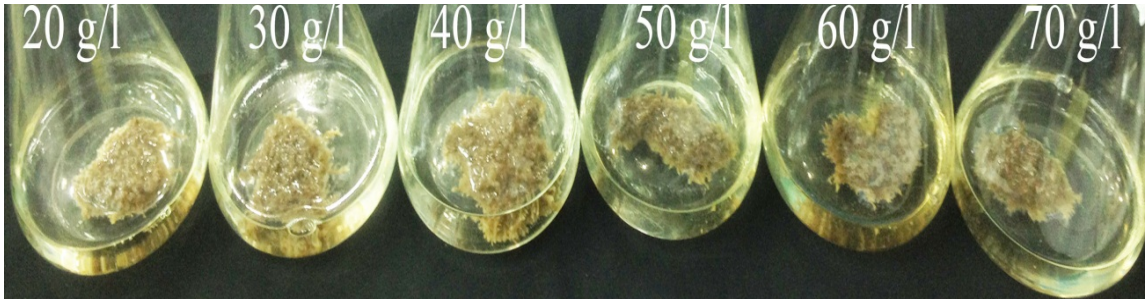

Figure 9. Effects of the sugar content on the root development of *P. bipinnatifidus*.

Table 6. Effects of the sugar content on the adventitious roots development of *P. bipinnatifidus*.

Sucrose content (g/L)	20	30	40	50	60	70
Fresh weight (g/75 ml)	1.989^{c}	2.168^{b}	2.318^{a}	2.198^{ab}	1.987^{c}	1.931^{c}
Dry weight (g/75 ml)	0.199^{ab}	0.215^{a}	0.220^{a}	0.211^{ab}	0.199^{ab}	0.193^{b}

The average score with different letters are significantly different at p = 0.01 level. Letters a, b, c, d, e and f in the same column represent the differences among treatments by t tests (LSD).

the root development through an ion exchange process through the membrane. According to "developing hypotheses about the acid", the size of cells were manipulated by its environment's pH, where it decreases with decreased pH (Cosgrove, 1999). Winch and Pritchard (1999), Evans (1976) and Edwards and Scott (1974) concluded that environemtal pH induced root elongation. The effects of pH on the development of adventitious roots were proved by Ling et al. (2009) while culturing *Orthosiphon stamineus* roots. pH 6.0 was the best among pH 4.0; 5.0; 5.8 and 7.0 and pH 6.0 was also appropriate for the adventitious roots development and saponin accumulation in *P. ginseng* (Kim et al., 2005).

Effects of sugar content on *P. bipinnatifidus* adventitious root development

Adventitious roots was cultured on liquid MS media, with different sucrose concentration (20, 30, 40, 50, 60 and 70 g/L), supplemented with 2 mg/L IBA at pH 6.0 in dark condition, 25±2°C and shaked at 80 rpm. Fresh weight and dry weight of root cultures were recorded after 6 weeks of culture (Table 6 and Figure 9).

The results (Table 6) show that culture media supplemented with 40 g/L sucrose were optimal for *P. bipinnatifidus* adventitious roots development. Carbohydrate was essential for plant metabolism *in vitro,* therefore sugar content in the media had a crucial impact on the root induction and development. Sugar plays an important role in the regulation and expression of the transcription of photosynthesis genes (Sheen, 1990) and also in signals of abscisic acid and ethylene (Leon and Sheen, 2003). Cheng et al. (1992) reported sucrose concentration at 2-3% positively affected the root induction of *Eucalyptus sideroxylon*. Moreover, sugar concentration was reported to have effects on adventitious roots induction of *O. stamineus* and *Scopoliaparviflora* at concentration of 30 and 50%, respec-

tively (Ling et al., 2009; Min, 2007). *Echinacea angustifolia* adventitious roots were cultured on bioreactor on MS media supplemented with 2 mg/l IBA and 50 g/L sucrose. The initial sugar concentration (50 g/L) gave the best result in the mass development and saponin content of *P. ginseng* adventitious roots (Thành and Yoeup, 2008).

Conclusion

This study showed the possibilities of *P. bipinnatifidus* adventitious root development from callus. Results indicated variable effects on root development, forming the basis for further investigations of culturing *P. bipinnatifidus* adventitious roots to collect valuable metabolites.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

This research received financial support from National Key Laboratory of Plant Cell Technology, Institute of Tropical Biology.

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