Kasetsart J. (Nat. Sci.) 47 : 748 - 759 (2013)

Growth Characteristics and Production of Physalins from *Physalis minima* Hairy Roots in Shake Flasks

Jualang Azlan Gansau[1,*] and Marziah Mahmood[2]

ABSTRACT

This report described the growth characteristics and production of physalins from *Physalis minima* hairy roots in shake flask culture. The presence of lateral branches in the inoculum had a negligible effect on the final root biomass dry weight (DW), specific growth rate (μ), doubling time (t_d) and production of physalins. However, excising the primary and lateral root tips reduced μ and the total root length but not the final biomass. Mature root tissues were observed to accumulate more physalin B and F (1.55 and 3.74 mg.g^{-1} DW, respectively) compared to the root tips (0.65 and 1.47 mg.g^{-1} DW, respectively). Increasing the number of root tips from 2 to 12 and the medium volume significantly reduced μ and extended t_d. Decreasing the medium volume with a small number of inocula reduced t_d, improved the biomass and production of physalins and μ. Using a 100 mL flask, four root tips cultured in 25 mL medium provided the optimum conditions for biomass (0.24 g DW) and production of physalins (1.68–3.5 mg.g^{-1} DW).

Keywords: *Physalis minima*, hairy roots, physalin, inoculum morphology, inoculum size, medium volume

INTRODUCTION

Physalis minima plants are tetraploid with globular fruits enclosed in an inflated bladder-like calyx and belong to the Solanaceae family (Burkill, 1966; Nayeemulla *et al*., 2006). The plants have been used traditionally to remedy headache, earache, fever, ulcers, spleen disorder, wound pustules, intestinal pains and gonorrhoea and as a purgative, a diuretic and a tonic and to restore flaccid breasts (Burkill, 1966; Kirtikar *et al*, 1975; Sethuraman and Sulochana, 1988; Ashok Kumar *et al*., 2010). *Physalis minima* contains several physalin (13,14-seco-16, 24-cycloergostane) compounds (Sen and Pathak, 1995; Kawai *et al*., 1996; Jualang *et al*., 2002, 2005); some of these compounds (especially physalin B and F) were reported to have great potential for use as an anti-inflammatory (Vieira *et al*., 2005; Pinto *et al*., 2010), antimycobacterial (Pietro *et al*., 2000, Januário *et al*., 2002), antimicrobial (Silva *et al*., 2005), antileishmanial (Elisalva *et al*., 2009), and anti-cancer (Antoun *et al*., 1981; Chiang *et al*., 1992a, b; Hemerson *et al*., 2006; Ooi *et al*., 2010; Hsu *et al*., 2012).

Hairy root cultures offer high and stable production of valuable secondary metabolites in many plants cultures (Guillon *et al*., 2006;

1 School of Science and Technology, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia.

2 Department of Biochemistry and Microbiology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400 Serdang, Selangor D.E., Malaysia.

* Corresponding author, e-mail: azlanajg@ums.edu.my

Received date : 04/02/13 Accepted date : 31/05/13

Makhzoum *et al*., 2013). Hairy roots grow rapidly and are highly differentiated, show plagiotropic growth and are highly branched on phytohormone free medium (Tenea *et al*., 2008; Saravanakumar *et al*., 2012). For the past 25 years, hairy roots have been investigated as a biological system for the production of valuable compounds from medicinal plants. However, the main constraint for commercial exploitation cultivations of hairy root is the development and scaling up to a bioreactor that permits the mass growth and production of secondary metabolites (Cui *et al*., 2011; Makhzoum *et al*., 2013). Emphasis has focused on optimizing the parameters to culture the delicate and sensitive plant hairy roots. The shake flask culture is a very common and useful tool for small-scale submerged studies. Using shake flask cultures has many advantages, as they are easy to manipulate, require little material and manpower expense and it is possible to carry out several experiments concurrently especially for studies like primary screening, testing of culture media, strain selection and growth evaluation (Van Suijdam *et al*., 1978; Kanokwaree and Doran, 1997a, b; Hu and Du, 2006).

The relationship between the inoculum morphology and culture performance is an important consideration in the development of large-scale processes using hairy root culture (Falk and Doran, 1996; Sun *et al*., 2012). The root inoculum prepared by mechanical chopping or partial homogenization often consist of a mixture of mature roots with tips, roots with lateral branches with all tips intact, and roots with excised or damaged tips (Ramakrishnan *et al*., 1994; Falk and Doran, 1996; Sun *et al*., 2012). The growth rate of hairy roots depended on a high rate of linear extension (root elongation), the formation of new growing points (lateral branches), and a secondary increase in root diameter (Rhodes *et al*., 1990). Previous work with *Atropa belladonna* hairy roots has shown that the specific growth rate and biomass yield varied with the root morphology. It has also been reported that the specific tissues that accumulated higher secondary metabolites are in mature roots compared to those in the tips (Falk and Doran, 1996). The inoculum density of the hairy root and medium volume also contributed to the effect of oxygen and nutrient utilization (Kanokwaree and Doran, 1997a). It is well known that shake flask cultures are greatly limited by the nutrient supply, total oxygen supply and oxygen mass transfer in the roots and by the need to limit the intensity of agitation in order to protect the biomass from shear damage (Van Suijdam *et al*., 1978; Kanokwaree and Doran, 1997a, b). Therefore, the aim of this study was to determine whether the inoculum morphology, inoculum density and medium volume exert an influence on the growth and physalins production in hairy root cultures of *P. minima*. The general site of the accumulation of physalins in hairy root cultures of *P. minima* was also considered.

MATERIALS AND METHODS

Hairy root cultures

Leaf-derived hairy roots of *Physalis minima* were initiated by using *Agrobacterium rhizogenes* strain LBA9402 (Jualang *et al*., 2002). The bacterium-free hairy roots were aseptically cultured in 25 mL of B5 hormone-free basal medium (Gamborg *et al*., 1968), pH 5.7, supplemented with 3% (weight per volume, w/v) sucrose and incubated in the dark at 25 ± 2 ^{0}C on an orbital shaker (110 rpm). In subculturing, 3-6 primary root tips, aged 8 d and 5.0 ± 0.5 cm in length were used as the inoculum. Experimental units were carried out in 10 replicates using 100 mL flasks and grown under similar conditions.

Inoculum root morphology

Five different inoculum root morphologies were denoted as culture types A, B, C, D and E (Figure 1) consisting of: the control (primary root with tip); the primary root with tip (0.5–0.7 cm)

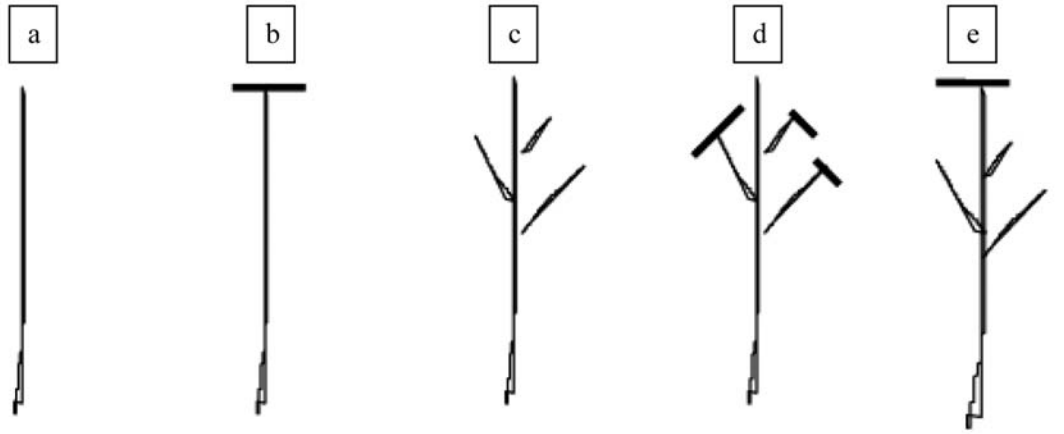

Figure 1 Different inoculum morphologies of *Physalis minima* hairy root cultures: (a) Control (primary root with tip) designated as A; (b) Primary root with tip excised designated as B; (c) Primary root tip with lateral branches designated as C; (d) Primary root tip intact with lateral branches and root tips excised designated as D; and (e) Primary root tip excised with lateral root tips intact designated as E.

excised; the primary root tip with lateral branches; the primary root tip intact with lateral branches root tips excised; and the primary root tip excised with lateral root tips intact, repsectively. For each treatment, one root tip of 5.0 ± 0.5 cm in length was inoculated into a flask containing 25 mL medium. The cultures were monitored daily for 8 d to measure the total root length and number of root tips, and harvested at day 15 to determine the biomass dry weight (DW) and physalins content. The morphological parameter of root growth unit was calculated according to Yu and Doran (1994) and Falk and Doran (1996)

Localization of physalin accumulation in hairy roots

One root tip of 5.0 ± 0.5 cm in length per flask of primary root with meristem tip (morphology type - A) was used as the inoculum. The tips (0.7–1.0 cm) and the remaining mature part of the roots without tips were then collected and analyzed separately after 15 d of culture. The roots with tips intact were used as the control.

Inoculum size and medium volume

Different numbers of root tips (1, 2, 3, 4, 6, 8, 10 or 12) of the primary root with meristem tip (morphology type - A, 5.0 ± 0.5 cm in length) were inoculated into 12.5, 25 and 50 mL of medium. The total root length, number of lateral roots (LR) and LR length were measured at 6 d of culture, while the biomass DW and physalins content were determined at 15 d after culture.

Kinetics growth analysis

The mathematical kinetic growth analysis of the hairy roots in the shake flask cultures was undertaken by adapting methods by Stanbury and Whitaker (1984) and Hjortso (1997) using the biomass DW as the variable parameter.

Extraction procedures and high performance liquid chromatography analysis

Two-gram samples of dry powdered tissue were extracted with methanol (MeOH) at room temperature under dark conditions. The crude methanolic extracts diluted with the same

volume of distilled water were partitioned twice by hexane and $CHCl_3$. The $CHCl_3$ partition was evaporated and dissolved in 65% (v/v) MeOH (spectra grade) and finally filtered through a Sep-Pak Classic Cartridge (Waters Corp.; Milford, MA, USA) for analysis using high performance liquid chromatography (HPLC) (Jualang *et al*., 2002). HPLC was performed on a Waters Associates model Baseline 810 high performance liquid chromatography attached to a Waters 501 solvent delivery system and detected at 220 nm using model 486 tunable absorbency detector (Waters Associates Inc.; Milford, MA, USA). A 3.9 × 150 mm I.D. Nova Pak C18 60Å steel cartridge column; fitted at 4 μm (Waters Associates Inc.; Milford, MA, USA) containing dimethyloctadecylsilyl-bounded amorphous silica and methylalcohol was used with the mixtures of methanol-water (65:35, volume per volume, v/v) as the eluents at a flow rate of 1.0 mL.min^{-1}. The mixtures consisted of methanol aqueous isocratic solvent (Uvasol, spectroscopy grade, Merck Group; Darmstadt, Germany) and double reverse-phase distilled water. Chromatograms of individual physalins were referred to as the authentic compounds.

Statistical analysis

The results were analyzed by one-way analysis of variance and the mean values were compared at $P < 0.05$ by Duncan's multiple range test (DMRT) to find the significant difference using the General Linear Model Procedure of the SAS version 9.1 (SAS Institute, NC, USA).

RESULTS AND DISCUSSION

Effects of inoculum roots morphology on culture performance

Table 1 and Figure 2 show the growth characteristics of *P. minima* hairy roots in 100 mL flasks containing 25 mL medium. Generally, the μ value of the different inoculum morphologies did not differ much and varied between 0.37 and 0.41 d^{-1}. The same value of μ obtained in cultures A and C indicated that the presence of lateral root tips did not significantly influence root growth. Meanwhile, excising of root tips resulted in 7.9–10.8% reduction of the μ value, with the lowest value being in culture B (0.37 d^{-1}). These results were different from those reported by Falk and Doran (1996) in *Atropa belladonna* hairy

Table 1 Effect of different inoculum morphologies on growth characteristics of *P. minima* hairy root cultures.

Inoculum morphology	μ (d^{-1})	t_d (d)	Total root length (cm)	No. of root tips	Biomass (g DW)	Physalins production (mg per gram DW)	
						Physalin B	Physalin F
A: Control (primary root with tip)	0.41	1.7	10.7±0.7^a	39.0±4.0^b	0.15±0.10^a	1.75±0.10^a	4.00±0.21^a
B: Primary root with tip excised	0.37	1.9	6.9±0.5^c	46.0±8.0^b	0.11±0.02^b	1.68±0.20^a	3.90±0.30^a
C: Primary root tip with lateral branches	0.41	1.7	11.3±0.5^a	87.0±7.0^a	0.17±0.11^a	1.90±0.12^a	4.20±0.16^a
D: Primary root tip intact with lateral branches root tips excised	0.38	1.8	11.1±0.3^a	52.0±6.0^b	0.13±0.00^b	1.83±0.05^a	4.10±0.30^a
E: Primary root tip excised with lateral root tips intact	0.40	1.7	9.40±0.4^b	93.0±5.0^a	0.17±0.01^a	1.70±0.16^a	3.90±0.25^a

μ = Specific growth rate; t_d = Doubling time, DW = Dry weight.

$^{a, b, c}$ = Mean values ± SD in the same column with the same lowercase letter are not significantly different at $P < 0.05$ by Duncan's multiple range test (n =10).

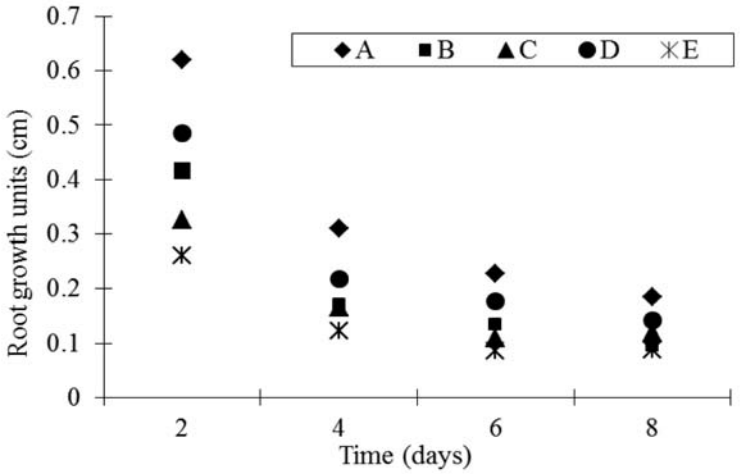

Figure 2 Effect of different inoculum morphologies on root growth units of *P. minima* hairy roots observed after 8 d in shake flask cultures. (A = the control (primary root with tip); B = primary root with tip (0.5–0.7 cm) excised; C = primary root tip with lateral branches; D = primary root tip intact with lateral branches root tips excised; and E = primary root tip excised with lateral root tips intact.)

roots, where μ was increased up to 25% when the inoculum consisted of roots with the primary root tips excised (culture E).

During the exponential growth phase, the root DW tissue was doubled from X_0 to $2X_0$ at regular intervals, named as the doubling time (t_d). The t_d varied between 1.7 and 1.9 d (Table 1) with cultures A, C and E showing the fastest growth development (1.7 d, respectively). Longer t_d values were obtained in cultures B (1.9 d) and D (1.8 d). Culture C produced the longest root length (11.3 cm), but this was not significantly different from cultures A and D. The elongation of root growth was reduced significantly when the primary tip was excised in cultures B and E. Even then, the number of root tips in both cultures was 12.5 and 24.1% higher than that in the original roots of cultures A and C, respectively. In the case of horseradish, madder (Kino-oka *et al*., 1999), *Nicotiana* (Aoki and Syōno, 2000) and *Calotropis* (Sun *et al*. 2012) hairy roots, it was proved that injured cells will stimulate the expression of genes that were homologous to *rolB, rolC* and open reading frames (ORFs) in the cellular T-DNA region of the genome. Therefore, the results obtained from the current study may explain the fluctuation in the expression of the *rol* genes and ORFs due to wounding of the hairy root tissues (Aoki and Syōno, 2000; Bettini *et al*., 2010; Verma *et al*., 2012). In particular, it has been reported that the meristematic tips require ten times more oxygen than other parts of root tissue (Falk and Doran, 1996) and yet oxygen supply may be a limiting factor for continued growth proliferation. This could explain why the specific root growth rate of culture E was lower than for culture C.

Among the root morphologies, type A had a higher root growth unit, while the lowest was in culture E for the first 2 d of culture incubation (Figure 2). However, the ratio of the root length to the number of root tips reduced following the culture time. No significant difference was observed in root growth unit between the root morphologies after culturing for 6 d. The root growth unit is a morphological parameter indicating the 'branchiness' of the root system

(Yu and Doran, 1994). The root growth unit obtained in this result was much higher than that in *Atropa belladonna* hairy root cultures (Falk and Doran, 1996); that is, *P. minima* hairy root cultures formed many lateral roots rather than undergoing root elongation. For most of the measurement period, the ratio of the root length to the number of tips ranged from 0.05 to 0.65 cm for all root morphologies. The lateral roots grew 2.5–4.5 cm in length without forming any new lateral branches; formation of a new tip occurred mainly on the primary root over the 6 d of culture. New lateral root branches only appeared after day 7, particularly in the fastest-growing cultures of A, C and E. Variation in root morphology has been proven to be closely related to other root characteristics such as the growth rate, production level and adaptability to liquid culture and could be an important factor in the design of reactors for large-scale hairy root culture production (Lenk *et al*., 2012; Neelwarne, 2012).

Culture C also produced the highest biomass DW (0.17 g per flask) at 15 d, but this was not significantly different from cultures A and E. The lowest biomass DW was obtained in culture B (0.11 g per flask). These results indicated that there was no significant difference between roots containing lateral branches (culture C) or without lateral branches (culture A) in terms of the final biomass production. Physalin B and F synthesis was not significantly affected by the inoculum root morphology. This finding was in line with Falk and Doran (1996) for tropane alkaloid production in *Atropa belladonna* hairy roots.

Localization of physalin accumulation in hairy roots

Physalin B and F accumulation (Table 2) was found to be higher in the mature root tissues (1.55 ± 0.26 and 3.74 ± 0.12, respectively) compared to the root tips (0.65 ± 0.20 and 1.47 ± 0.44, respectively). Mature roots often contain higher concentrations of secondary metabolites than the root tips (Falk and Doran, 1996; Kuźma *et al*., 2009; Gangopadhyay *et al*., 2011). Taking into account the biomass DW and specific physalins content, the amounts of physalin B and F in the whole roots were equal to the sum of the amounts in the tips and mature parts within experimental error. Although culture E produced more root tips than the A, B, C or D, the specific physalin levels were not affected significantly since a proportionally greater mass of mature root tissue was also generated. Because the length of the root growth unit was similar in each culture, preferential accumulation of physalins in particular parts of the roots did not result in different specific physalin contents from the different inoculum morphologies. This result however, did not identify the site of synthesis of the physalins, because at present, it is not clear where the biosynthesis, translocation and storage processes of physalins occur in the roots.

Table 2 Localization and accumulation levels of physalin B and F *in P. minima* hairy roots after 15 d in shake flask cultures.

Root part	DW (g)	Physalins production (mg per gram DW)	
		Physalin B	Physalin F
Whole root (control)	0.15 ± 0.02[a]	1.90±0.50[a]	4.22±0.35[a]
Mature part (tips excised)	0.10 ± 0.02[b]	1.55±0.26[a]	3.74±0.12[b]
Tips	0.05 ± 0.02[c]	0.65±0.20[b]	1.47±0.44[c]

Dw = Dry weight.

Mean values ± SD in the same column with the same lowercase letter are not significantly different at $P < 0.05$ by Duncan's multiple range test (n = 3).

Effects of inoculum number and medium volume on culture performance

Figure 3 and Table 3 show the relationship between inoculum number, medium volume and growth performance of *P. minima* hairy root cultures. The number of lateral roots (LR), the length of LR, and total root length did not significantly differ between the different inoculum numbers. However, the trend of these growth parameters appeared to reduce when the inoculum number increased from 3 to 12 tips per flask. The highest number of lateral root tips was obtained when the inoculum contained 3 root tips which was similar to *Beta vulgaris* hairy root culture (Carvalho *et al*., 1997). The increased number of inoculum root tips also substantially reduced the root growth unit, while the μ in 12.5 mL inoculated with 12 tips was 29.6% lower than with 4 tips; and in 50 mL, 12 tips growth was 5.9% lower than with 4 tips. These results agreed with the growth characteristic of *Atropa belladonna* (Yu and Doran, 1994; Kanokwaree and Doran, 1997a) and *Panax ginseng* (Jeong *et al*., 2009) hairy root cultures. The variations in the growth rate of hairy root cultures are not simply related to the number of inoculated root tips per milliliter of medium. When the inoculum density was maintained at a constant level, but the number of root tips and the amount of liquid volume were changed, the growth rate of the roots was also altered significantly. For example, the initial μ of 4 tips in 25 mL was 14.7% higher than with 8 tips in the 50 mL medium. Similar results have also been reported in different plant species (Hilton and Rhodes, 1993; Doran, 1994; Kanokwaree and Doran, 1997a; Jeong *et al*., 2009; Saravanakumar *et al*., 2012). The inoculum size of different medium volumes also influenced the doubling time (t_d) values. The t_d values of culture inoculated with 4 root tips were 30.0, 22.2 and 5.3% faster than those of 12 root tips in the 12.5, 25.0 and 50.0 mL medium, respectively. Evaluation of the t_d value of *P. minima* (1.5–2.6 d) hairy roots and other plant species such as *A. belladonna* (1.3–2.3 d; Kanokwaree and Doran, 1997a) and *Catharanthus roseus* (3.1–5.9 d; Bhadra and Shanks, 1995) hairy root cultures have suggested that the specific oxygen demand of *P. minima* and *A. belladonna* was considerably higher than that in *C. roseus* hairy root cultures.

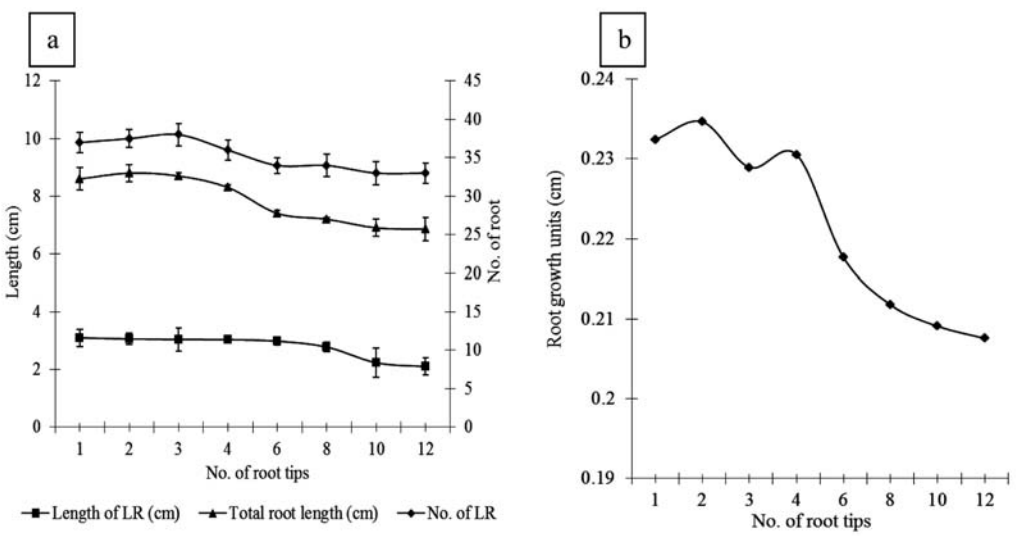

Figure 3 Effect of inoculum size on: (a) Growth performance (total root length, number (No.) of lateral roots (LR), length of LR); and (b) Root growth units of *P. minima* hairy roots after 6 d in shake flask cultures. (Vertical bars show the SD.)

Table 3 Effect of inoculum number and medium volume on growth characteristics of *P. minima* hairy roots.

Medium volume (mL)	Number of inoculum root tips	Maximum growth (g DW)	Biomass yield from total sucrose ($g.g^{-1}$)	μ (d^{-1})	t_d (d)	Physalins production (mg per gram DW)	
						Physalin B	Physalin F
12.5	2	0.15^{c}	0.40	0.45	1.5	1.51±0.01^{b}	3.51±0.50^{a}
	4	0.13^{c}	0.35	0.35	2.0	1.72±0.03^{a}	3.63±0.23^{a}
	8	0.13^{c}	0.35	0.28	2.5	1.13±0.10^{c}	3.01±0.68^{a}
	12	0.13^{c}	0.34	0.27	2.6	0.92±0.06^{d}	2.62±0.12^{b}
25.0	2	0.24^{b}	0.32	0.46	1.5	1.47±0.03^{b}	3.49±0.35^{a}
	4	0.24^{b}	0.32	0.39	1.8	1.68±0.10^{a}	3.50±0.75^{a}
	8	0.22^{b}	0.30	0.33	2.1	1.40±0.08^{b}	3.45±0.68^{a}
	12	0.25^{b}	0.29	0.32	2.2	1.30±0.04^{bc}	3.10±0.52^{a}
50.0	2	0.33^{a}	0.22	0.40	1.7	1.35±0.10^{bc}	2.65±0.67^{b}
	4	0.35^{a}	0.23	0.36	1.9	0.98±0.15^{d}	1.60±0.64^{c}
	8	0.43^{a}	0.28	0.34	2.0	0.93±0.07^{d}	1.45±0.38^{c}
	12	0.38^{a}	0.25	0.34	2.0	0.76±0.09^{d}	1.25±0.30^{c}

μ = Specific growth rate; t_d = Doubling time, DW = Dry weight.

Mean values ± SD in the same column with the same lowercase letter are not significantly different at $P < 0.05$ by Duncan's multiple range test (n =5).

Biomass DW increment for different inoculum numbers with a constant medium volume was only observed with 1 to 2 inoculum root tips, and the addition of more root tips did not significantly influence the final biomass productivity. In addition, *P. minima* root cultures do not appear to display a minimum inoculation density as observed for most plant cell suspensions (Jualang *et al*., 2005; Mathur and Shekhawat, 2013). Hence, it is possible to conduct growth studies at very low densities (Neelwarne, 2012). Increasing the number of inoculum root tips with a constant medium volume would be expected to promote more rapid conditioning of the medium and therefore, better growth, yet the experimental results showed the opposite relationship between the root growth and the number of inoculum tips (Ono and Tian, 2011; Saravanakumar *et al*., 2012). The absence of a lag phase in the cultures even when inoculated with the smallest number of tips supported the conclusion that 'medium conditioning' effects do not play a significant role (Kanokwaree and Doran, 1997a) in *P. minima* hairy root cultures. Biomass DW production in 25.0 mL (0.24 g per flask) was about 82.7% higher compared to that in 12.5 mL (0.13 g per flask). However, when the volume doubled from 25.0 ml to 50.0 mL, the increase was only 44.0%. In terms of economic processes, high yields of biomass with lower medium support would reduce the maintenance cost (Ono and Tian, 2011; Georgiev *et al*., 2012). The ratio of the biomass DW to the total initial sucrose was also reduced when the medium volume increased from 12.5 mL to 50.0 mL and the inoculum size increased from 2 to 12 root tips. This result suggested that an increase in the medium volume up to the optimum conditions would reduce the growth efficiency with respect to substrate utilization.

Specific physalin B and F production were also significantly affected by the number of inocula and the medium volume. The yields of physalin B and F reduced up to 25 and 20%, respectively, when the number of inocula increased from 4 to 12 root tips and the medium volume doubled from 25 mL to 50 mL. Taking the biomass DW yield

into account, the highest physalin B and F yields (17.6 and 38.4 mg.L^{-1} culture, respectively) were obtained in cultures containing 25.0 mL medium inoculated with 4 root tips. This result was in line with ginsenosides production in *Panax ginseng* hairy root culture (Jeong *et al*., 2009).

CONCLUSION

This work demonstrated that the presence of lateral branches with or without root tips used as inoculum has negligible influence on growth and the production of physalins in *P. minima* hairy roots. However, excising the primary and lateral root tips reduced μ and the total root length but not the final biomass. Physalin B and F were mostly accumulated in the mature root tissues (1.55 and 3.74 mg.g^{-1} DW, respectively) rather than in the root tips (0.65 and 1.47 mg.g^{-1} DW, respectively). Increasing the number of inoculum root tips from 2 to 12 and increasing the medium volume significantly reduced the μ and t_d values. However, decreasing the medium volume with a small number of inocula reduced t_d, improved the biomass and production of physalins and μ. Using a 100 mL flask size, 25 mL of medium with four root tips was the optimum condition for growth and physalin production. Hairy root morphology type A showed easier and more homogeneous production among the replicates. In addition, culture A produced the same performance as cultures C and E in terms of the biomass and production of physalins. Thus, it was recommended that 25 mL of medium with four root tips of morphology type A should be used as the optimum conditions for shake flask cultures.

ACKNOWLEGEMENTS

The authors thank the Malaysian Government for providing scholarships during this research study. The assistance of Prof. Dr. Masao Kawai, Department of Applied Chemistry, Nagoya Institute of Technology, Japan is acknowledged for providing the reference physalins and guiding the HPLC analysis.

LITERATURE CITED

Antoun, M.D., D. Abramson, R.L. Tyson, C.J. Chang, J.L. McLaughlin, G. Peck and J.M. Cassady. 1981. Potential antitumor agents XVII Physalin B and 25,26-epidihydrophysalin C from *Witheringia coccoloboides*. **J. Nat. Prod.** 44: 579–585.

Aoki, A. and K. Syōno. 2000. The roles of Ri *rol* and Ng *rol* genes in hairy root induction in *Nicotiana debneyi*. **Plant Sci.** 159(2): 183–189.

Ashok Kumar, G., K.P. Shivalinge Gowda and C.M. Mahesh. 2010 Antiulcer effect of aqueous extract of *Physalis minima* in ethanol induced acute gastric ulcer in rats. **J. Pharm. Res.** 3(4): 671–674.

Bettini, P., R. Baraldi, F. Rapparini, L. Melani, M.L. Mauro, D. Bindi and M. Buiatti. 2010. The insertion of the *Agrobacterium rhizogenes rolC* gene in tomato (*Solanum lycopersicum* L.) affects plant architecture and endogenous auxin and abscisic acid levels. **Sci. Hortic.-Amsterdam** 123(3): 323–328.

Bhadra, R. and J.V. Shanks. 1995. Statistical design of the effect of inoculum condition on growth of hairy root cultures of *Catharanthus roseus*. **Biotechnol. Tech.** 9: 681–686.

Burkill, I.H. 1966. **A Dictionary of the Economic Products of Malay Peninsula, Vol II**. Ministry of Agriculture and Cooperatives. Kuala Lumpur, Malaysia. 2443 pp.

Carvalho, E.B., S. Holihan, B. Pearsall and W.R. Curtis. 1997. Effect of root morphology on reactor design and operation for production of chemicals, pp. 151–167. *In* P. Doran, (ed.). **Hairy Roots Culture and Applications**. Harwood Academic Publishers. Amsterdam, Netherlands.

Chiang, H.C., S.M. Jaw and P.M. Chen. 1992a. Inhibitory effects of physalin B and physalin F on various human leukemia cells *in vitro*. **Anticancer Res.** 12: 1155–1162.

Chiang, H.C., S.M. Jaw, C.F. Chen and W.S. Kan. 1992b. Antitumor agent, physalin F from *Physalis angulata* L. **Anticancer Res.** 12: 837–844.

Cui, X.H., H.N. Murthy, Y.X. Jin, Y.H. Yim, J.Y. Kim, K.Y. Paek. 2011. Production of adventitious root biomass and secondary metabolites of *Hypericum perforatum* L. in a balloon type airlift reactor. **Bioresource Technol.** 102(21): 10072–10079.

Doran, P.M. 1994. Production of chemicals using genetically transformed plant organs. **Ann. NY. Acad. Sci.** 745: 426–441.

Elisalva, T.G., S.L. Milena, A.S. Luana, M.R. Ivone, B.C.T. Therezinha, R. Ribeiro dos Santos, L.C. Washington dos Santos and B.P.S. Milena. 2009. Activity of physalins purified from *Physalis angulata* in *in vitro* and *in vivo* models of cutaneous leishmaniasis. **J. Antimicrob. Chemoth.** 64: 84–87.

Falk, L.R. and P.M. Doran 1996. Influence of inoculum morphology on growth of *Atropa belladonna* hairy roots and production of tropane alkaloids. **Biotechnol. Lett.** 18: 1099–1104.

Gamborg, O.L., R.A. Miller and K. Ojima. 1968. Nutrient requirement of suspension cultures of soybean root cells. **Exp. Cell Res.** 50: 151–158.

Gangopadhyay, M., S. Dewanjee, D. Chakraborty and S. Bhattacharya. 2011. Role of exogenous phytohormones on growth and plumbagin accumulation in *Plumbago indica* hairy root and conservation of elite root clones via synthetic seeds. **Ind. Crop. Prod.** 33(2): 445–450.

Georgiev, V.G., T. Bley, and A.I. Pavlov. 2012. Bioreactors for the cultivation of red beet hairy roots, pp. 251–281. *In* B. Neelwarne, (ed.). **Red Beet Biotechnology**. Springer-Verlag. USA.

Guillon, S., J. Trémouillaux-Guiller, P.K. Pati, M. Rideau and P. Gantet. 2006. Hairy root research: Recent scenario and exciting prospects. **Curr. Opin. Plant Biol.** 9(3): 341–346.

Hemerson I.F.M., R.T. Márcia, L.V. Costa-Lotufo, M.O. De Moraes, C. Pessoa, M.L. Veras, O.D. Loiola Pessoa, E.R. Silveira and A.P.N. Nunes Alves. 2006. *In-vitro* and *in-vivo* antitumour activity of physalins B and D from *Physalis angulata* L. **J. Pharm. Pharmacol.** 58(2): 235–241.

Hilton, M.G. and M.J.C. Rhodes. 1993. Factors affecting the growth and hyoscyamine production during batch culture of transformed roots of *Datura stramonium*. **Planta Med.** 59: 340–344.

Hjortso, M.A. 1997. Mathematical modeling of hairy roots growth, pp. 169–178. *In* P.M. Doran, (ed.). **Hairy Roots Culture and Applications**. Harwood Academic Publishers. Amsterdam, Netherlands.

Hsu, C.C., Y.C. Wu, L. Farh, Y.C. Du, W.K. Tseng, C.C. Wu, F.R. Chang. 2012. Physalin B from *Physalis angulata* triggers the NOXA-related apoptosis pathway of human melanoma A375 cells. **Food Chem. Toxicol.** 50(3–4): 619–624.

Hu, Z.B. and M. Du. 2006. Hairy root and its application in plant genetic engineering. **J. Integr. Plant Biol.** 48(2): 121–127.

Januário, A.H., E. Rodrigues Filho, R.C.L.R., Pietro, S. Kashima, D.N. Sato and S.C. França. 2002. Antimycobacterial physalins from *Physalis angulata* L. (Solanaceae). **Phytother. Res.** 16(5): 445–448.

Jeong, C.S., H.N. Murthy, E.J. Hahn, H.L. Lee and K.Y. Paek. 2009. Inoculum size and auxin concentration influence the growth of adventitious roots and accumulation of ginsenosides in suspension cultures of ginseng

(*Panax ginseng* CA Meyer). **Acta Physiol. Plantarum.** 31(1): 219–222.

Jualang Azlan, G., M. Marziah, M. Radzali and R. Johari. 2002. Establishment of *Physalis minima* hairy roots culture for the production of physalins. **Plant Cell Tiss. Org.** 69: 271–278.

_____. 2005. Accumulation of physalin in cells and tissues of *Physalis minima* (Linn.). **Acta Horti.** 676: 53–59.

Kanokwaree, K, and P.M. Doran. 1997a. Effect of inoculum size on growth of *Atropa belladonna* hairy roots in shake flasks. **J. Ferment. Bioeng.** 84: 378–381.

_____. 1997b. The extent to which external oxygen transfer limits growth in shake flask culture of hairy roots. **Biotechnol. Bioeng.** 55: 520–526.

Kawai, M., B. Makino, H. Yamamura and Y. Butsugan. 1996. Upon 'physalin L' isolated from *Physalis minima*. **Phytochemistry** 43: 661–663.

Kino-oka, M., Y. Hitaka, K. Ninomiya, M. Taya and S. Tone. 1999. Segmentation of plant hairy roots promotes lateral root emergence and subsequent growth. **J. Biosci. Bioeng.** 88: 690–692.

Kirtikar, K.R., B.D. Basu and I.C.S. An. 1975. **Indian Medicinal Plants, Vol, 3.** 2nd ed. Bishen Singh Mahandra Pal Singh and Periodical Experts. New Delhi, India. 2793 pp.

Kuźma, L., E. Bruchajzer, and H. Wysokińska. 2009. Methyl jasmonate effect on diterpenoid accumulation in *Salvia sclarea* hairy root culture in shake flasks and sprinkle bioreactor. **Enzyme Microb. Tech.** 44(6–7): 406–410.

Lenk, F., M. Vogel, T. Bley and J. Steingroewer. 2012. Automatic image recognition to determine morphological development and secondary metabolite accumulation in hairy root networks. **Engin. Life Sci.** 12(6): 588–594.

Makhzoum, A.B., P. Sharma, M.A. Bernards, and J. Trémouillaux-Guiller. 2013. Hairy roots: An ideal platform for transgenic plant production and other promising applications. **Recent Adv. Phytochem.** 42: 95–142.

Mathur, S., and G.S. Shekhawat. 2013. Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: An *in vitro* approach for production of stevioside. **Acta Physiol. Plant.** 35(3): 931–939.

Nayeemulla, S., M.S. Sudarshana, S. Umesha and P. Hariprasad. 2006. Antimicrobial activity of *Rauvolfia tetraphylla* and *Physalis minima* leaf and callus extracts. **Afr. J. Biotechnol.** 5(10): 946–950.

Neelwarne, B. 2012. Red Beet Hairy Root Cultures, pp. 199–249. *In* B. Neelwarne, (ed.). **Red Beet Biotechnology.** Springer-Verlag. USA.

Ono, N.N., and L. Tian. 2011. The multiplicity of hairy root cultures: Prolific possibilities. **Plant Sci.** 180(3): 439-446.

Ooi K.L., T.M. Tengku Sifzizul and S. Shaida Fariza. 2010. Growth arrest and induction of apoptotic and non-apoptotic programmed cell death by, *Physalis minima* L. chloroform extract in human ovarian carcinoma Caov-3 cells. **J. Ethnopharmacol.** 128(1): 92–99.

Pietro, R.C.L.R., S. Kashima, D.N. Sato, A.H. Januário and S.C. Franca. 2000. *In vitro* antimycobacterial activities of *Physalis angulata* L. **Phytomedicine** 7(4): 335–338.

Pinto, N.B., T.C. Morais, K.M.B. Carvalho, C.R. Silva, G.M. Andrade, G.A.C. Brito, M.L. Veras, O.D.L. Pessoa, V.S. Rao and F.A. Santos. 2010. Topical anti-inflammatory potential of Physalin E from *Physalis angulata* on experimental dermatitis in mice. **Phytomedicine** 17: 740–743.

Ramakrishnan, D., J. Salim and W.R. Curtis. 1994. Inoculation and tissue distribution in pilot-scale plant root bioreactors. **Biotechnol. Tech.** 8: 639–644.

Rhodes, M.J.C., R.J. Robins, J.D. Hamill, A.J. Parr, M.G. Hilton and N.J. Walton. 1990. Properties of transformed root cultures, pp. 201–225. *In* B.V. Charlwood and M.J.C. Rhodes, (eds.). **Proceedings of the Phytochemical Society of Europe Secondary Products from Plant Tissue Culture.** Clarendon Press. Oxford, UK.

Saravanakumar, A., A. Aslam. and A. Shajahan. 2012. Development and optimization of hairy root culture systems in *Withania somnifera* (L.) Dunal for withaferin-A production. **Afr. J. Biotechnol.** 11(98): 16412-16420.

Sen, G. and H.D. Pathak. 1995. Physalin L, a 13,14-*seco*-16,24 cyclosteroid from *Physalis minima*. **Phytochemistry** 39: 1245–1246.

Sethuraman, V. and N. Sulochana. 1988. The anti-inflammatory activity of *Physalis minima*. **Fitoterapia** 59: 335–336.

Silva, M.T.G., S.M. Simas, T.G.F.M Batista, P. Cardarelli and T.C.B Tomassini. 2005. Studies on antimicrobial activity, *in vitro*, of *Physalis angulata* L. (Solanaceae) fraction and physalin B bringing out the importance of assay determination. **Mem. Inst. Oswaldo Cruz.** 100(7): 779–782.

Stanbury, P.F. and A. Whitaker. 1984. **Principles of Fermentation Technology.** Pergamon Press. Oxford, UK. 255 pp.

Sun, J., J. Xiao, X. Wang, X. Yuan and B. Zhao. 2012 Improved cardenolide production in *Calotropis gigantea* hairy roots using mechanical wounding and elicitation. **Biotechnol. Lett.** 34(3): 563–569.

Tenea, G.N., A. Calin, L. Gavrila and N. Cucu. 2008. Manipulation of root biomass and biosynthetic potential of *Glycyrrhiza glabra* L. plants by *Agrobacterium rhizogenes* mediated transformation. **Rom. Biotech. Lett.** 13(5): 3922–3932.

Van Suijdam, J.C, N.W.F. Kossen and A.C. Joha. 1978. Model for oxygen transfer in a shake flask. **Biotechnol. Bioeng.** 20: 1695–1709.

Verma, P., A.K. Mathur, and K. Shanker. 2012. Growth, alkaloid production, *rol* genes integration, bioreactor up-scaling and plant regeneration studies in hairy root lines of *Catharanthus roseus*. **Plant Biosyst.** 146(1): 27–40.

Vieira, A.T., V. Pinho, L.B. Lepsch, C. Scavone, I.M. Ribeiro, T. Tomassini, R.R. Santos M.B.P. Soares, M.M. Teixeira and D.G. Souza. 2005. Mechanisms of the anti-inflammatory effects of the natural secosteroids physalins in a model of intestinal ischaemia and reperfusion injury. **Brit. J. Pharmacol.** 146(2): 244–251.

Yu, S. and P.M. Doran. 1994. Oxygen requirement and mass transfer in hairy root culture. **Biotechnol. Bioeng.** 44: 880–887.