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RESEARCH PAPER

Influence of Mist Intervals and Aeration Rate on Growth and Second Metabolite Production of *Pseudostellaria heterophylla* Adventitious Roots in a Siphon-mist Bioreactor

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Abstract Plant adventitious root culture in bioreactors is a promising alternative for the efficient production of medicinal herbs. Adventitious roots of *Pseudostellaria heterophylla* were induced from callus and then cultivated in a siphon-mist bioreactor. An orthogonal test established that the optimal medium for adventitious root induction was MS medium supplemented with 1.0 mg/L naphthaleneacetic acid and 2.0 mg/L 3-indolybutyric acid. Under these conditions, the average root number was more than 14 on each 1.0 cm diameter callus and the rooting rate reached 100%. The bioreactor was equipped with an integral siphon-spraying device designed to automatically supply the liquid medium. The operation parameters of the bioreactor were assessed by varying the mist interval and the aeration velocity. The mist interval was negatively related to average growth rate of the adventitious roots and positively related to saponin and polysaccharide content. A relatively high aeration rate was necessary to achieve the maximum biomass production, but the secondary metabolite production was not enhanced by increasing the aeration velocity.

Keywords: adventitious roots, bioreactor, mist, *Pseudostellaria heterophylla*, siphon

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1. Introduction

Pseudostellaria heterophylla is a Chinese medicine that has long been valuable in aiding the recovery from chronic illnesses. *P. heterophylla* roots sold in markets are usually collected from farms. Field cultivation is time-consuming, and the yield and quality are often visibly reduced due to virus diseases. Plant adventitious root culture in bioreactors, which has been studied for other medicinal herbs [1-5], has been considered as a promising alternative source. This potential is hampered by a lack of similar research on *P. heterophylla*.

Reactors used for adventitious roots culture can be classified as either liquid-phase or gas-phase [6]. Conventional stir-tank and bubble column reactors are liquid-phase reactors because the explants are submerged in the growth medium. Nutrient liquid, trickle-bed and nutrient mist reactors are gas-phase reactors, as explants are intermittently exposed to ambient air [7]. Compared with the liquid-phase bioreactor, the mist reactor improves gas exchange and reduces shear force on plant adventitious roots.

For *in vitro* root culture, the maximum biomass density that can be achieved is dependent on the delivery of oxygen and other nutrients into the dense matrix [8]. A study reported that normal plants grown using increasing misting times displayed higher fresh weight and the maximum amount of rooting [8]. However, *in vitro* experiments have indicated that roots are unable to use the extra nutrients supplied by longer misting to produce additional biomass, because the nutrient mist rapidly passes by the plant tissues and coalescences [9]. The gas composition and density surrounding tissues are also important in the culture and secondary metabolite productivity of *in vitro* cultivated roots [10-12]. Roots may be more capable of compen-

sating for poor liquid dispersion than for poor gas dispersion within reactor systems [13]. Thus, serial experiments were presently undertaken to optimize the culture conditions by varying the mist intervals and aeration rate.

Ultrasonic mist reactors and acoustic window mist reactors can be prone to electrical problems [7,14,15], complicated operation and high cost. In the present study, a novel siphon mist bioreactor was devised and was used for the continuous cultivation of *P. heterophylla* adventitious roots. The key component of the system is siphon spray device, which delivered automatic spraying. The results demonstrate that the bioreactor culture system is stable, convenient, energy efficient, and inexpensive.

2. Material and Methods

2.1. Obtaining aseptic seedlings

Fresh buds of *P. heterophylla* obtained from China Pharmaceutical University were surface disinfected for 8 min using a 0.1% $HgCl_2$ solution and immersion in 75% ethanol for 30 sec. After three rinses in sterile distilled water, the growth points with two leaf primordia were placed on MS medium [16] supplemented with 30 g/L sucrose, 5.5 g/L agar, and 0.5 g/L 6-benzylaminopurine (6-BA) to obtain aseptic seedlings. The medium pH was adjusted to 5.8 prior to autoclaving and the cultures were maintained at 25°C in darkness.

2.2. Induction of callus and adventitious roots

To induce callus, stem segments with a single node were inoculated on the aforementioned MS medium that was also supplemented with 2.0 mg/L 2,4-dinitrophenylhydrazine (2, 4-D). Cultures were conducted as described above. For adventitious root differentiation, callus (diameter 1.0 cm) was transferred to solid MS medium containing various concentrations of 6-BA, 3-indolybutyric acid (IBA), and naphthaleneacetic acid (NAA) (Table 1). To optimize the growth regulators, an experiment was carried out with a L_9 (3^4) orthogonal array. This experiment incorporated four variables at three different settings with three replicates. The number of adventitious roots formed in each explants was recorded 15 days after transfer and culture at 25°C in the dark.

Table 1. Effect of different phytohormone concentration on the differentiation of adventitious roots from callus

No.	A 6-BA (mg/L)	B NAA (mg/L)	C IBA (mg/L)	D Blank control	Mean number of roots from each callus
1	1 (2.0)	1 (2.0)	1 (2.0)	1	6.3 ± 0.9
2	1	2 (1.0)	2 (1.0)	2	5 ± 0.5
3	1	3 (0)	3 (0)	3	1 ± 0.4
4	2 (1.0)	1	2	3	6.3 ± 0.5
5	2	2	3	1	5.3 ± 0.3
6	2	3	1	2	5.3 ± 0.5
7	3 (0)	1	3	2	10 ± 1.6
8	3	2	1	3	14.3 ± 1.7
9	3	3	2	1	8.3 ± 0.3

The experiments were designed by using orthogonal test of L9 (3^4), and all results were repeated for three times.

2.3. Adventitious roots culture in bioreactor

Adventitious roots (10 g fresh weight) were transferred to a 500 mL flask containing 100 mL MS medium supplemented with 30 g/L sucrose, 0.1 mg/L 6-BA, and 3 mg/L IBA for proliferation. Flasks were shaken at 120 rpm in the dark. Bioreactor cultures were also performed using 4.0 L MS medium containing the same phytohormone concentration as the flask culture, using an initial inoculation density of 0.5 g/L dry weight. The bioreactor was placed in the dark at 25°C for 24 days. The schematics of the siphon mist bioreactor system are depicted in Fig. 1. This bioreactor is a compound system with a working volume of 4.8 L (total volume 6.0 L). The stir tank promotes gas-liquid mixture. Two model BT100-1F liquid pumps (Baoding Lange Constant Flow Pump, Baoding, China) separately control the rates of medium addition and liquid circulation. A 4-blade impeller provides agitation

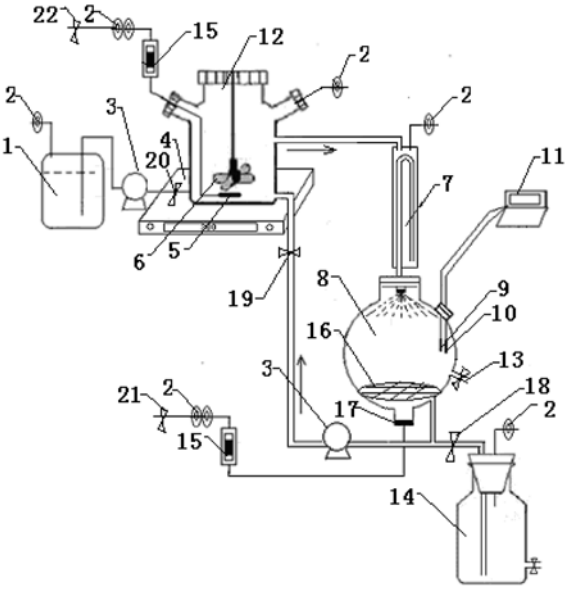

Fig. 1. Schematic diagram of the mist bioreactor system. 1. medium reservoir, 2. air filter, 3. pump, 4. magnetic churn-dasher, 5. air sparger, 6. agitator blade, react tank, 7. spray device, 8. growth chamber, 9. DO probe, 10. pH probe, 11. computer, 12. stirring tank, 13. sampling and harvest port, 14. discarded medium reservoir, 15. air flowmeter, 16. supporting net, 17. air sparger, 18 and 19. valve.

and a microporous glass flake embedded in the pipe acts as the gas sparger. A model PANsys 3000 microprocessor (Shanghai Linhai Biological Technology, Shanghai, China) monitors the dissolved oxygen (DO) and pH values. The key component of the system is a 20 mL siphon automatic spray device. The bubble column mode is used as a control. Since it proved difficult to uniformly distribute roots in the growth chamber with mist run without manual loading, the device was initially run as a bubble column (open valve 21, close valves 18, 19, 20, and 22) for 3 days to allow the roots to disperse, after which the medium in the culture chamber was drained into the agitated tank and the mist mode (open valves 18, 22, and 20; close valves 19 and 22) was initiated. When the reactor was operated in a mist mode, the exhausted medium was transferred out of the bioreactor continuously as a perfusion culture. The growth comparison between the mist mode and the bubble column mode was done by determining the changes in root dry weight during the culture period. For the mist run, the mist feeding lasted for 5 min followed by a mist-off period that depended on the rate of medium feeding. To determine the appropriate misting cycle for root growth, a series of perfusion liquid feed rates were used with different aeration rates. The mist intervals used were 30, 12, and 6 min, with corresponding respective medium feed rates of 20, 50, and 100% per day. To investigate the effect of the aeration rate on the growth and metabolite accumulation of the adventitious roots, the air flow rates were varied from 0.1 to 0.7 vvm by adjusting the gas pump.

2.4. Determination of root dry weight and average growth rate

Adventitious roots were separated from the medium by passage through a stainless steel sieve. Dry weight was measured after drying for 24 h at 60°C. The average root growth rate was calculated as:

(Maximum dry root weight – 1 Initial dry root weight) / (Initial dry root weight × Δt)

Where Δt refers to the cultivation period during which the maximum dry root mass was obtained [18]. A time-course test of perfusion was conducted for 24 days, with samples taken every 3 days. The batch culture was designed at the same mist cycle (mist feeding for 5 min followed by a 7.5 min mist-off period) and an aeration rate of 0.3 vvm as the perfusion run, except for the omission of medium exchange.

2.5. Determination of specific oxygen uptake rate (SOUR)

SOUR was measured at 25°C. Five grams of fresh root was added to a 350 mL chamber full of air-saturated water and equipped with a dissolved oxygen probe. The chamber was quickly closed with a silica gel stopper. The adventitious roots were kept in suspension using a magnetic stirring bar, and the decrease in the DO was recorded. SOUR was calculated from the root dry weight and the DO slope against time [18].

2.6. Determination of saponin and polysaccharide

To obtain the saponin content, samples of dry roots were ground to a fine powder in a mortar, and filtered through a 100-mesh sieve. Ether (100%) was added to the filtrated powder and extracted in a model SXT-02 soxhlet (Shanghai Hongji Equipment, Shanghai, China) for 120 min at 40°C. Ether was removed by evaporation and the samples were extracted for 3 ~ 4 h with methanol at 80°C. After evaporation of the solvent under reduced pressure, the methanol extract was redissolved in 30 mL distilled water followed by extraction with water-saturated n-butanol, which was removed by distillation. After redissolution in methanol and dilution to 10 mL, the absorbance of samples was measured at 560 nm using a model TJ270-30 spectrophotometer (Tianjin Top Instrument, Tianjin, China). The standard sample was a 0.952 mg/mL saponin solution of *P. heterophylla* [19]. For polysaccharide determination, 0.1 g of sample was put into a conical flask and reflux extracted with 80% ethanol for 120 min at 90°C. After immediate filtration, the residue was washed by 80% ethanol, and then extracted with 80 mL distilled water for 60 min at 90°C. The sample solution that was obtained after another filtration was adjusted to 0.5 mL. One milliliter of benzene was added to the sample along with 5 mL of oil of vitriol, followed by heating in boiling water for 15 min. The absorbance was measured at 490 nm by using a model TJ270-30 spectrophotometer (Tianjin Top Instrument). The standard was a 9.84% glucose solution [19].

3. Results and Discussion

3.1. Optimization of adventitious root induction

The developmental process of rooting generally involves root initiation and root elongation [20]. In root initiation, specific cells de-differentiate to form root meristems [21], which then elongate. Root initiation and root elongation have different optima for plant growth regulators. The optimum factorial combination for *P. heterophylla* adventitious root induction was $A_3B_2C_1$ (MS medium supplemented with 1.0 mg/L NAA and 2.0 mg/L IBA); under this condition the average rooting number reached 14.3 in each callus (Table 1).

An analysis of variance indicated that the root number increased in response to higher concentrations of IBA and NAA, but that 6-BA had a significant ($P = 0.01$) negative

Table 2. Variance analysis of adventitious roots differentiation

Source of variance	Square of deviance	Degree of freedom	F value	$F_{0.01}$
S_A	75.527	2	14.315	99.000*
S_B	18.667	2	35.421	99.000
S_C	15.86	2	30.095	99.000
S_D	0.53	2		

*Means significantly ($P < 0.01$) different. S_A, S_B, S_C, and S_D refer to the source of variances from the concentration of 6-BA, NAA, IBA and blank control, respectively.

Table 3. Adventitious roots growth and secondary metabolism under different aeration and medium feed rate

Aeration rate (vvm)	Medium feed rate (/day)	Average growth rate (g/day)	Saponins content (%)	Polysaccharide content (%)
0.1	20%	0.34 ± 0.02^{a}	0.23 ± 0.08^{a}	10.72 ± 1.83^{a}
	50%	0.37 ± 0.06^{a}	0.29 ± 0.05^{a}	9.23 ± 0.22^{a}
	100%	0.36 ± 0.03^{a}	0.22 ± 0.03^{a}	10.00 ± 1.23^{a}
0.3	20%	0.37 ± 0.01^{a}	0.25 ± 0.06^{a}	14.05 ± 0.09^{A}
	50%	0.35 ± 0.05^{a}	0.20 ± 0.1^{a}	10.3 ± 0.53^{B}
	100%	0.39 ± 0.01^{a}	0.24 ± 0.05^{a}	11.02 ± 0.63^{B}
0.7	20%	0.34 ± 0.08^{a}	0.27 ± 0.03^{a}	11.12 ± 0.62^{bA}
	50%	0.40 ± 0.03^{a}	0.26 ± 0.05^{a}	12.8 ± 0.35^{aA}
	100%	0.42 ± 0.06^{a}	0.21 ± 0.03^{a}	5.99 ± 0.44^{cB}

Within each column means followed by the same letter are not significantly different at the $p = 0.05$ level by Duncan's multiple range test.

influence on adventitious root induction (Table 2), which is consistent with other reports [22-24]. Variation in rooting performance may be genetic or can be attributed to the inhibitory effect of 6-BA on the endogenous levels of auxin present in plant cells, although details of the mechanism remain unclear.

3.2. Effect of mist cycle on adventitious root growth and secondary metabolite production

The mist cycle determines the delivery of nutrients to the roots and also controls the gas exchange in a bioreactor. A study conducted using *Aartemisia annua* determined that an extended misting period can preserve the plant roots, while short-term misting results in browning and necrosis [25]. Presently, the mist interval was negatively related to the growth rate of adventitious roots (Table 3). As the medium feeding rate increased from 20 to 100% per day, adventitious roots increased in biomass, consistent with results obtained with *Hyoscyamus niger* roots upon culture of the plants in a mist bioreactor [9]. Presently, the saponin and polysaccharide content of adventitious roots decreased with the augmentation of the perfusion medium feeding rate. A possible reason is that part of the metabolites were released into the medium and then transferred out of the bioreactor in the exhausted medium.

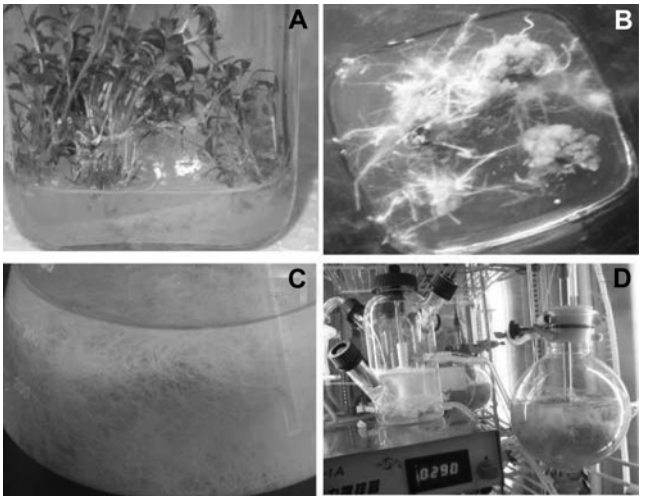

Fig. 2. Development and culture of *P. heterophylla* adventitious roots. (A) Plantlets of *P. heterophylla*, (B) adventitious roots developing from callus, (C) adventitious roots cultivated in shake flask, and (D) adventitious roots cultivated in bioreactor.

3.3. Effect of air flow rate on adventitious root growth and secondary metabolite production

Gas composition and concentration in plant cells and tissue cultures are important factors affecting plant physiology. In one study, aeration significantly affected biomass and alkaloid production of *Scopolia parviflora* adventitious roots [26]. Presently, when the bioreactor was operated at 0.5 vvm, the root balls floated and grew better. However, roots became gray in color and with no formation of tangled root balls at 0.1 vvm. Also, biomass formation increased with increased air flow rate (Table 3); thus, relatively high aeration rates were necessary to achieve maximum biomass production. Saponin and polysaccharide content of adventitious roots were not increased by an increase in the aeration from 0.1 to 0.7 vvm. The combination of high biomass increase and high metabolites accumulation remains

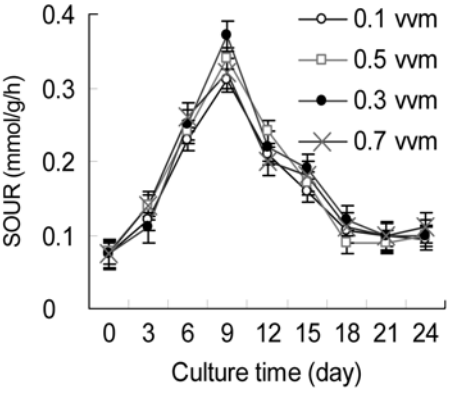

Fig. 3. SOUR of adventitious roots with different aeration rates. These experiments were conducted in perfusion mode and all determinations were repeated three times.

a challenge.

SOUR of adventitious roots increased on day 9, then declined, and finally stabilized, which paralleled the average growth rate of adventitious roots (Fig. 3). The variation in aeration from 0.1 to 0.7 vvm had no obvious effect on SOUR and secondary metabolites. The critical DO concentration can be described as DO concentration above which no further increase in SOUR can be observed [5]. When oxygen levels are below the critical DO concentration, cellular metabolism may be affected due to decreased in energy (ATP) levels [5]. In this study, the DO in the whole period with different aeration velocities remained higher than 30 percent of air saturation (data not shown), which may be higher than the critical value of hypoxia stress. Therefore, the variation in aeration from 0.1 to –0.7 vvm had no obvious effect on SOUR and secondary metabolites.

3.4. Growth comparison of *P. heterophylla* Adventitious roots between mist mode and bubble column mode

When cultured in the bubble column mode, the color of adventitious roots gradually deepened from white to grey as the root culture proceeded and then died, which might be attributed to a deficiency of mineral nutrients and oxygen. Compared with the bubble column mode, the mist run produced greater biomass (Fig. 4). For mist culture, nutrition and oxygen were continuously fed to the culture chamber through constant supplementation of fresh medium as perfusion culture.

For a bubble column run, there are "dead areas" in growth chamber and parts of the roots immersed in medium are in an oxygen-poor state for a prolonged time [27]. Mist culture was conducted to alleviate this problem. After thorough mixing, the gas-liquid mixtures were sprayed on the root surface. Intermittent spraying avoided long-time immersion of plant tissues in culture media.

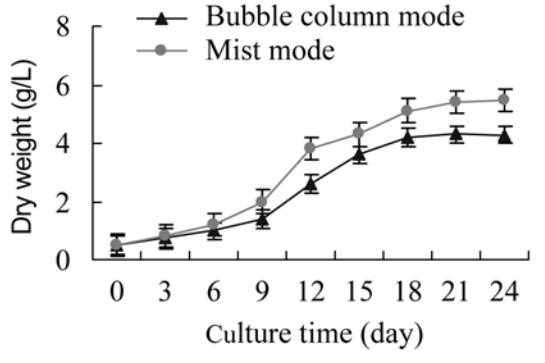

Fig. 4. Growth comparison of *P. heterophylla* adventitious roots between mist mode and bubble column mode. The mist culture with media feed rate of 50%/day was conduct under the same aeration rate (0.3 vvm) and mist cycle (mist-off interval of 7.5 min) as bubble column run. All determinations were repeated three times.

As the root biomass increased during culture, the headspace of the vessel decreased and the subsequent growth rate of adventitious roots decreased. In continuous cell and tissue cultures, manipulation of the biomass harvest is effective in achieving high growth rates and maintaining them at a constant density [28]. In this study, the harvest pipe was designed in the bioreactor, but no further research was conducted.

Of the reactors available to culture roots, the nutrient mist reactor is unique because the gas composition and density in the reactor environment can be controlled precisely, and also the shear rate is low [29]. This bioreactor culture system is stable, convenient, and inexpensive, with potential industrial applications. For herbs used in traditional Chinese medicine, roots are often used as medicinal organs. Adventitious roots induced by *in vitro* methods display a high rate of proliferation and active secondary metabolism [30,31], and have the potential to be substitute products of mother plants.

We plan further studies on scale-up and volume increase of this bioreactor system and to apply it to achieve continuous biomass harvest.

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