Factors affecting induction of shoot cluster of *Artemisia annua*

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Abstract **Object** To study the factors affecting the induction of shoot cluster of *Artemisia annua*. **Methods** Conventional plant tissue culture technology was applied to regulate the biosynthetic metabolism of secondary metabolism product, artemisinin of *A. annua*. **Results** Effects of genotype, hormones, basal medium on the induction of *A. annua* shoot cluster were notable, while that of light intensity ranging between 1 000— 6 000 lx and temperature ranging between 20℃— 30℃ were not. Among the five *A. annua* strains used, the induction ratio of strain 025 was the best. The optimal concentration for induction of shoot cluster was 6-BA 2.0 mg/L and NAA 0.15 mg/L. Ions were also found to be very important in the shoot cluster induction and the biosynthesis of artemisnin. **Conclusion** Effective regulation of induction of *A. annua* shoot cluster and artemisinin biosynthesis can be carried out *via* the regulation of physical and chemical factors.

Key words *Artemisia annua* L.; shoot cluster; induction ratio; artemisnin

青蒿丛生芽诱导影响因素的研究

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摘 要: 目的 对影响青蒿丛生芽诱导因素进行基础性研究。方法 把常规的植物组织培养技术应用于调控青蒿中次生代谢产物青蒿素的生物合成代谢。结果 青蒿的基因型,激素和基本培养基对丛生芽的发生有显著影响,而光强在 1 000~ 6 000 lx和温度在 20℃~ 30℃对丛生芽的发生影响不大;在 5种基因型的青蒿中,025丛生芽的诱导率最高;诱导丛生芽的激素组合是 6-BA 2.0 mg/L和 NAA 0.15 mg/L;另外,离子在青蒿丛生芽的诱导和青蒿素的生物合成过程中起着非常重要的作用。结论 组织培养条件下,青蒿丛生芽的诱导及青蒿素的生物合成可以通过理化因子有效地进行调控。

关键词: 青蒿;丛生芽;诱导率;青蒿素
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1 Introduction

In recent years, applied studies have been more and more extensively used in plant biotechnology, so as to modify the plant characteristics, especially medicinal plants[1,2]. Artemisinin is a colorless, needle form of crystal, its melting point is 156 ℃— 157 ℃, its molecular formula is $C_{15}H_{22}O_5$, and it is a sesquiterpene lactone with a peroxy group[3], which is easy to decompose by heat. Artemisinin has characteristics of fast curative effects and low side-effect in malaria[4]. Currently, there are two billion people suffering from the infection of malarial parasites every year in the world. In tropical regions, especially in the Southeastern Asia and Western Africa, chloro-quine-resistant malaria had become the great enemy of public health and has also become the main cause of death[5]. Artemisinin has been a recommended medicine by the World Health Orga- nization. Artemisinin also is immunode-pressive, cell immunological enhancing, and an effector of anti-influenza virus[4].

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Recently, medicinal artemisinin was directly extracted from the leaves and buds of *Artemisia annua* L. The extractive was done with complex links, taking much time and great effort the quality of *A. annua* in different regions also made a great difference. Although artemisinin could be synthesized artificially, it could not be put into production due to high cost and strong side-effect. The methods of callus and cell culture have been tested, but the synthesis of artemisinin, because of the nondifferentiation status of its cells, could only be achieved after tissue differentiation. The experimental results were disappointing[6]. Woerdenbag used buds for cultivation. The content of artemisinin could be up to 0.08% of dry weight of the culture, but the growth cycle would last weeks[7]. In this paper, by using the explants of *A. annua*, the shoot cluster culture system was established and also the existence of artemisinin in the cultures was detected, which probably will be a new method for the industrialized production of artemisinin.

2 Materials

Plant materials. High artemisinin producing strains 001, 014, 021, 025, 032 were collected from Sichuan Province of China. The seeds were germinated in agar aseptically and sterile seedlings of *A. annua* were cultured on the Murashige and Skoog[8] basal medium with 0.7% Sigma agar containing 3% sucrose in growth chambers at 26 ℃ for 16 h day length. Sterile seedlings were used for inducing shoot clusters in MS basal medium supplemented with hormones in different concentrations (Table 1).

Table 1 Effect of plant hormones in different concentration induced ratio of *A. annua* on multiple shoots

Hormones /(mg·L^{-1})						Explants[a] and multiple shoots formation explants with shoots /%[b]			
6-BA	KT	ZT	2,4-D	NAA	IBA	LD[c]	L	LS	SF
0.0	0	0	0	0	0	0	0	0	0
0.4	0	0	0	0.8	0	75.2	68.6	76.0	48.2
0.8	0	0	0	0.4	0	89.1	80.3	91.1	56.5
1.0	0	0	0	0.2	0	93.5	85.8	94.4	63.3
2.0	0	0	0	0.15	0	96.8	89.4	95.8	68.7
2.0	0	0	0	0.1	0	97.4	92.5	97.8	74.2
2.0	0	0	0	0.05	0	98.7	93.9	99.8	76.1
2.0	0	0	0	0	0	79.2	72.5	83.4	50.0
1.0	0	0	0	0	0	48.2	44.7	55.7	38.1
0	1.0	0	0	0.05	0	72.1	65.2	76.0	55.0
0	1.0	0	0	0.5	0	68.4	61.3	71.8	46.3
0	2.0	0	0	0.1	0	62.2	54.3	66.6	42.2
0	4.0	0	0	0.2	0	53.2	48.9	55.4	38.9
0.8	0	0	0.1	0	0	86.3	81.5	89.5	75.8
1.0	0	0	0.5	0	0	78.5	72.3	81.7	67.4
2.0	0	0	1	0	0	66.2	62.8	69.9	57.8
0	0	1.0	0	0.1	0	80.2	76.4	82.5	64.1
0	0	2.0	0	0.2	0	78.5	73.1	80.6	69.7
0	0	4.0	0	0.5	0	74.1	70.4	78.0	65.8
1.0	0	0	0	0	0.5	56.3	54.0	59.7	50.9
1.0	0	0	0	0	1.0	45.2	40.2	49.8	36.3
2.0	0	0	0	0	0.5	60.9	57.4	63.3	55.4
2.0	0	0	0	0	1.0	58.4	57.6	64.1	56.5

[a] All results were analyzed from 200- 300 explants [b] Data were taken after 4 weeks of culture in every case

[c] LD, L, LS, and SF represented leaf disc, leaves, leaf with leafstalk and stem fragment

3 Methods

3.1 Measurement of shoot cluster growth: Four shoot clusters, about 0.5 g (fresh weight, FW) induced in the same condition were inoculated into each triangle flask weighted in advance (W_1). The triangle flask after inoculating was weighed (W_2), the difference of the two weight is that of inoculation material ($\Delta W = W_2 - W_1$). FW of shoot cluster was obtained in the same growth condition, the fresh shoot cluster was dried at 50 ℃ and oven

dried to constant weight, and the dry weight (drying weight, DW) was got so the DW of the inoculation could come up in the ratio of FW to DW (FW/DW). The shoot clusters were sub cultured for 4 weeks later and harvested after cultured in the subculture medium for 6 weeks.

Accounting method for the shoot clusters growth:

Growth increment (GI, g/flask) = Harvest weight (HW, g/flask, DW or FW) - Inoculating weight (IW, g/flask, DW or FW)

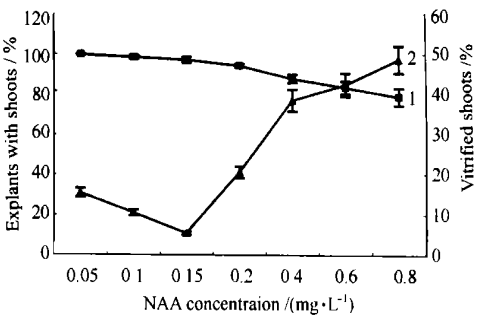
3.2 Measurement of light and temperature effect on induction of shoot clusters: Solid induction medium 30— 40 mL was contained and 28 leaf discs were inoculated in each 90 mm cultivation disc. 1. To leave the disc in growth chamber at (25± 1) ℃ for 16 h day length with 1 000, 2 000, 3 000, 4 000, 5 000, and 6 000 lx light intensity and darkness separately, then analyze the effect of light on shoot cluster induction ratio; 2. To leave the disc in growth chamber at 3 000 lx light intensity for 16 h day length at 15℃, 20℃, 25℃, 30℃, 35℃, 40℃ separately, then analyze the effect of temperature on shoot cluster induction ratio. Best growth of 4 weeks old shoot clusters were used for subculture test.

3.3 Measurement of different factors in the effect on growth and artemisinin biosynthesis of shoot clustres: Clusters 3— 4 of induced shoot cluster were inoculated into solid subculture medium with different combination. Cultivated in growth chamber at (25± 1) ℃ for 16 h day length with 1 000 lx light intensity. Effect on the growth of shoot clusters was concluded according to the GI difference. In the same time, content of artemi- sinin was measured. So differ- ent factors in different concentration affecting the growth and artemisinin biosynthesis could come out.

3.4 Measurement of artemisinin content: UV artemisinin determination was performed as described by Shen *et al*[9].

4 Results

4.1 Effects of different hormone concentration on induction ratio of shoot clusters: Put leaf discs, leaves, leaves with stems and stem fragments from seedlings of 2— 3 weeks old into the MS medium supplemented with different hormones at different concentration (Table 1). Results indicated that shoot cluster induction ratio was best using 6-BA and NAA combination to different explants. Results also told us that shoot clusters induction ratio could reach to 98.7%, 97%, 96.8% when 6-BA 2.0 mg/L was combined with NAA 0.05 mg/L, NAA 0.1 mg/L, and NAA 0.15 mg/L, respectively. Further study showed that growth of shoot cluster was good, but verification at differ- ent degrees was found when concentration varied (Fig. 1). Verification ratio of shoot cluster was about 15.4%, 10.5%, and 5.4% when NAA concentration was 0.05, 0.1, and 0.15 mg/L res- pectively. So combination of 6-BA 2.0 mg/L and NAA 0.15 mg/L was preferred when induc- tion and verification ratio were taken into account. In this paper, MS medium supplemented with 6-BA 2.0 mg/L and NAA 0.15 mg/L was called M-I1.



1-explants with shoots /% 2-vitrified shoots /%

Fig. 1 Effects of different NAA concentration in combination with 6-BA at 2.0 mg/L on shoot cluster induction ratio and vitrified shoot ratio of *A. annua*

4.2 Effects of *A. annua* with different genotype on induction ratio of shoot clusters: Put leaf disc explants of different genotype at same age into M-I1 medium, the results were included in Fig. 2, Fig. 2 revealed that shoot cluster induction ratio of 025 strain could be 98%, that of 032 line about 80%, and that of the three other lines between 60%— 70%. Results indicated that the same genus plants with different genotype possessed different perception ability to the same exogenous hormone.

So in order to get efficient induction ratio of shoot cluster, different hormone combination should be used to induced shoot cluster according to different genotype plant of the same genus.

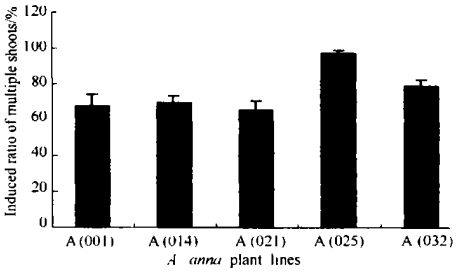


Fig. 2 Shoot cluster induction ratio of *A. annua* with different genotype

4.3 Effects of different explant age on induction ratio of shoot clusters Put leaf disc explants from different part of 025 line of 1— 8 weeks old, respectively into M-I1 medium, the results displayed in Fig. 3. Fig. 3 demonstrated that induction ratio of shoot cluster in M-I1 medium could reach up to more than 95% using explants between 1 and 8 weeks old, which indicated that the age of seedlings had less effect on the induction of shoot clusters. Fig. 4 a and b demonstrated that induction ratio was the same, almost 100%, using young and old seedlings as explants.

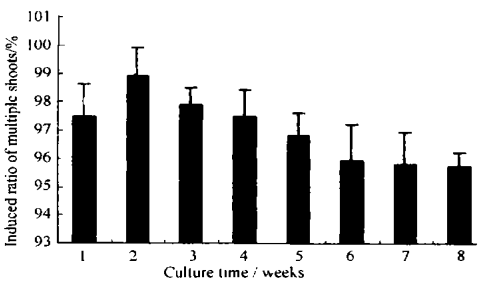


Fig. 3 Effects of culture time of explant plant on shoot cluster induction ratio of *A. annua*

4.4 Effects of light and temperature on induction ratio of shoot clusters Laid leaf disc explants on M-I1 medium in different culture condition from darkness to 6 000 lx light intensity, at 25 ℃, effect of light intensity on induction ratio of shoot cluster was recorded in Fig. 5. Results indicated that shoot cluster induction ratio of M-I1 medium was almost dependent on light intensity. Just when in darkness, induction ratio of shoot cluster

a. shoot cluster induction from leaf disc explants aged 3 weeks; b. shoot cluster induction from leaf disc explants aged 8 weeks; c. shoot cluster induced in light; d. shoot cluster induced in darkness; e. shoot cluster induced from leaf disc of seedlings with normal culture condition; f. shoot cluster induced from leaf disc of seedlings with continuous light culture condition; g. shoot cluster induced from leaf disc of seedlings treated at normal light length anew after continuous light culture

Fig. 4 Shoot cluster induction

was a little lower than that induced in light, also could be more than 80%. Shoot cluster induced in darkness grew faster than that in light, but shoot clusters in darkness was slimmer and each shoot cluster contained fewer little shoot compared with that of in light (Fig. 4 c and d). Also, laid leaf disc explants on M-I1 medium in different culture

condition from 15 ℃ to 40 ℃, in 6 000 lx light intensity, effect of temperature on induction ratio of shoot cluster was recorded (Fig. 6). Results showed that too high or low temperature had strong effect on induction ratio of *A. annua* shoot cluster, while the induction ratio was the highest, up to 98%, at 25 ℃. Further research indicated that variation ratio of ± 3 ℃ had little effect on the induction of shoot cluster.

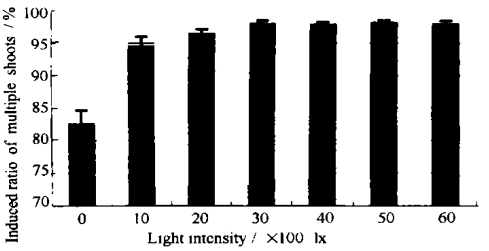


Fig. 5 Effects of light intensity on shoot cluster induction ratio of *A. annua*

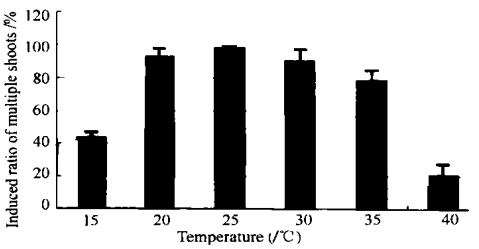


Fig. 6 Effects of temperature on shoot cluster induction ratio of *A. annua*

4.5 Effect of explants from seedlings treated in different light length on the shoot cluster induction ratio. Before inoculation, put sterile seedlings in growth chamber with different light length 8, 12, 16, 20 for 24 h at 25 ℃ for 4 weeks. Result of shoot cluster induction was included in Table 2. Table 2 revealed that continuous light on seedlings could badly restrain the occurrence of shoot clusters, even the 6-BA concentration in the induction medium was increased up to 8 mg/L, but result was unalterable. However, treating the seedlings exposed by long light length in the growth chamber for 2 weeks at 16 h light length, shoot clusters could be induced from leaf disc explants (Fig. 4 e, f, and g). Probable reason of the result is that continuous light inhibited the expression of cytokinin receptor or receptor-related gene in *A. annua* seedlings, so the leaf disc exhibited defects in exogenous cytokinin-mediated shoot induction. Yet when normal light length resumed, the restrainability of cytokinin receptor or receptor-related gene expression was released from the continuous light and the shoot cluster could also be induced successfully. In *Arabidopsis* study, two mutants crel and wol also were found to exhibit defects in cytokinin-mediated shoot induction from callus and root vascular morpho- genesis[10,11], which demonstrated that extracellu- lar cytokinin could activated AHK4, a cytokinin receptor. In addition, explants from seedlings in relative short light length cultivation could lead to low induction ratio of shoot cluster. Maybe genetic, genomic, and biochemical experiments will elucidate the details in cytokinin perception and target gene expression essential in formation of shoot cluster.

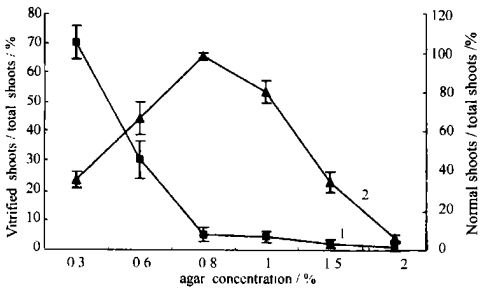
Table 2 Effect of light cycle on shoot cluster induction of *A. annua*

Leaf disc LL*** / h	Seedlings LL*** / h				
	8	12	16	20	24
8	62* /126**	79/130	94/139	76/132	0/132
12	68/125	86/132	121/136	108/134	0/135
16	76/128	92/129	139/142	124/129	5/178
20	75/126	93/130	121/128	111/123	2/154
24	70/129	89/125	112/118	109/119	1/128

* Inducing multiple shoot number; ** Explant number; *** LL-light length

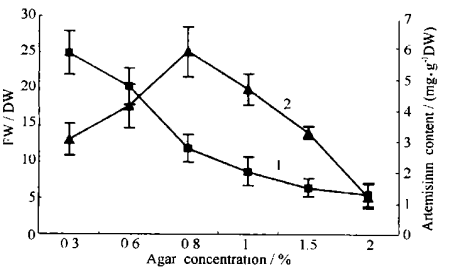
4.6 Effect of agar concentration on the induction of shoot cluster. Agar was used as not only solidifying material in medium in common use but also regulator to regulate the humidity in culture flask. Because, verified shoots were detained by agar in tissue culture efficiently through regulated humidity in medium[12]. So, in present study, agar was used as a regulator to regulate the induction, growth of shoot cluster, and artemisinin biosynthesis. Fig. 7 and Fig. 8 elucidated that 0.8% agar concentration favorited the induction and growth of shoot cluster, normal shoot cluster ratio was about 95%. When the concentration of agar was too high, shoot cluster was short of water and verification ratio was low, moreover, shoot cluster would scorch rapidly. When the concentration ratio was too low, ratio of FW/DW would be over

20, shoot cluster showed water-logged station and verifi- cation was serious. Determination of artemisinin suggested that artemisinin content be the highest in 0.8% agar concentration when shoot cluster ratio of FW/DW was about 10, which indicated that biosynthesis of artemisinin was closely related to the growth of shoot cluster. It seemed complicated why verification was decreased when the concentration of agar was increased. Debergh *et al*.[12] found that the increase of agar could lead to remarkable increase of Ca^{2+}, K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Cu^{2+}.



1-vitrified shoots/total shoots /%
2-normal shoots/total shoots /%

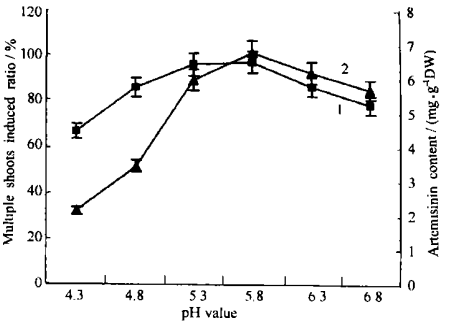
Fig. 7 Effect of agar on growth of *A. annua* shoot cluster



1-FW/DS 2-artemisinin content

Fig. 8 Effect of agar concentration on growth and artemisinin biosynthesis of *A. annua* shoot clusters

4.7 Effect of pH value on the shoot cluster induction and artemisinin biosynthesis Inoculated leaf discs in induction medium with different pH value, result was summarized in Fig. 9. Fig. 9 elucidated that the induction of shoot cluster was improved when pH value was between 5.5 and 5.8. However induction of shoot was restrained when pH value was higher than 5.8, or lower than 5.5. Biosynthesis of artemisinin could be promoted when pH value of medium was 5.8, other pH values inhibited artemisinin biosynthesis in differ- ent degree, especially lower than 5.3.



1-induced ratio of multiple shoots /% 2-artemisinin/(mg· g⁻¹)

Fig. 9 Effect of initial pH value on shoot cluster induction ratio of *A. annua* and artemisinin biosythesis

5 Discussion

Optimal physical and chemical factors of affecting the induction of *A. annua* shoot clusters were found in the present study, MS basal medium supplemented 6-BA 2.0 mg/L, NAA 0.15 mg/L, 0.8% agar, and pH 5.8 was found to be efficient in growth chamber in 16 h light length, 3 000 lx light intensiy, and at 25℃. Seedlings used as explants should be treated in about 16 h light length 2 weeks before induction of shoot clusters. Only in this condition, could shoot cluster grow well and production of artemisinin also be im- proved. In previous study, the rule that the biosynthesis of artemisinin could only be achieved after a certain degree of differentiation for the *A. annua* tissue was revealed[1,13]. The synthesis of artemisinin has been studied in suspension cell[14], callus[6,15], shoot[16], and hairy root culture[17]. All related studies indicated that the artemisinin production obtained by dedifferentiated cultures of *A. annua* is very poor, a certain degree of develop-ment is a prerequisite for artemisinin biosynthesis. In the present study, shoot clusters in optimal condition could also become differen- tiated and synthesis artemisinin. And shoot clusters in different growth stage and culture condition also produced different content of artemsinin. Further study of *A. annua* shoot cluster will be to discover proper medium for the growth of shoot cluster and biosynthesis of artemisinin respectively, which maybe the founda-

tion of the industrialized pro- duction of artemisinin through two-step method.

References

[1] Geng S, Ma M, Ye H C, *et al*. Effects of *ipt* gene expression on the physiological and chemical characteristics of *Artemisia annua* L. [J]. *Plant Sci*, 2001, 160: 691-698.

[2] Geng S, Ye H C, Li G F, *et al*. Flowering of *Artemisia annua* L. test-tube plantlets and artemisinin production with shoot clusters induced from flower organ explants [J]. *Chin J Appl Environ Biol* (应用与环境生物学报), 2001, 7(3): 201-206.

[3] Klayman D L. Qinghaosu (artemisinin): an antimalarial drug from China [J]. *Science*, 1985, 228: 1049-1055.

[4] Gabor A B. Artemisinin and its derivatives, an important new class of antimalarial agents [J]. *Pharmacol Therapeut*, 2001, 90: 261-265.

[5] WHO. Roll Back Malaria. *A Global Partnership* [M]. Geneva: World Health Organization, 1998.

[6] He X C, Zeng M X, Li G F, *et al*. Induction and differentiation of *Artemisia annua* L. calli and variation of artemisinin content [J]. *Acta Bot Sin* (植物学报), 1983, 25(1): 87-90.

[7] Woerdenbag H J, Luers J F J, Uden W Van, *et al*. Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. [J]. *Plant Cell Tiss Organ Cult*, 1993, 32(3): 247-257.

[8] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures [J]. *Physiol, Plant*, 1962, 15: 473-497.

[9] Shen X K, Yan K D, Luo Z Y. Determination of artemisinin content by ultraviolet meter [J]. *Chin Pharm Anal* (药物分析杂志), 1983, 3(1): 24-26.

[10] Inoue T, Higuchi M, Hashimoto Y, *et al*. Identification of CRE1 as a cytokinin receptor from *Arabidopsis* [J]. *Nature*, 2001, 409(6823): 1060-1063.

[11] Mahonen A P, Bonke M, Kauppinen L, *et al*. A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root [J]. *Genes Dev*, 2001, 14(23): 2938-2943.

[12] Debergh P C. Effects of agar brand and concentration on the tissue culture medium [J]. *Physiol Plant*, 1983, 59: 270-276.

[13] Ferreira J F S, Janick J. Production and detection of artemisinin from *Artemisa annua* [J]. *Acta Hort*, 1995, 390: 41-49.

[14] Nair M S R, Acton N, Klayman D L, *et al*. Production of artemisinin in tissue culture of *Artemisia annua* [J]. *J Nat Prod*, 1986, 49(4): 504-507.

[15] Tawfiq N K, Anderson L A, Robrets M F, *et al*. Antiplasmodial activity of *Artemisia annua* plant cell cultures [J]. *Plant Cell Rep*, 1989, 8(5): 425-428.

[16] Fulzzle D P, Sipahimalani A T, Heble M R. Tissue culture of *Artemisia annua*: organogenesis and artemisinin production [J]. *Phytother Res*, 1991, 5: 149-153.

[17] Qin M B, Li G Z, Ye H C, *et al*. Induction of hairy root from *Artemisia annua* with *Agrobacterium rhizogenes* and its culture *in vitro* [J]. *Acta Bot Sin* (植物学报), 1994, 36: 165-170.

特异性真菌作用于龙血树材质形成血竭的研究

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摘 要: 目的 探讨剑叶龙血树血竭的形成与微生物活动之间的关系。方法 用分离自剑叶龙血树根部的内生真菌 9568D镰孢霉接种于剑叶龙血树材质(经灭活处理)。结果 保湿培养 4~ 5个月后,在接种部位有红色血脂颗粒形成,经 UV、IR光谱分析及抗菌活性实验,初步证实该血脂与来自剑叶龙血树的天然血竭无本质差异。结论 特异性真菌作用于龙血树材质可促成血竭的形成。

关键词: 9568D镰孢霉;龙血树材质;血竭

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Study on formation of Dragon's blood in *Dracaena cochinchinensis* inoculated with *Fusarium* 9568D

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Key words *Fusarium* 9568D; *Dracaena cochinchinensis* (Lour.) S. C. Chen; Dragon's blood

血竭(Dragon's blood)是中药传统品种之一, 其性味甘、温、咸、平,兼有活血、化瘀、止血、补血的

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