Forest Chemicals Review
www.forestchemicalsreview.com
ISSN: 1520-0191
Nov-Dec 2021 Page No. 152-158
Article History: Received: 05 October 2021, Revised: 02 November 2021, Accepted: 25 November 2021, Publication: 31 December 2021

Induction of Adventitious Bud Regeneration from Root Explants in Sambong (*Blumea balsamifera* (L.) DC.)

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Abstract:

Root explants were used for the induction of adventitious bud regeneration in sambong (*Blumea balsamifera* (L.) DC.), and different medium formulas, such as different concentrations of Murashige and Skoog basal medium, 6-benzyladenine, naphthaleneacetic acid, and sucrose were compared for the effects in this research. The results showed that roots could be used as explants for high efficient induction of adventitious bud regeneration in sambong. The optimal medium composition for the induction of adventitious bud regeneration was Murashige and Skoog basal medium supplemented with 1.0 mg/L 6-benzyladenine, 0.05 mg/L naphthaleneacetic acid, and 30 g/L sucrose. In this study, we not only optimized the tissue culture system but also reduced the generation of chimeras in the process of tissue culture. The protocol established in the present experiment provides a technical basis for further research such as artificial induction of mutation and transgenic manipulation in sambong.

***Keywords**: Sambong; ainaxiang; Blumea balsamifera; root; adventitious buds; tissue culture*

I. INTRODUCTION

Sambong (*Blumea balsamifera* (L.) DC.), also known as ainaxiang, borneol wormwood, is a perennial herb of the Compositae family widely distributing in southeast Asian countries and many provinces such as Hainan, Guangdong, Guangxi, Guizhou, Yunnan, Taiwan in China[1, 2]. Sambong is also one of the important plant sources for obtaining natural *l*-borneol (Ai pian), and its power and volatile oil is also the raw material to produce Chinese traditional patent medicines “Golden Throat Health Spray” and “Yan Li Shuang Mouth Dropping Pills”, those output value exceeds 15 million dollars. Sambong branches, leaves, twigs, and roots are rich in volatile oil and flavonoids, and were used conveniently for medicinal purposes[3-5].

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The germination rate of tiny sambong achene is not higher than 30% and usually less than 6%, and the propagation coefficient of the traditional ramet propagation method is also not high[6]. In order to increase the propagation efficiency, Yang and Yang used stems with axillary buds as explants and tested methods for sterilization of explants, bud proliferation, and different medium formulas[7]; Yan et al. used leaf explants for the study of various factors affecting the responses in tissue cultures[8]. Tang et al. also used stem segments with axillary bud points as explants to study the effects of hormones on the callus induction, sub-generation proliferation and rooting[9]. although there have been many predecessors conducting various experiments for optimizing the tissue culture techniques in sambong, the results are still not satisfactory in increasing the growth coefficient and the survival rate of tissue culture seedlings, and can not yet meet the technical needs for large-scale propogation.

The existing technical solution for plant tissue culture in sambong is either to promote the growth of existing bud points on the stem[7, 9], or to obtain adventitious buds through callus differentiation[8]. The former method is to increase the growth of existing buds, without inducing adventitious buds, and cannot be used in biotech breeding. In the latter method, callus is induced from leaves. The callus may produce chimerism or uncontrolled mutations during the callus induction process, which increases the uncertainty of the final product[10]. Therefore, in this experiment, the root segment of the plant tissue culture seedling was used as the explant, and the adventitious bud was formed by direct differentiation through induction. The research aims to improve the efficiency of plant tissue culture, while reducing the production of chimeras during the tissue culture process, and to provide a technical basis for artificial mutagenesis and genetic modification of sambong.

II. MATERIALS AND METHODS

2.1 Materials

The test material is the sterile seedlings obtained by tissue culture of sambong plants in the resource nursery. Before induction, they were inoculated in a rooting medium based on 1/2 MS medium. The cultivation environment was: 26℃, light intensity It is 1500 Lx, and the light duration is 12 h/d.

2.2 Methods

2.2.1 Screening of Murashige and Skoog (MS) basic medium concentration

Choose MS, 1/2 MS, and 3/4 MS to inoculate the roots of the tissue cultured seedlings of Sambong in the ultra-clean workbench, inoculate 8 bottles for each concentration, and 8 roots per bottle. Section (the length of the root section is about 1.0-2.0 cm), put them into the tissue culture room after inoculation, and observe and record the culture regularly.

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2.2.2 Screening of 6-Benzyladenine (6-BA) concentration

Using the best medium selected by "1.2.1" as the basic medium, add 6-BA solution of different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L), and inoculated the sambong roots in the ultra-clean workbench. The root segments of the seedlings are transferred in, 8 bottles are inoculated at each concentration, and each bottle has 8 root segments (the length of the root segment is about 1.0-2.0 cm). After the inoculation, they were placed in the tissue culture room for culture, and the culture situation is regularly observed and recorded.

2.2.3 Screening of Naphthaleneacetic acid (NAA) concentration

Add different concentrations of NAA solution (0.025, 0.05, 0.075, and 0.1 mg/L) to the best basic medium and the best 6-BA concentration solution screened by "1.2.1" and "1.2.2", with a concentration of 0 mg/L as the control group. Transfer the root segments of the sambong seedlings in a clean bench, inoculate 8 bottles at each concentration, and 8 roots per bottle (the length of the roots is about 1.0-2.0 cm). Then put them into the tissue culture room for culture, observe and record the culture regularly.

2.2.4 Screening of sucrose concentration

Add different concentrations of sucrose solution (10, 20, 30, 50, and 70 g/L), with 0 mg/L as the control group, transfer the roots of the sambong tissue culture seedlings in a clean bench, inoculate 8 bottles at each concentration, and 8 roots per bottle (root length Approximately 1.0-2.0 cm). After the inoculation is completed, place them in the tissue culture room for culture. Observe and record the culture on a regular basis.

2.2.5 Culture conditions

The temperature of the tissue culture room was controlled at 24 ± 3 °C, the photoperiod was 12 h/d, the illumination intensity was 2000 Lx, and the relative humidity was 60%. On the 30th days post inoculation, count the number of remaining uncontaminated bottles, the number of effective root segments, and the number of adventitious buds obtained, calculate the number of buds per unit bottle and the number of buds per unit root. The formula is as follows:

(1) Valid buds No. per bottle = the total number of adventitious buds / the number of valid bottles;

(2) Valid buds No. per root segment = the total number of adventitious buds / the number of valid root segments.

III. RESULT AND ANALYSIS

3.1 The effects of different concentrations of MS basic medium on adventitious bud induced with root explants

The influence of different concentrations of MS basic medium is shown in Table 1. It was found that when MS is used as the basic medium, the total number of adventitious buds is the highest (36), the number of valid buds per bottle (7.20) and the number of valid buds per root segment (0.900) are both

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higher than those in media with other concentration of MS. By comparing the total number of adventitious shoots, the number of shoots per unit bottle, and the number of shoots per unit root, it can be known that the order of the pros and cons of each medium is MS>3/4MS>1 /2MS, it is determined that MS is the best basic medium.

TABLE 1. The effects of different concentrations of MS basic medium on adventitious bud induced with root explants

Basic medium	Bottles No.	Valid bottles No.	Valid roots No.	Adventitious buds No.	Valid buds No. per bottle	Valid buds No. per root segment
1/2MS	8	5	40	19	3.80	0.475
3/4MS	8	6	48	29	4.83	0.604
MS	8	5	40	36	7.20	0.900

3.2 The effects of different concentrations of 6-BA on adventitious bud induced with root explants

Using MS as the basic medium and adding different concentrations of 6-BA, the results of its effect on root-induced adventitious bud differentiation were shown in Table 2. When the BA concentration is 1.0 mg/L, the number of valid buds per bottle (6.20) and the number of valid buds per root segment are both the highest (0.775). In addition, when the concentration of 6-BA is 1.5 mg/L, the total number of buds is 22, and when the concentration of 6-BA is 2.0 mg/L, the total number of buds drops to 20, which is the same as the budding at the concentration of 1.0 mg/L. Comparing the numbers, the order of the size of the three is: 1.0 mg/L>1.5 mg/L>2.0 mg/L, indicating that when the BA concentration is greater than 1.0 mg/L, it will promote adventitious buds due to the high concentration of growth regulators. The effect of differentiation gradually weakened. Therefore, it is determined that the optimal concentration of 6-BA is 1.0 mg/L.

TABLE 2. Effects of different concentrations of 6-BA on adventitious bud induced with root explants

BA (mg/L)	Bottles No.	Valid bottles No.	Valid roots No.	Adventitious buds No.	Valid buds No. per bottle	Valid buds No. per root segment
0.5	8	6	48	31	5.17	0.646
1.0	8	5	40	31	6.20	0.775
1.5	8	5	40	22	4.40	0.550
2.0	8	5	40	20	3.33	0.417

3.3 The effects of different concentrations of NAA on adventitious bud induced with root explants

After confirming that MS is the best basic medium, 1.0 mg/L is the best 6-BA concentration, use this as the basic medium to add different concentrations of NAA solution, and the results of its effect on root-induced adventitious bud differentiation are shown in Table 3. When the NAA concentration is 0.05 mg/L, the total number of adventitious buds is the largest (33), the number of valid buds per bottle (6.60) and the number of valid buds per root segment are both the highest (0.825). From an overall point of view, the five NAA concentrations are 0.05 mg/L as the dividing point. When the NAA concentration is lower

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than 0.05 mg/L, the total number of adventitious buds obtained increases with the increase of NAA concentration, indicating that NAA differentiates adventitious buds at this time The promotion effect and the effect gradually increase; when the NAA concentration is higher than 0.05 mg/L, the total number of adventitious buds obtained decreases with the increase of NAA concentration, indicating that NAA promotes the differentiation of adventitious buds at this time, but the effect is gradually weakened. Therefore, the optimal NAA concentration is determined to be 0.05 mg/L.

TABLE 3. The effects of different concentrations of NAA on adventitious bud induced with root explants

NAA (mg/L)	Bottles No.	Valid bottles No.	Valid roots No.	Adventitious buds No.	Valid buds No. per bottle	Valid buds No. per root segment
0	8	5	40	18	3.60	0.450
0.025	8	6	48	23	4.60	0.575
0.05	8	5	40	33	6.60	0.825
0.075	8	4	32	22	4.40	0.550
0.1	8	5	40	19	3.17	0.475

3.4 The effects of different concentrations of sucrose on adventitious bud induced with root explants

After confirming that MS is the best basic medium, the best 6-BA concentration is 1.0 mg/L, and the best NAA concentration is 0.05 mg/L, use this as a basal medium to add different concentrations of sucrose solution, which can induce adventitious buds from root segments. The results are shown in Table 4 below. When the sucrose concentration is 30 g/L, the total number of adventitious buds is the highest (22), the number of valid buds per bottle (4.40) and the number of valid buds per root segment are both the highest (0.550). As an energy substrate and osmotic regulator, sucrose has an inducing effect on the morphogenesis of tissue cultured seedlings, but it has a certain threshold [5]. Throughout the 5 kinds of sucrose, the concentration is 30g/L as the demarcation point. When the sucrose concentration is less than 30 g/L, the higher the sucrose concentration, the greater the total number of adventitious buds; when the sucrose concentration is greater than 30 g/L, the higher the sucrose concentration, the less the total number of adventitious buds, indicating that the number of adventitious buds has exceeded the threshold of sucrose tolerance, so the optimal sucrose concentration is determined to be 30 g/L.

TABLE 4. The effects of different concentrations of sucrose on adventitious bud induced with root explants

Sucrose (g/L)	Bottles No.	Valid bottles No.	Valid roots No.	Adventitious buds No.	Valid buds No. per bottle	Valid buds No. per root segment
10	8	5	40	11	2.20	0.275
20	8	5	40	14	2.80	0.350
30	8	5	40	22	4.40	0.550
50	8	5	40	12	2.40	0.300
70	8	5	40	8	1.60	0.200

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IV. CONCLUSION

The micropropagation of sambong were usually uses stems with bud points and leaves as explants[7-9]. When using stems with bud points, no adventitious bud was induced. It's merely a process of growing up of the original bud point on the stem. When leaves were used, it needs to undergo two steps of differentiate. First, dedifferentiation and form callus and the second, differentiation and form adventitious buds. Adventitious bud induction is a complex physiological process regulated by explants, culture media, and plant growth hormones, which makes the process of inducing adventitious buds more uncertain[11]. In this paper, the roots of sambong seedlings were used as explants, and only one step of dedifferentiation was happened and directly induce the formation of adventitious buds without the need for dedifferentiation to form callus, which reduces the production of chimera during the tissue culture process of sambong.

Usually, to directly formation of adventitious buds without differentiation of callus, radicle or hypocotyl of plantlets were used as explants. For example, Liang used sterile seedling hypocotyls and radicles as explants to establish the in vitro regeneration system of *Atractylodes macrocephala*, which directly induces adventitious buds[12]. The best medium for inducing adventitious bud differentiation in this plant was determined as MS + TDZ 1.5 mg/L + NAA 0.2 mg/L, and the differentiation rate of hypocotyl adventitious bud was as high as 91.18%. Yanhong Zhang took the hypocotyls of Isatis indigotica seeds as explants, and the adventitious bud induction rate was 77. 7% at the concentration of 1.0 mg/L 6-BA + 0.5 mg/L NAA[13]. In this research, the root segments of the aseptic seedling of sambong were used as the explants. Compared with the former two, the transfer of the root segments is less difficult than the inoculation of the radicle, and the process is simpler.

There are many factors that affect plant tissue culture, such as varieties, explants, culture media, culture conditions, etc.[14]. We compared the concentration of MS basic medium, BA, NAA, and sucrose to induce adventitious bud differentiation from sterile seedling roots. To determine the best root-induced adventitious bud differentiation medium composition: MS + 1.0 mg/L 6-BA + 0.05 mg/L NAA + 30 g/L sucrose. Yang used the first-generation medium of MS + 2.0 mg/L 6-BA to induce adventitious bud grown on stems with bud points of sambong as explants[7], and Yan used sambong leaves as the primary medium to induce callus[8]. The medium used to induce callus from leaf explants is MS + 3.0 mg/L 6-BA + 0.1 mg/L NAA. Compared with the former two, the plant hormones concentration used in this article is lower. The reason is that the root segments of the sterile seedling is less resistant to plant hormones than the stem and leaves of the plant. If the plant hormones concentration are too high, the differentiation process of adventitious buds will be inhibited.

In this study, the root segments of the tissue cultured seedlings of sambong were used as explants to directly induce differentiation to form adventitious buds. In another words, the somatic cells do not need to dedifferentiate to form callus and then differentiate to form adventitious buds. Adventitious buds are directly produced by somatic cells dedifferentiation. As a result, the production of chimeras was reduced.

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There are many factors that affect the differentiation of adventitious buds, such as the culture medium, the types of plant hormones, and the concentration of hormones. In our experiments, we compared different concentration of MS media, phytohormones, and sucrose. The best medium composition for root-induced adventitious bud differentiation was determined as follows: MS + 1.0 mg/L 6-BA + 0.05 mg/L NAA + 30 g/L sucrose.

ACKNOWLEDGMENT

This work was supported by the special start-up funding project for the reform of non-profit scientific research institutions of the Institute of Tropical Crop Variety Resources, Chinese Academy of Tropical Agricultural Sciences (#PZS2021016), Hainan Province Science and Technology Project Funding (#ZDYF2020167), Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (#1630032020002), 2019 Hainan Province Basic and Applied Basic Research Program (Natural Science Field) High-level Talent Project Funding (#2019RC310), and Hainan provincial Natural Science Foundation of China (#2019CXTD414).

REFERENCES

[1] Wang H., et al., Research Progress of *Blumea balsamifera* L. DC. and Dosage form Development Value Discuss. Modern Chinese Medicine, 2014. 16(11): p. 953-956.

[2] Pang Y.X., et al., *Blumea balsamifera* - A Phytochemical and Pharmacological Review. Molecules, 2014. 19(1): p. 9453-9477.

[3] Bhuiyan N.I., et al., Chemical components in volatile oil from *Blumea balsamifera* (L.) DC. Bangladesh Journal of Botany, 2009. 38(1): p. 107-109.

[4] Ali D.M., et al., Flavonoids from *Blumea balsamifera*. Fitoterapia, 2005. 76(1): p. 128-30.

[5] Chen M., et al., Flavonoids from *Blumea balsamifera*. Natural Product Research, 2010(22): p. 991-994.

[6] Ou G.T., et al., Seedling growing method for *Blumea balsamifera* seeds. Chinese Patten, CN201310194066.4, 2013.

[7] Yang S., et al., Research on Tissue Culture Technology of *Blumea balsamifera*. Modern Agricultural Science and Technology, 2012. 18: p. 151-153.

[8] Yan M., et al., Tissue culture of Ainaxiang. Jiangsu Agricultural Sciences, 2014. 42(2): p. 33-35.

[9] Tang S., et al., Study on rapid propagation technology of *Blumea balsamifera* seedlings. Journal of Guizhou Normal College, 2015. 31(3): p. 38-40.

[10] Tilney-Bassett R., Plant Chimeras. Quarterly Review of Biology, 1986. 4.

[11] Hicks G., Shoot induction and organogenesis in vitro: A developmental perspective. In Vitro Cellular & Developmental Biology-Plant, 1994.

[12] Liang Y., et al., Direct shoot regeneration in vitro for *Atractylodes macrocephala* using epicotyl and radicle as explants. Journal of Hebei University (Natural Science Edition), 2020. 40(2): p. 171-175.

[13] Zhang Y., et al., Studies on Plant Regeneration of *Isatis indigotica* via Adventitious Bud. Modern Chinese Medicine, 2020. 22(10): p. 1679-1683.

[14] Bhoite H.A., et al., Plant Tissue Culture: A Review. World Journal of Pharmaceutical Sciences, 2014. 2(6): p. 565-572.