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Organogenesis and Regeneration of *Symplocos paniculata* From Immature Zygotic Embryos *in vitro*

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Abstract:Taking immature zygotic embryos of *Symplocos paniculata* as materials,using modified MS medium,the organogenesis and regeneration were researched. The results showed that the mMS medium could induce callus greatly,the highest induction rate 92.5% was obtained on the medium with 0.2 mg/L 6-BA+0.1 mg/L NAA. 72.4% of calluses initiated green bud-like structures when they were cultured on mMS plus 0.25 mg/L 6-BA+0.15 mg/L NAA. The calluses with green bud-like structures were subcultured onto mMS medium without plant growth regulators for 15 days,76.6% of them developed into shoots. Shoots elongated to 1.5 cm long on 1/2 mMS medium plus 1.5% sucrose 20 days after culture.

Keywords:*Symplocos paniculata*;immature zygotic embryos;organogenesis;regeneration

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Symplocos paniculata (Thunb.) Miq. belongs to *Symplocaceae*. It is native in mixed forests at an elevation of 800－2 500 m in the Himalayas,and eastern Asia,including China,Japan,and Korea[1]. Sapphire berry is a deciduous shrub or small tree with rough yellowish-brown bark,creamy white fragrant flowers,and blueberry-like fruits. This plant is known for edible and medicinal usages. The bark is used for producing a kind of yellow dye[2],and the juice of the bark is applied externally to sprains and muscular swellings[3]. The plant has been used in traditional medicines for the treatment of inflammation[4],menorrhagia,bowel complaints,eye diseases,ulcers[5],and typeⅡ diabetes[6]. It is also used as a gargle for giving firmness to spongy and bleeding gums[5]. The fruit is edible and can be used in jams,jellies,and sauce[7]. In addition,the seed has oil up to 36.6% with unsaturated fatty acids,such as oleic acid and linoleic acid[8]. This plant has recently been investigated as an ideal raw material for preparation of biodiesel[8-10]. The domestication and cultivation of this wild plant is necessary for protecting the natural genetic resources from over-exploitation.

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Propagation from seed germination requires stratification[11]. It is better to sow seeds in a cold frame in late winter,and they need 12 months to germinate[11]. Seed propagation of sapphire berry is limited due to long period of stratification,and propagation of a specific cultivar is impossible because of seedling variability. Semi-hard wood cuttings,7－10 cm long with a heel,can form roots in about 4 weeks and have a good percentage in July or August[12-13]. However,vegetative propagation of sapphire berry by cuttings may be subject to seasonal variations. There is an urgent need to develop a stable and efficient protocol for the large-scale propagation of sapphire berry in order to meet its ever-increasing demand and conserve its germplasm and genetic diversity.

Tissue culture provides a fast clonal regeneration for reforestation projects,nursery production and preservation of natural genetic resources[14]. Tissue culture of *Symplocos tetagona* had been conducted[15]. They found that the calluses formed on 1/2 MS medium supplemented with 2,4-D (2,4-Dinitrophenylhydrazine) 2 mg/L when mature zygotic embryos were cultured for 25 days,and axillary shoots developed on WPM medium plus 6-BA or kinetin (N^6-furfuryladenine) at the concentration of 0.2－1.5 mg/L. However,little information is available for plant regeneration of *Symplocos paniculata*. The aim of this study is to develop *in vitro* regeneration protocol for *Symplocos paniculata* from immature embryos.

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1 MATERIALS AND METHODS

1.1 Test materials

Green immature fruits (4－5 mm in diameter) of sapphire berry were collected in mid-to-late August (about 12 weeks after pollination) from an approximately 20-year-old superior tree grown in Dawei Mountain (Liuyang, Hunan).

The induction medium consisted of Murashige and Skoog[16] basal medium, modified MS (mMS) or woody plant medium[17] supplemented with 30 g/L sucrose, 0.65% (w/v) agar, and plant growth regulators. Modified MS (mMS) was prepared by adding 50 mg/L Na_2SO_4 in inorganic salt and reducing $Zn_2SO_4 \cdot 7H_2O$ to 8 mg/L in trace elements.

1.2 Test methods

1.2.1 Test materials treatment Fruits were thoroughly washed for 4 hours in running tap water. They were surface disinfected for 5 minutes with 10%(v/v) commercial bleach (5.25% sodium hypochlorite) and then rinsed in distilled water 5－6 times. Seeds excised from fruits were soaked in 75%(v/v) ethanol for 5 minutes, and subsequently in 50% (v/v) commercial bleach containing 0.1% Tween-20 for 15 minutes, and rinsed 5－6 times with sterile distilled water. The pericarp and testa of all seeds were removed.

1.2.2 Explants inoculation The white immature zygotic embryos (1.5－2.0 mm in length) were transferred to Pyrex glass flasks containing 25 mL induction medium. 6-BA (6-benzylaminopurine) at 0.15 mg/L, 0.20 mg/L, 0.25 mg/L or 0.30 mg/L plus 0.10 mg/L NAA (α-naphthaleneacetic acid) was tested. All media were autoclaved at 121℃ for 20 minutes after adjusting pH 5.8 using NaOH or HCl. All cultures were maintained under dark condition in a culture room at (25±2)℃. A total of 20 explants (immature zygotic embryos) were used for each treatment, and each treatment was repeated 3 times. 20 days after cultured, the quality and the presence or absence of calluses were recorded.

1.2.3 Organogenesis and plant regenerationm MS basic media supplemented with 6-BA at 0.20 or 0.25 mg/L and NAA at 0.10 mg/L, 0.15 mg/L or 0.20 mg/L were used for callus differentiation and plant regeneration. mMS without any plant growth regulators was prepared as the control. All medias were added with 30 g/L sucrose and 0.65% (w/v) agar, and pH was adjusted to 5.8. A total of 20 flasks with 5 calluses (5 mm in diameter) per flask were used for each treatment, and each treatment was repeated 3 times. 30 days after cultured under dark condition, the days for calluses to differentiate, and the presence or absence of calluses with green bud-like structures were recorded. Differentiated calluses were cultured on mMS basic medium without phytohormones under the culture room for 15 days to elongate the shoots. Regenerated shoots were transferred to the mMS basic medium at 1/2 mMS plus 15 g/L sucrose and kept in culture under light condition with a 12 hours photoperiod and 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from cool white fluorescent tubes.

1.3 Item determination

The percentage of explants with calluses and calluses with bud-like structures were calculated. The callus induction rate was calculated using the equation, induction rate(%)=(explants with calluses/total explants cultured)×100; the callus differentiation rate was calculated using the equation, differentiation rate (%) = (differentiated calluses/total calluses cultured)×100.

1.4 Data analysis

Analysis of variance (ANOVA) was performed using JMP® 9.0 (SAS Institute, Inc., Cary, NC). Tukey's Honestly Significantly Different (HSD) test at $P<0.05$ was applied for means separations.

2 RESULTS ADN ANALYSIS

2.1 Callus induction

The immature zygotic embryos began to expand 5 days after culture (Fig. 1A). Calluses with globular structures formed on the hypocotyl and radicle of zygotic embryos 10 days after culture (Fig. 1B, C), and the entire embryo differentiated to calluses with horn-shaped organogenesis tissue 15 days after culture (Fig. 1D, E, F). 20 days after culture, the number of immature embryos with calluses was counted while the color and texture of the calluses were also recorded (Table 1).

Both basic medium and plant growth regulator significantly affected callus induction; however, no interactive effects occurred (Table 1). The majority of immature embryos cultured on MS (average 64.6%) and mMS (average 85.1%) media formed calluses, while 32.4% (average) of immature embryos cultured on WPM media had calluses. Most of the calluses formed on immature embryos that cultured on MS and WPM media were watery or soft in texture. This kind of callus was difficult to further differentiate and eventually died during subculture. However, the calluses formed on immature embryos that

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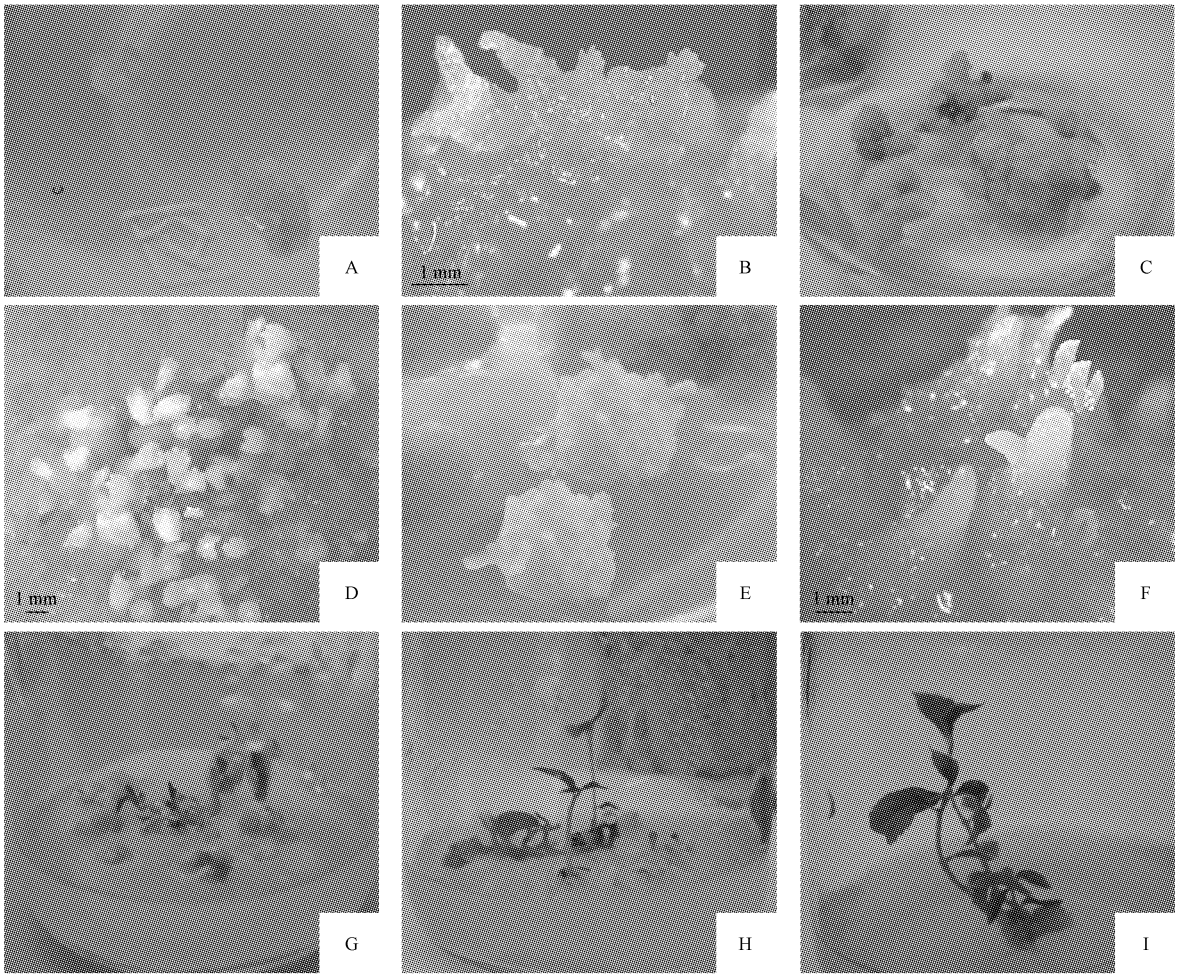
cultured on mMS medium were usually loose, milky white with strong differentiation capacity. As the concentration of 6-BA increased, there was a quadratic trend for the percentage of calluses formed on immature embryos. When immature embryos cultured on mMS supplemented with 0.20 mg/L 6-BA and 0.10 mg/L NAA, the percent of embryos with calluses was the maximum, 92.5%.



Note: A, Calluses induced from immature zygotic embryo 5 days after culture; B and C, Globular calluses formed and proliferated 10 days after culture; D, E and F, Leaf primordia initiated 15 days after culture; G, Light green leaf primordia developed on the mMS basal medium containing 0.25 mg/L 6-BA and 0.15 mg/L NAA; H, Green shoots regenerated on mMS mediums without plant growth regulators for 15 d; I, Shoots elongated on 1/2mMS medium plus 1.5% sucrose 20 days after culture.

Fig. 1 Regeneration of *Symplocs paniculata* from immature zygotic embryo via organogenesis

Table 1 Callus induction of *Symplocos paniculata* on different media supplemented with 6-BA and NAA

Basal medium	Concentration of 6-BA/(mg·L^{-1})	Concentration of NAA/(mg·L^{-1})	Induction rate of explants/%	Callus morphology
MS	0.15	0.10	50.1±0.3gd	Yellowish white, watery texture
	0.20	0.10	73.2±1.3c	Green, firm texture
	0.25	0.10	69.8±2.4c	Green with white tissue scattered on the surface, soft texture
	0.30	0.10	65.3±0.9c	White, watery texture
mMS	0.15	0.10	81.2±1.0bc	Light yellow, firm texture
	0.20	0.10	92.5±0.9a	Milky white, loose texture with granular tissue
	0.25	0.10	86.3±0.8ab	Milky white, soft texture
	0.30	0.10	80.3±0.4bc	Yellowish green, soft texture
WPM	0.15	0.10	21.2±0.6f	Milky white, watery texture
	0.20	0.10	37.9±0.5e	White with slightly green, soft texture
	0.25	0.10	37.0±0.3e	White, soft texture
	0.30	0.10	33.5±0.9e	Brown, watery texture

Note: Different lowercase letters in the same column show significant difference at 0.05 level. The same below.

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2.2 Organogenesis and plant regeneration

Milky white calluses in loose texture were subcultured (Table 2). It took 18 days for the calluses that cultured on the medium containing 0.25 mg/L 6-BA and 0.15 or 0.20 mg/L NAA to differentiate into green bud-like structures (Fig. 1G), whereas 22 days for those on the medium plus 0.20 mg/L 6-BA and 0.10 mg/L, 0.15 mg/L or 0.20 mg/L NAA. However, there was no sign of differentiation of calluses observed on the medium without any hormones. This data was excluded for analysis. The percent of calluses that differentiated into bud-like structureswas significant among 6-BA levels, NAA levels, and the interactive effects were also significant (Table 2). The percent of calluses with bud-like structures responded quadratically to the increased 6-BA. Regardless of the NAA concentration, the percent of calluses with bud-like structures was 2—3 times higher, compared to those cultured on the medium with 0.2 mg/L 6-BA. The maximum value of differentiation rate was 72.4% when the calluses were cultured on 0.25 mg/L 6-BA and 0.15 mg/L NAA.

The calluses with green bud-like structures were subcultured on to mMS medium without plant growth regulators for 15 days. A total of 76.6% of calluses with bud-like structures developed into shoots (Fig. 1H). They were then transferred on to 1/2 mMS medium plus 1.5% sucrose. Shoots elongated, and the height of shoots was 1.5 cm after 20 days of culture (Fig. 1I).

Table 2 *Symplocos paniculata* calluses differentiated on modified MS supplemented with 6-BA and NAA

Concentration of 6-BA /(mg·L^{-1})	Concentration of NAA /(mg·L^{-1})	Time of differentiation /d	Rate of calluses with shoot/%
0.20	0.10	22	23.3±1.5e
0.20	0.15	22	25.7±1.8d
0.20	0.20	22	25.6±0.8de
0.25	0.10	22	66.0±1.5b
0.25	0.15	18	72.4±1.0a
0.25	0.20	18	57.9±0.6c

3 CONCLUSION

In conclusion, calluses were induced from immature embryos cultured on mMS supplemented with 0.2 mg/L 6-BA and 0.1 mg/L NAA with callus induction percentage of 92.5%. The induced calluses were cultured on mMS plus 0.25 mg/L 6-BA and 0.15 mg/L NAA, and 72.4% of the calluses differentiated into green bud-like structures. These tissues were subcultured on to mMS medium without plant growth regulators for 15 days, 76.6% of which developed into shoots. Shoots elongated to 1.5 cm long on 1/2 mMS medium plus 1.5% sucrose 20 days after culture. Shoots were still under test to develop roots. This protocol might provide a new approach for the rapid propagation of *symplocos paniculata*.

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云南栽培金盏花遗传多样性RAPD分析

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摘 要:用采自云南昆明、曲靖和玉溪等地的8份金盏花种质资源为试材,利用改良CTAB法提取DNA,筛选引物,优化PCR反应体系,对其进行遗传多样性RAPD(random amplified polymorphic DNA)和NTSYS2聚类分析,以建立金盏花亲缘关系分析方法,明确分布在云南省各地的金盏花种质资源之间的亲缘关系和遗传多样性。结果表明:优化的PCR反应体系能达到良好的扩增效果,30条RAPD引物在8份金盏花种质资源中共产生165个位点,其中45个位点具有遗传多态性,约占27.28%。CK与7号的相似性最小,用CK和7号作为亲本的后代会有较大的分离。试验表明金盏花种植资源遗传多样性丰富,遗传关系与其地理关系密切相关,但并非严格的限聚在一起,需结合表型更深入地分析。

关键词:金盏花;RAPD;遗传多样性

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金盏花(*Calendula officinalis* L.)属菊科金盏菊属一年生草本植物,又名金盏菊、山金菊等,适合于我国大多数地区栽培,具有较高的经济价值。首先作为园林植物中最为重要的观赏花卉之一,其在城市园林造景,局部区域美化中具有独特作用,其次以金盏花的植株和花为原料可提取叶黄素和精油,不但是化妆品中一种天然的、具有良好抗氧化功能的原料,还可用于治疗多种皮肤疾病,如外伤、皮疹、皮肤破裂等,具有抗感染和杀菌消炎等功效[1]。近些年来,国内外学者主要开展金盏花授粉结实和环境条件的关系[2]、水肥措施对经济性状的影响[3]、盐胁迫对金盏花生长和逆境的生理响应机制[4]、雄性不育两用系选育及植物学特性研究[5],而关于金盏花种质资源的研究尚鲜见报道。

分子标记技术是分析种质亲缘关系的有力工具,其中RAPD分子标记是建立在PCR基础之上。用筛选的有10个碱基的单链随机引物,对基因组DNA进行PCR扩增以检测其多态性位点,具有操作简单、无需专门设计引物、对模板DNA质量要求不高、不受材料生长情况限制,能反应材料的遗传信息差异等优点;虽然RAPD分子标记有重复性不佳的缺点,但可以通过改良PCR反应体系加以改善[6]。已有学者为了在金盏花雄性不育的育种过程中使用RAPD分子标记技术,通过条件优化

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白檀未成熟胚的器官发生和植株再生

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摘 要:以白檀未成熟胚为试材,以改良MS为培养基,研究了白檀未成熟胚的器官发生和植株再生。结果表明:改良MS(mMS)能够很好的诱导愈伤组织,在添加了0.2 mg/L 6-BA＋0.1 mg/L NAA的培养基中,诱导率高达92.5%;胚性愈伤组织分化最佳培养基为mMS＋0.25 mg/L 6-BA＋0.15 mg/L NAA,分化率为72.4%;分化出的胚性愈伤组织在空白mMS培养基上继代培养15 d,76.6%的组织分化出芽,再转入1/2mMS＋1.5%蔗糖培养基上培养20 d,芽长至1.5 cm。

关键词:白檀;未成熟胚;器官发生;植株再生

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