Study on Induction and Culture of Callus of Citrus grandis cv. 'Hongmianmiyou'

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Abstract. Citrus grandis cv. 'Hongmianmiyou' is mutated from 'Guanximiyou', which has been widely cultivated in Sichuan Province because of the attractive appearance, rich nutritional ingredients and trace elements. To effectively cultivate more Hongmianmiyou seedlings for production, we established the callus tissue induction system. The basic nutrient medium consisted of MS medium with 3% of sucrose and 0.8% of agar, adjusted to pH 5.8. Various concentrations of 6-benzylaminopurine and 2,4-dichlorophenoxyacetic, as well as polyvinyl pyrrolidone and active carbon, and culture condition were carried out to develop protocols for the in vitro propagation of pummelo. The appropriate formula was MS medium supplemented with 1 mg•L⁻¹ of 2,4-D, 0.5 mg•L⁻¹ of 6-BA and 0.05% of active carbon. Explants were cultured maintained under a 16 h photoperiod provided by cool white fluorescent light 1200 Lux after cultured at dark for 7 days. Light green compact callus induced from semi-lignified stems explant of spring shoots in 'Hongmianmiyou', reaching 97% induction rate.

1. Introduction

Pummelo[Citrus grandis(L.) osbeck]belongs to genus Citrus L., family Rutaceae, which is an important fruit tree endowed with high economic and nutritional values [1]. China is one of major centers of origination and genetic diversity, possessing abundant pummelo germplasms. The cultivation history of pummelo can be traced back to 3000 years ago [2], including three major cultivation areas, Southeast coastal, South China and Southwest China [3]. Until now, more than 200 pummelo varieties have been selected and cultivated in China [4]. Citrus grandis cv. 'Hongmianmiyou' is mutated from 'Guanximiyou', which has been widely cultivated in Fujian and Sichuan Provinces because of the attractive appearance, rich nutritional ingredients and trace elements. Compared with 'Guanximiyou', the maturity period of 'Hongmianmiyou' has been advanced 10 to 15 days, representing great advantages in the fresh fruit market.

In the pummelo production, propagation methods contain seedling, grafting and layering. It is time and money consuming to propagate by seedling and grafting, which cannot meet the demand of large number of seedlings. Moreover, the propagated seedlings in the field carry a lot of viruses and pathogens, which make the pummelo trees weakly grow, reduce the yield and degrade the fruit quality. It is, therefore, necessary to establish in vitro propagation to propagate virus-free mother trees.
This study attempted to establish induction and culture of callus of ‘Hongmianmiyou’. The system will provide abundant materials for the pummelo production and study the mechanism of incompatible between rootstock and scion.

2. Materials and methods

2.1 Source of tissue and preparation of explants
Explants of *Citrus grandis* cv. ‘Hongmianmiyou’ were collected in Pujiang County, Chengdu City. Mature stems, semi-lignified stems and young stems of spring shoots and summer shoots were gathered on 10, 20, 30 March 2016, 10, 20, 30 June 2016, respectively.

Growing shoot tips (~ 5.0 cm long) were soaked in distilled water for 20 min and washed three times with sterile distilled water. Shoot tips were dipped in 70% of ethanol for 1 min after washing, then surface sterilized with 0.1% of HgCl$_2$ for 7 min with gentle stirring. Shoot tips were rinsed three times in sterile distilled water. The young leaves and basal portions of shoots injured by sterilant were removed and 1.0 cm long shoot-tip explants were cultured.

2.2 Induction and culture of callus

The basic nutrient medium consisted of Murashige and Skoog (MS) basal medium [5] with 3% of sucrose, adjusted to pH 5.8 with 0.1 N of NaOH and solidified with 0.8% of agar. Each 60 mL jar included 15 mL medium and was sterilized by autoclaving for 30 min at 121 °C, 105 kPa.

In the first experiment, freshly prepared explants were cultured on medium containing 6-benzylaminopurine (BA) (0.3, 0.5, 1mg/L) and 2,4-dichlorophenoxyacetic acid (2,4-D)(0.5, 1.0, 1.5 mg/L). A total of five experiments were conducted and replicated for three times (Table 1). Cultures were maintained at 22± 2 °C under a 16 h photoperiod provided by cool white fluorescent light 1200 Lux after cultured at dark for 7 days. The induction rates were calculated after 14 days. In the second experiment, similar explants were cultured on 0.1% of polyvinyl pyrrolidone (PVP) and 0.05% of active carbon to observe the brown status. In the third experiment, explants were cultured at light after 7 days at dark, and all light, respectively. The induction rate and culture of callus was summarized after 14 days.

2.3 Data analysis

Significant differences between the means of the treatments were determined with 95% confidence (p < 0.05) limit by Duncan multiple range test using SPSS18.0 (IBM, USA). Data are shown as the means of three replicates.

3. Results

3.1 Effect of plant growth regulators on induction of callus

Table 1 shows the induction rate of callus for each combination of the plant growth regulators 6-BA and 2,4-D. Various concentration of plant growth regulators dramatically affected the induction rate of callus. The induction rate exhibited an increasing trend and then a decreasing change with the increasing concentrations of 6-BA and 2,4-D. It is better to use the semi-lignified stems of both spring and summer shoots as explants than mature and young stems. The callus induction rate of MS medium with 0.5 mg•L$^{-1}$ of 6-BA and 1.0 mg•L$^{-1}$ of 2,4-D reach 97% using semi-lignified stems of spring shoots as explants, significant higher than other concentrations. The light green callus shows vigorous growth with protuberance and a few of buds (Fig. 1).
Table 1. Effect of 6-BA and 2,4-D on the callus induction using spring and summer shoots of *Citrus grandis* cv. 'Hongmianmiyou'

| Code | Concentration of hormone | Callus induction rate using spring shoots/% | Callus induction rate using summer shoots/% |
|------|--------------------------|---------------------------------------------|---------------------------------------------|
|      | 6-BA/2,4-D | Mature stems | Semi-lignified stems | Young stems | Mature stems | Semi-lignified stems | Young stems |
| 1    | 0.3/0.5    | 13.3bc/+    | 20.0c/#            | 0.7d/*    | 0.7d/+    | 26.0c/#            | 0.7d/*    |
| 2    | 0.5/0.5    | 26.7b/++    | 53.0b/###          | 33.3b/**  | 13.3bc/++ | 46.0b/###          | 26.7b/++  |
| 3    | 0.5/1.0    | 73.3a/+++   | 97.0a/###          | 86.7a/*** | 66.7a/+++ | 95.0a/###          | 80.0a/*** |
| 4    | 1.0/1.0    | 20.0b/++    | 60.0b/###          | 46.7b/*** | 33.3b/++  | 53.0b/###          | 40.0b/++  |
| 5    | 1.0/1.5    | 0.7d/+      | 26.0c/#            | 13.3c/*   | 20.0b/+   | 33.0bc/            | 13.3bc/*  |

Note: The different normal letters at column indicate significant difference at 0.05 level. The same as below.

+/#/*: Light green small callus with poor growth;
++++/#####/****: Light green compact callus with a few of protuberance;
+++++/#####/****: Light green vigorous callus with protuberance and a few of buds.

3.2 Effect of anti-browning agents on the growth of callus

0.1% PVP and 0.05% active carbon was used to restrain browning of explants. Among 40 explants, only 5 explants were browned by 0.05% of active carbon, while 28 explants were browned by 0.1% of PVP. This indicated that active carbon had significant effect on anti-browning of explants.

3.3 Effect of culture on the induction of callus

We also carried out the effect of culture condition on the induction of callus. The induction rate of callus reached 95% (38/40) in light culture after dark culture, while obtained just 30% (12/40) in all light culture. It was helpful for the callus formation in dark culture.

4. Discussion

Previous studies often used semi-lignified stems of citrus to establish in vitro culture due to its strong regeneration ability [6-8]. For 'Guanximiyou', the success rate of semi-hardened stem segments was highest, reaching 83.3% by Zeng et al. [7]. This study also confirmed that induction rate of callus by using semi-lignified stems were significantly higher than mature and young stems of 'Hongmianmiyou'. In the pre-experiments, different degree of browning was observed in the explants, which was related to the injury and chemicals. Antioxidants, including vitamins C, E and PVP, metal-chelator, and adsorbent, could alleviate browning of explants [9]. This study indicated that active carbon could effectively alleviate browning rather than PVP.
It has been reported that callus could be divided into three types, loose light yellow callus, compact light yellow callus, and white paste [10, 11]. Goh et al. [12] reported that the leaf explants of *Citrus grandis* cultured on medium with 4.52 μM of 2,4-D and 4.44 μM of BA produced yellowish-green, compact callus. Huang et al. [11] showed that 2,4-D alone could induce green compact callus of which about 87.5% could differentiate. He also suggested that 2,4-D and NAA have different roles on callus induction for various citrus species as well as at different culture stages [11]. Here, we obtained light green, compact callus, which might be due to the higher concentration of 2,4-D. Lower concentration of 6-BA probably induce the formation of buds. Overall, the appropriate formula for callus induction of 'Hongmianniyou' was MS basal medium supplemented with 0.5 mg*L*^{-1} 6-BA, 1 mg*L*^{-1} of 2,4-D and 0.05% active carbon in dark culture.

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