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INDUCTION OF CALLUS FORMATION FROM DIFFERENT PARTS OF *CITRUS GRANDIS* (OSBECK) FLOWERS

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ABSTRACT

The objectives of the present work were to study the capability of pomelo's floral tissues to produce callus and to investigate the influence of plant growth regulators on callus induction and development. Various parts of flower namely petal, sepal, style, ovary, pistil and cup base were cultured onto Murashige and Skoog (MS) basal medium supplemented with different levels of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and 6-Furfurylaminopurine (kinetin). It was found that different parts of flowers favored different levels of hormone for callus induction. The highest formation of callus were obtained from petal and sepal cultured on MS media supplemented with 1.5 mg/l kinetin. A 50 $\pm$ 2.3% explants from style and pistil had initiated callus when cultured on MS media supplemented with 0.05 mg/l and 0.10 mg/l BAP, respectively. On the other hand, sucrose agar (SA) media alone had managed to induce callus formation from almost every part of the flowers with a success rate between 7.69 $\pm$ 0.32 to 50 $\pm$ 3.18%. The uncut part of flowers initiated high percentage of callus (14.6 $\pm$ 0.35%) as shown in the study on petal.

Keywords: *Citrus grandis,* callus induction, flowers, auxin, cytokinin

INTRODUCTION

Citrus grandis also known as pomelo is a member of Rutaceae family. Pomelo is famous as the largest fruit among citrus. The flowers are fragrant, and described as having a floral-, jasmine- and orange-like fragrance that showed great potential in fragrance industry (Svoboda & Greenaway 2003). It has borne singly or in clusters of 2 to 10 in the leaf axils; the 3 to 7 petals are yellowish-white that arise from the base of the sexual column, somewhat hairy on the outside and dotted with yellow-green glands. The flowers are in small clusters in the leaf axils of a preceding growth flush but single in the axils of a just-completed growth flush. The staminate portion consists

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of 20 to 40 upright white filaments, sometimes united into several groups at the base, with yellow anthers on the tip (Morton 1987).

At the end of 1960s, plant cell cultures technology was introduced as a possible tool for studying and producing secondary metabolites. Cell culture systems represent a potential of renewable sources of valuable compounds for a large scale in controlled conditions to ensure the continuous supply of the compounds that used for flavors, fragrances and other applications (Dixon 1985). The use of cell culture for producing these natural products has long been considered as an attractive solution to the problem on extracting the metabolites compounds from the whole plant, since the cell can be cultured in large quantities and harvesting of the desired product could be less problematic. Typically, the cell cultures need specific conditions to accumulate large amount of secondary compounds like manipulating the parameters of the environment and medium conditions (Vanisree & Hsin 2004). The basis of all nutrients is a mixture of mineral salts combining the essential macro- and micro-element together with source of carbon. The usual supplements are vitamins, amino acids, sugar alcohols and plant growth regulator like auxin, cytokinin, abscisic acid, gibberellin and ethylene. All these elements play role in contributing to rapid proliferation of the cell (Dixon 1985).

In the early years of tissue culture works on citrus, researchers were more focused on the morphological aspects of cell development in the culture (Hidaka *et al.*1981; Smith 1992). Later, other studies have been done focusing on the production of secondary metabolite especially flavonoid and monoterpenes in callus and suspension cultures (Tisserat *et al.*1989; Berhow *et al.* 1994; Siti Mahyuni *et al.* 1997). Currently, there has been no report on the cell culture development from pomelo's flowers. The objectives of this research were to study the capability of pomelo's floral tissues to produce callus and to investigate the influence of plant growth regulators on callus induction and development. This is an initial study of possibility to produce fragrance compounds in the callus that would be conducted in other study.

MATERIALS AND METHODS

Plant Material

About 2-week-old fresh flower buds of pomelo were collected from established plantation in Kodiang, Kedah. The buds were carefully chosen to be free from symptoms of necrosis and disease infections. The buds were kept in the fridge and used within 24 hours to ensure the viability of the cells.

Explants Sterilisation and Preparation

The collected buds were firstly washed under running tab water for 30 sec. to remove dust, dirt and small insects that might attached to them. The buds were undergoing a treatment with 70% ethanol for 5 min. and followed by 0.525% sodium hypochlorite for 5 min. Later, the buds were rinsed three times in sterile distilled water. Six different parts of buds, namely the petals, sepals, styles, ovaries, pistils and cup bases were selected as the source of explants.

Media Preparation for the Study of Callus Induction

Study on the effect of explant sizes on callus induction was carried out using three different sizes of petal. The sizes used were 2.5 ± 0.24 cm for the uncut, 1 cm and 2 mm for the dissected petal and were cultured onto MS media supplemented with 1.50 mg/l kinetin. To investigate the effect of hormone on the callus formation, different concentrations of 2,4-D, BAP and kinetin were used. Murashige & Skoog (MS) medium with hormone modification were prepared into 14 combinations with addition of 30 g/l sucrose and 7 g/l agar powder in the disposable petri dishes. The control media consisted of 30 g/l sucrose and 7g/l agar powder and referred as the sucrose agar media. The combination of 2,4-D (0 mg/l and 0.025 mg/l) was designed in a factorial combination with 0 mg/l, 0.025 mg/l, 0.05 mg/l and 0.10 mg/l BAP. The same method of study was carried out between 2,4-D (0 mg/l and 1.0 mg/l) with 0.5 mg/l, 1.0 mg/l and 1.5 mg/l kinetin. The pH of the media was adjusted to 5.7 and autoclaved at 121 ºC, 15 psi for 15 min before poured into sterile petri dishes in a laminar air flow. Cultures were maintained on the open shelves in the laboratory with 12 hours light at 320 lux and 12 hours dark at room temperature of 25 ± 2 ºC. Data on percentages of callus induction were recorded after 5 days in culture for each treatment.

Statistical Analysis

Means and standard errors were used throughout and statistical significance among values were assessed using ANOVA incorporating the post hoc Tukey-Honestly Significantly Different (Tukey-HSD) test using SPSS (Statistical Package for Social Science) 13.0 software. A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSIONS

The effect of explant sizes on callus formation as shown in Figure 1 demonstrated that callus formation from uncut petal was higher (14.6 ± 0.35%) compared to the dissected petal. This result provided guidance on the suitable size for the next treatment on the effect of hormone and applied for different explants used.

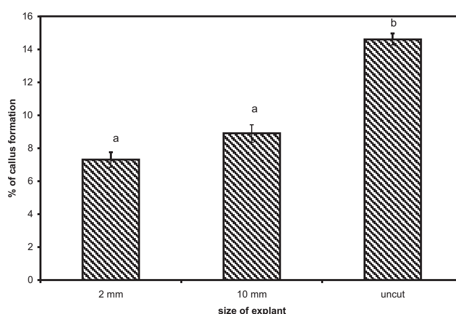
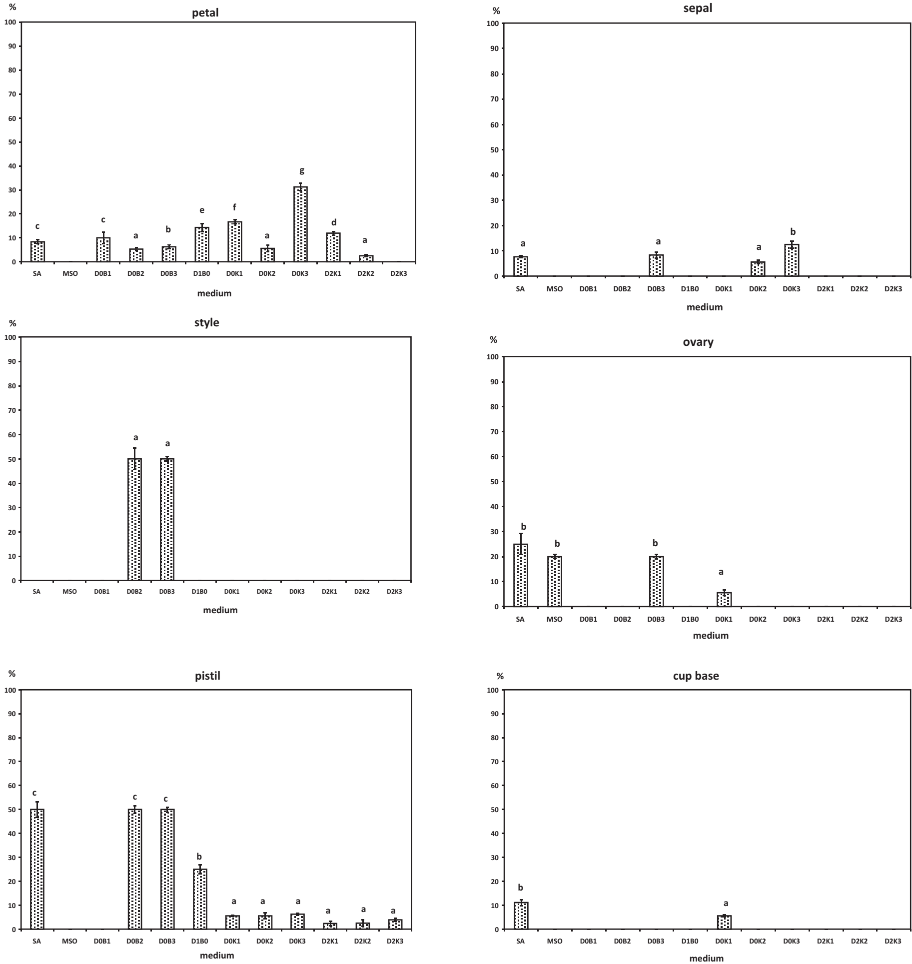


Figure 1. Percentage of petal producing callus in different size. Each treatment has more than 30 replicates and was repeated for three times. Data were collected on day 5 after culture on MS media supplemented with 1.50 mg/l kinetin. Different alphabets between bars indicate a significant difference at $P<0.05$ according to Tukey HSD test. Bar indicates the standard error of mean.



SA Sucrose agar
MSO MS basal medium
D0B1 0.025 mg/l BAP
D0B2 0.050 mg/l BAP
D0B3 0.100 mg/l BAP
D1B0 0.025 mg/l 2,4-D
D0K1 0.5 mg/l kinetin
D0K2 1 mg/l kinetin
D0K3 1.5 mg/l kinetin
D2K1 1 mg/l 2,4-D + 0.500 mg/l kinetin
D2K2 1 mg/l 2,4-D + 1.000 mg/l kinetin
D2K3 1 mg/l 2,4-D + 1.500 mg/l kinetin

Figure 2. Percentage of explants producing callus in various concentration of 2,4-D, BAP and kinetin. Each treatment has more than 20 replicates and was repeated for three times. Data were collected on day 5 after culture. Data was not shown for treatments with nil callus formation for any of the flower parts. Different alphabets between bars indicate a significant difference at $P<0.05$ according to Tukey HSD test. Bar indicates the standard error of mean.

Figure 2 illustrates the results on the effect of modified MS media with various concentrations of 2,4-D, BAP and kinetin on callus initiation from various parts of flowers (Plate 1). For the petal, the highest percentage of explants (31.25 ± 1.51%) that formed callus was obtained when cultured on MS medium supplemented with 1.5 mg/l kinetin without 2,4-D. Interestingly, callus also were able to initiate from petal cultured on sucrose agar medium at the production rate of 8.33±0.88%.

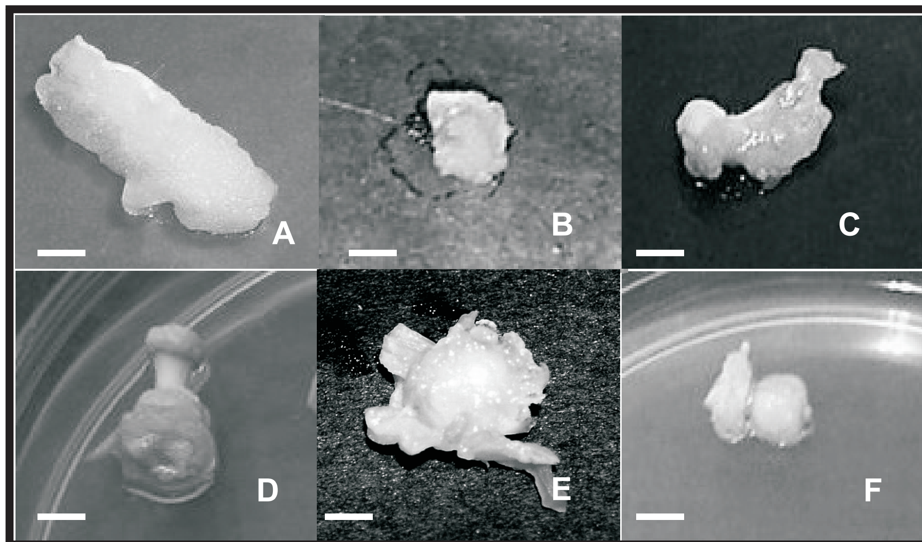


Plate 1 : Callus formation of pomelo floral tissues: (A) petal cultured on MS + 1.500 mg/l kinetin medium (bar = 4.5 mm), (B) sepal cultured on MS + 1.5 mg/l kinetin medium (bar = 5 mm), (C) style cultured on MS + 0.050 mg/l BAP (bar = 2.5 mm), (D) pistil cultured on MS + 0.050 mg/l BAP (bar = 5 mm), (E) cup base cultured on sucrose agar (bar = 3 mm), (F) ovary cultured on sucrose agar (bar = 3.3 mm). The calluses formed were white friable callus. Photos were taken at day 5 after cultured.

Study on sepal showed that MS medium with 1.5 mg/l kinetin without addition of 2,4-D was the most suitable medium that enabled to initiate callus formation with the percentage of 12.50 ± 1.36. This was followed by MS medium supplemented with 0.10 mg/l BAP only (8.33 ± 1.24%). The addition of cytokinin has slightly increased the ability of sepal to initiate callus. Sepal that cultured on sucrose agar media with the absence of exogenous hormone, nutrient elements and mineral salts were still enabled to form callus at a comparable rate which is 7.69 ± 0.32%.

In the case of style, it was found that modified MS media with addition of 0.05 to 0.10 mg/l BAP is most favorable for callus initiation as the percentage obtained for both concentrations were about 50%. This was considered the highest percentage of explants cultured on media with hormone that enabled to form callus compared to other parts of flowers. Therefore, it can be suggested that cells from the style were more viable and actively divide in culture condition. Thus, it was presumed that BAP is needed for the stylar tissues to trigger the callus formation. Explants from ovary were found not much influenced by the exogenous hormone and nutrient elements in

initiating the callus. This finding was in agreement with effort to grow callus of *Eucalyptus camaldulensis* by *in vitro* cultures (Giamakis *et al.* 2001). Callus formation from explants cultured on sucrose agar medium was found to be the highest (25 ± 4.19%). The other three media; MS medium without addition of hormone, modified MS medium with 0.10 mg/l BAP and 0.5 mg/l kinetin, were still unable to form callus but at a lower percentage (20 ± 0.81%, 20 ± 0.89% and 5.56 ± 1.13%), respectively.

From the observation on pistil, sucrose agar was found sufficient to initiate callus formation up to 50%. This result was found as good as 50% results obtained with style cultured on BAP supplemented media. One explanation on this phenomena is, the location of the pistil below the style may explain why endogenous cytokinin (BAP) in pistil was higher as cytokinin was transfered from the bottom to the top of the plant. Cytokinins produced in the root of a plant reach their target tissues by moving up the plant in the xylem sap (Campbell & Reece 2005). MS medium supplemented with 0.05 mg/l and 0.10 mg/l BAP was both found to have similar capability as the sucrose agar medium in initiating the callus. Medium with 0.05 mg/l 2,4-D only managed to obtain 25 ± 1.84% callus formation from the total explants. Callus were formed from the cup base when cultured on sucrose agar medium with success rate at 11.11 ± 1.29% and from medium with addition of 0.5 mg/l kinetin without 2,4-D at 5.56 ± 0.39% .

It was interesting to find out that some of the explants from various parts of flowers were able to initiated callus even on sucrose agar medium. From this observation it was believed that addition of hormone is not crucial but favour for callus induction. It was assumed that the presence of endogenous hormone in the excised explant is sufficient for callus to develop. Kayim *et al.* (2006) reported that in citrus, somatic embryognesis is induced either by the lack of growth regulators or reduction of sucrose concentration in the culture medium in contrast to other plant species. This also indicates that callus induction was not affected by the concentration of the additional hormone in the culture medium. More importance, there was a potential to develop new cells from the bud flowers *in vitro* for any means of study for example in the study of fragrance compounds production in citrus flowers.

As reported in many studies, the key to establishing callus and cell suspension cultures is the choice of the optimum culture medium components, proper explant source and plant growth regulator concentrations (George & Sherrington 1984). The result was also supported by Carimi *et al.* (1999) that explants cultured on MS media could produce callus without addition of hormones. In another way, it is believed that nutrient supply was obtained from the explants itself and source of carbon, which is sucrose, is sufficient for callus induction. In this study, most of the explants show callus formation and growth up to day 5 of the culture period and demonstrated necrosis after day 7 and for the consequences, they have failed to endure further growth.

CONCLUSIONS

Pomelo floral tissues had a capability to initiate callus formation but were barely responsive to the callus initiation effort with variation of hormone that has been used.

Successful callus initiation of 50% was obtained from the style when cultured on MS media with addition of 0.05 and 0.1 mg/l BAP and from pistil when cultured on sucrose agar medium. The MS modified media were sufficient for callus initiation only and failed to promote further growth of the callus.

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