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EFFECT OF EXPLANT TYPE AND DIFFERENT PLANT GROWTH REGULATORS ON CALLUS INDUCTION AND GROWTH IN *Citrus sinensis* (L.) OSBECK

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ABSTRACT. *In this study, we analyzed the effect of using different explants (zygotic embryos, leaves, stems, and cotyledons) on callus induction and the effect of different PGRs on the growth response of the callus. The results showed that callogenesis was faster when all explants were incubated in the dark than in light incubation. Maximum callus induction was observed in zygotic embryos (compared to other explants) and in the group cultured in 3 mg/L 2,4 D and 1 mg/L 6- BAP combination. Similarly, 3 mg/L 2,4 D and 1 mg/L BAP PGRs were also the effective concentrations for callus induction from cotyledons and leaves. However, a 2 mg/L 2,4 D and 1 mg/L BAP combination was the most potent in inducing callus from stems. In addition, our results indicated that the best hormonal combination for callus multiplication of callus derived from zygotic embryos was 1 mg/L 2,4 D and 1 mg/L BAP. The inhibition of ethylene activity improved callus growth during the multiplication stage in two groups being treated with 1 mg/L 2,4 D alone or with 2 mg/L 2,4 D in combination with 1 mg/L BAP. In contrast, ethylene synthesis inhibitors had insignificant effects on callus multiplication in any of the groups cultured in different PGRs combinations. The calluses obtained in all treated groups were friable, loose, and had a white to yellow color.*

KEYWORDS: *Citrus sinensis, 6-benzyl amino purine, 2,4 dichloro phenoxy acetic acid, callus, ethylene.*

INTRODUCTION

Citrus sinensis (L.) Osbeck (Common name: blood orange) belongs to the Rutaceae family and is one of the leading fruit crops favored for its juice, flavor, and nutritional value (Azim et al. 2011, Hasan et al. 2016). Several

types of research documented that the breeding of citrus plants (including *C. sinensis*) needs improvement due to the long juvenility phase in this plant and its sensitivity to pathogens as well as other environmental factors (Kiong et al. 2008, Azim et al. 2011). Accordingly, improving citrus breeding is required to cover the market's needs (Singh & Rajam 2009). Earlier reports depicted the effectiveness of the *in vitro* culture in generating citrus with better characteristics such as high yield and stress resistance (Guo et al. 2000, Koltunow 2002). In this regard, callus cultures are considered valuable tools for the propagation of plants. Accumulating lines of evidence showed that plant growth regulators (PGRs) and types of explants play vital roles in the induction and multiplication of callus (Savita et al. 2010). Multiple studies exhibited the effectiveness of using auxins (e.g., 2,4 dichloro phenoxy acetic acid; 2,4 D) and cytokinins (e.g., 6-benzyl amino purine; BAP) combinations in the production of callus (Savita et al. 2011). On the other hand, it has been well-recognized that the plant hormone ethylene has crucial effects on callus growth (Goren et al. 1979). Kumar et al. (2009) reviewed the studies that focused on the effect of an inhibitor of ethylene action, silver nitrate ($AgNO_3$), on plant growth modulation. $AgNO_3$ was useful in several *in vivo* and *in vitro* applications in plant growth regulations (Kumar et al. 2009).

Even though citrus is one of the most important crops in Jordan, there is a lack of studies conducted on callus production from this fruit. Also, there is a need for effective tissue culture protocols that can aid in citrus breeding. Accordingly, this research aimed to determine an efficient protocol for callus induction in citrus and the effect of PGRs on callus induction and growth.

MATERIALS AND METHODS

Media preparation

Two types of media were used in this experiment. Half Murashige & Skoog (MS) media (2.15 g/L MS) was selected for seed germination (Murashige & Skoog, 1962) and was supplemented with 1 mg/L or 3 mg/L gibberellic acid, 1.2% agar (pH 5.8). The media was prepared by mixing the previously mentioned components, then was autoclaved at 121 °C /15 pound-force per square inch (psi) for 30 min. In laminar flow, the media was poured into sterile test tubes.

For callus experiments, Murashige & Tucker (MT) basal media was used and prepared according to Carimi (2005). Briefly, 4.3 g/L MS media and 30 g/L sucrose

were dissolved in distilled water and then were supplemented with a mixture of 100 mg/L myoinositol, 5 mg/L nicotinic acid, 10 mg/L pyridoxine hydrochloride (HCl), 10 mg/L thiamine HCl and 2 mg/L glycine. The media was prepared by mixing the previously mentioned components with the PGRs, then was autoclaved at 121°C/15 psi for 30 min. In laminar flow, the media was poured into 100 x 15 mm sterile agar plates.

Seed germination

Citrus sinensis ripe fruits were brought from the commercial market in Amman, Jordan. In laminar flow, the seeds of *C. sinensis* were collected and dehusked by sharp blades. Good quality seeds were used in the study. The seeds were surface sterilized in 70% ethanol for 5 sec, washed with sterile distilled water, soaked in 5% sodium hypochlorite for 5 min, then rewashed at least three times with sterile distilled water. The seeds were dried on sterilized filter paper. After that, they were cultured in test tubes containing the MS seed germination media. The tubes were labeled and incubated in the dark at 25 ± 2°C. The cultured explants were observed daily to record the day of the seedlings' appearance and transfer the seedlings to light incubation (3000 lux intensity) at 25 ±2°C for 16 hr. Later, different parts of the seedlings (leaves from the second node, stems, and cotyledons) were used to produce callus. All experiments were conducted in triplicates.

Determining the effect of PGRs on callus induction

Culturing zygotic embryos for callus production

To expose embryos, *C. sinensis* were dehusked, surface sterilized, and cut individually and longitudinally into two halves. The seeds were cultured in MT media test tubes that contain combinations of PGRs (2,4 D and BAP) as presented in Table 1, according to Hasan et al. (2019). Half of the Petri dishes were incubated in the light, and half of the Petri dishes were kept in the dark at 25 ± 2°C. The Petri dishes were observed regularly to record the date of callus appearance and the characteristics of the produced callus. All experiments were conducted in triplicates.

Culturing cotyledons, leaves, and stems for callus production

MT media was used to initiate callus from cotyledons, leaves (at the second node), and stems that were aseptically excised from the cultured seedlings. The explants were cut into small pieces and distributed in different Petri dishes containing MT media supplemented with 2,4 D and BAP combinations, as shown in table 1. Half of the Petri dishes were incubated in the light, and half of the Petri dishes were kept in the dark at 25 ± 2°C. The Petri dishes were observed regularly to record the callus appearance date and the produced callus characteristics. All experiments were conducted in triplicates.

Table 1: The combinations of PGRs used in the experiments for callus production.

PGR combination	Zygotic embryos	Young leaves (from the second node)	Cotyledons	Stems	Light	Dark
2,4 D (1 mg/L)/ BAP (1 mg/L)	X				X	
2,4 D (1 mg/L)/ BAP (1 mg/L)		X			X	
2,4 D (1 mg/L)/ BAP (1 mg/L)			X		X	
2,4 D (1 mg/L)/ BAP (1 mg/L)				X	X	
2,4 D (1 mg/L)/ BAP (1 mg/L)	X					X
2,4 D (1 mg/L)/ BAP (1 mg/L)		X				X
2,4 D (1 mg/L)/ BAP (1 mg/L)			X			X
2,4 D (1 mg/L)/ BAP (1 mg/L)				X		X
2,4 D (2 mg/L)/ BAP (1 mg/L)	X				X	
2,4 D (2 mg/L)/ BAP (1 mg/L)		X			X	
2,4 D (2 mg/L)/ BAP (1 mg/L)			X		X	
2,4 D (2 mg/L)/ BAP (1 mg/L)				X	X	
2,4 D (2 mg/L)/ BAP (1 mg/L)	X					X
2,4 D (2 mg/L)/ BAP (1 mg/L)		X				X
2,4 D (2 mg/L)/ BAP (1 mg/L)			X			X
2,4 D (2 mg/L)/ BAP (1 mg/L)				X		X
2,4 D (3 mg/L)/ BAP (1 mg/L)	X				X	
2,4 D (3 mg/L)/ BAP (1 mg/L)		X			X	
2,4 D (3 mg/L)/ BAP (1 mg/L)			X		X	
2,4 D (3 mg/L)/ BAP (1 mg/L)				X	X	
2,4 D (3 mg/L)/ BAP (1 mg/L)	X					X
2,4 D (3 mg/L)/ BAP (1 mg/L)		X				X
2,4 D (3 mg/L)/ BAP (1 mg/L)			X			X
2,4 D (3 mg/L)/ BAP (1 mg/L)				X		X

Effect of PGRs on callus multiplication

Due to the availability and maximum production of callus from zygotic embryos compared to other explants, this callus was chosen to examine the effect of PGRs on callus multiplication and to assess the effect of ethylene inhibition on the growth of callus after 30 days of incubation. In more detail, the groups of calluses obtained from zygotic embryos were cut into small pieces, assigned numbers, and weighed (weight 1) under sterile conditions. The pieces of callus produced from every combination of PGRs were divided into three groups. Group 1 is the control group treated with MT media alone without PGRs or inhibitors, Group 2 is the group

treated with PGRs only, and Group 3 is the group treated with inhibitors/PGRs. In this part of the study, two inhibitors were used: 10 mg/L $AgNO_3$ (an inhibitor for ethylene activity) and 5 mg/L Aminooxyacetic acid (AOA) (an inhibitor for ethylene synthesis). After one month of callus subculturing, the weight of calluses (weight 2) was measured. The percentage increase in weight was calculated using the following equation: % Weight increase = [(weight 2-weight 1)/ weight 1]*100%. All experiments were conducted in triplicates.

Statistical analysis

The percent increase in callus weight was determined and presented as the Mean± standard error of the mean (SEM). The statistical significance of differences between groups was assessed by one-way analysis of variance (ANOVA) followed by the suitable posthoc test (Dunnett's test) using GraphPad Prism version 6. $p<0.05$ was considered significant.

RESULTS

Seed germination

Healthy seedlings were obtained after three weeks of culturing dehusked seeds in the dark in half MS media deprived of sucrose/myoinositol and supplemented with 3 mg/L GA, 1.2% agar (pH 5.8). Green seedlings were obtained after transferring the cultured seedlings into light incubation (Figure 1). They were used as a source of young leaves (obtained from the second node), cotyledons, and stem explants for callus production.

Figure 1. Seedlings of *C. sinensis* after their transfer from dark to light

Effect of different PGRs on callus induction

Our previous data showed that using 1 mg/L BAP in combination with different concentrations of 2,4 D was more effective than using 0.5 mg/L BAP in combination with different concentrations of 2,4 D. Accordingly, the concentration of 1 mg/L BAP in combination with 2,4 D was selected in this study. We tested the effect of PGRs combinations on callus induction in zygotic embryos, young leaves (from the second node), cotyledons, and stems obtained from the *in vitro* grown seedlings.

Figure 2 presents the calluses obtained from the best PGR combination in each explant when incubated in the dark. It was found that the best combination for callus induction in zygotic embryos was 3 mg/L 2,4 D and 1 mg/L BAP (Figure 2A), while the effective combination in callus multiplication was 1 mg/L 2,4 D and 1 mg/L BAP.

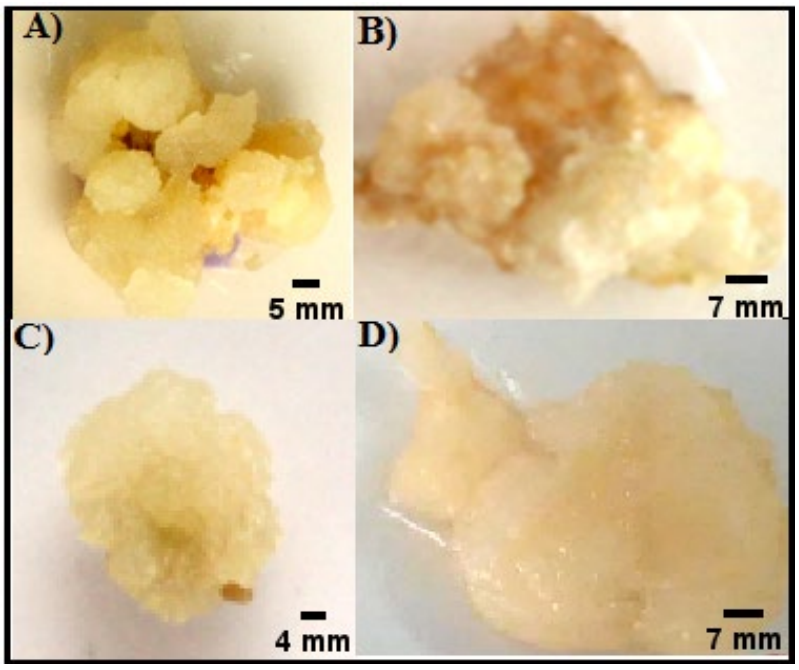

Figure 2. Callus production in dark incubation from zygotic embryos cultured in 3 mg/L 2,4 D and 1 mg/L BAP (A), young leaves (from the second node) cultured in 3 mg/L 2,4 D and 1 mg/L BAP (B), cotyledons cultured in 3 mg/L 2,4 D and 1 mg/L BAP (C) and stems cultured in 2 mg/L 2,4 D and 1 mg/L BAP (D) when incubated in the dark.

Among different combinations, the concentration of 3 mg/L 2,4 D and 1 mg/L BAP caused callus induction in young leaves obtained from the second node and cotyledons (Figures 2B and 2C). In contrast, 2 mg/L 2,4 D and 1 mg/L BAP were the best concentrations for callus induction from stems (Figures 2D). No callus was produced in zygotic embryos cultured in MT media deprived of PGRs (Control group). The produced callus had a loose, friable texture and white to yellow color. Table 2 presents the type and size of calli obtained from different explants after their culture with PGRs versus the control group. In all tested groups, callogenesis obtained from different explants cultured in the dark appeared faster (after two weeks) compared to the light incubation, whereby callus appeared after three weeks (data not shown).

Effect of PGRs on callus multiplication

Our findings showed that supplementing MT media with 1 mg/L 2,4 D and 1 mg/L BAP was the best combination for the multiplication of callus produced from zygotic embryos compared to other groups (Figure 3). The results showed that sub-culturing callus in MT media without PGRs or other additions caused a significant decrease in the weight percentage of callus compared to the callus that included PGRs in their culture and subculture.

Table 2. Type and size of calluses produced in different explants treated with different concentrations of PGRs and cultured in the dark.

Explant	Type of callus	Size of calluses at different concentrations of PGRs		
		2,4 D (1 mg/L) BAP (1 mg/L)	2,4 D (2 mg/L) BAP (1 mg/L)	2,4 D (3 mg/L) BAP (1 mg/L)
Zygotic embryos	Friable	XX	X	XXX
Young leaves (from the second node)	Friable	X	XX	XXX
Cotyledons	Friable	XX	X	XXX
Stems	Friable	X	XXX	XX

In our study, the effect of auxins on callogenesis in zygotic embryos was concentration-independent. In more detail, callus initiation and multiplication in the group treated with 1 mg/L 2,4 D and 1 mg/L BAP

combination was bigger than the group cultured in 2 mg/L 2,4 D and 1 mg/L BAP combination. Concerning the effect of ethylene inhibition on callus growth, the results showed an increase in callus development due to the inhibition of ethylene activity in two groups (1 mg/L or 2 mg/L 2,4 D in combination with 1 mg/L BAP). In contrast, inhibiting ethylene synthesis didn't affect the percentage of weight increase in callus in any of the treated groups. The results of callus multiplication in different groups are illustrated in Figure 4A-4C.

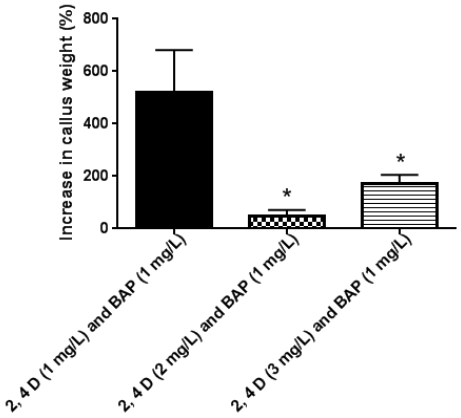

Figure 3. Effect of different concentrations of 2,4 D in combination with 1 mg/L BAP on the percentage increase in the weight of callus produced from zygotic embryos. (* Significant compared to 2,4 D (1 mg/L) and BAP (1 mg/L) treated group, $p<0.05$.)

DISCUSSION

Establishing a successful protocol for callus induction is very useful for plant regeneration, research, and biotechnology. The protocols for callus initiation differ according to many factors (e.g., species, place, type of explants). There is a lack of published reports about callus induction from *C. sinensis* in Jordan. The present investigation studied the effect of using different explants on callus induction. It assessed the impact of combining PGRs on the induction and growth of callus produced from *C. sinensis*. In the current study, the embryonic part of the seeds was cultured in MT

media to initiate callus.

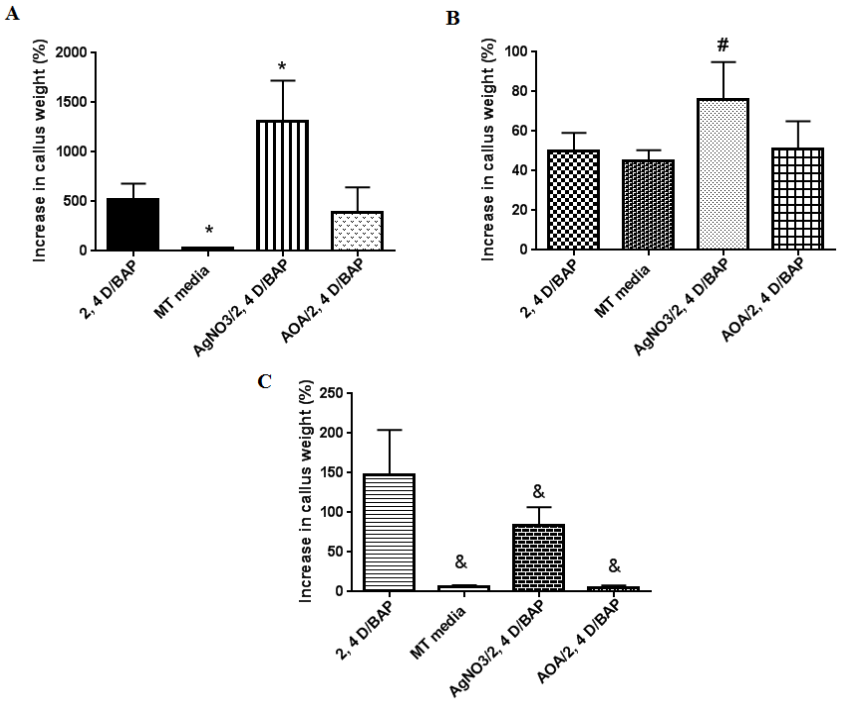

Figure 4. Effect of the administration of $AgNO_3$ or AOA on the percentage increase in the weight of callus produced from zygotic embryos cultured in MT media that contain 1 mg/L 2,4 D and 1 mg/L BAP combination. A. * Significant compared to 2,4 D (1 mg/L) and BAP (1 mg/L) treated group. B. # Significant compared to 2,4 D (2 mg/L) and BAP (1 mg/L). C. & Significant compared to 2,4 D (3 mg/L) and BAP (1 mg/L) group, $p<0.05$.

2,4 D and BAP combination was effective in inducing callus from different explants. In this regard, Ramdan et al. (2014) used different combinations of 2,4 D and BAP in five types of citrus (*Cleopatra mandarin, Rangpur lime, Citrus volkameriana, Trifoliate orange,* and *Citrus aurantium*). The authors reported pronounced effects of PGRs on the induction, physical appearance of callus, and its multiplication in a concentration-dependent manner. Similarly, 2,4 D and BAP combination accompanied by dark incubation was effective in initiating callus from

epicotyl explants obtained from seedlings of *Citrus acida* in India (Chakravarty & Goswami 1999).

Mayerni et al. (2020) used 2,4 D (0.5, 1, 1.5 and 2 mg/L) and BAP (1 mg/L) combination to induce callus from *Pogostemon cablin* Benth in Indonesia. In another study, Khan et al. (2015) recommended using a higher concentration of 2,4 D and a lower concentration of BAP to induce callus from grapes leaves. Further, earlier report showed that propagation of callus from *Pelargonium* x *domesticum* was only possible with combinations of 2,4-D and BAP (Haensch 2007). Furthermore, the callus obtained from all groups used in this study had a loose friable texture and white to yellow color. The appearance of this color can be explained by the fact that auxins reduce the amount of chlorophyll (Karatas et al. 2010). Wahyuni et al. (2017) used different combinations of PGRs to induce callus from *Justicia gendarussa* leaf explants. The authors reported that the color and texture of the produced calluses differ between the groups treated with 2,4 D/BAP combination and the group incubated with Indole-3-butyric acid (IBA)/BAP. Moreover, 2,4 D and BAP combination showed effectiveness in callus induction from the root, stem, cotyledon, and leaf explants from *Trachyspermum ammi* in Iran (Nasab 2018). Likewise, 13.50 µM of 2,4-D and 4.50 µM of BAP combination was optimum for producing callus, with a 93.75% success rate in a medicinal and ornamental plant (*Hymenocallis littoralis*) from Malaysia (Sundarasekar et al. 2012).

On the other hand, our data revealed that inhibiting ethylene activity but not synthesis enhanced callus production in the groups cultured in 1 mg/L or 2 mg/L 2,4 D combined with 1 mg/L BAP, indicating that ethylene, as expected, has a negative effect on the growth of callus. In contrast, using an inhibitor of ethylene action but not synthesis increased callus production in the groups of 3 mg/L 2,4 D accompanied by 1 mg/L BAP. Our possible explanation is that this decrease was attributed to accumulative stress and toxicity from maintaining high concentrations of auxins in the presence of the inhibitor during the multiplication stage that lasted for 30 days. Likewise, it was documented that adding 10-15 mg/L $AgNO_3$ improved callus induction in tomatoes (*Solanum lycopersicum* MILL.) (Shah et al. 2014).

In conclusion, we have developed an efficient protocol for callus induction from different explants. Based on that, we recommend a protocol of using equal concentrations of 2,4 D and BAP in MT media to culture citrus explants as this combination elicited callogenesis and was the most effective in the multiplication of callus. Another protocol that can be

recommended is to start with a high concentration of 2,4 D (e.g., 3 mg/L) accompanied by BAP (1 mg/L) to induce callus, then shift to a lower concentration of 2,4 D (e.g., 1 mg/L) accompanied with 1 mg/L BAP to maintain callus. In addition, we determined the suitable conditions for the propagation and the multiplication of callus. These protocols can open new avenues for crop improvement and biotechnology research.

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