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Effect of different concentrations of kinetin and 2,4,D on callus induction of citrus rootstock (Citrus sp.)

Seyedeh Zahra Hosseini*, Nadali Babaeian Jelodar**, Heshmatallah Rahimian** and Gholamali Ranjbar**

**Ph.D. Student, Sari Agricultural Sciences and Natural Resources University, IRAN*
***Professor, Sari Agricultural Sciences and Natural Resources University, IRAN*

(Corresponding author: Seyedeh Zahra Hosseini)
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ABSTRACT: The present investigation was undertaken to study the effect of different concentrations and combinations of 2, 4-dichloro-phenoxyacetic acid (2, 4-D), Kinetin (Kin) on callus induction of five citrus rootstocks (Sour orange, Citromelo, Citrange, Poncirus, Volkameriana) on MS basal medium. Different concentration of 2,4-D and Kin were tasted in order to obtain the best callus formation. Maximum callus induction response (100%) was obtained on MS medium with 2,4-D (2 mg/l) and Kin (3 mg/l) for Poncirus. Best callus induction response of Citrange (94.44%) was observed on MS medium with 2,4-D (3 mg/l) and Kin (6 mg/l). The highest frequency of the callus induction rate (100%) was occurred on MS medium supplemented with 2.4-D (3 mg/l) and Kin (6 mg/l) for Citromelo and Volkameriana. MS medium supplemented with 2,4-D (2 mg/l)and Kin (3 mg/l) showed maximum callus induction (66.67%) for Sour orange.

Key words: Callus induction, Citrus rootstocks, 2, 4-D, Kin.

INTRODUCTION

Citrus is one of the most important commercial crops of the world valued for its juice and other by products (Ramdan *et al*., 2014). Advances in tissue culture have generated new opportunities for citrus genetic improvement. In vitro propagation has therefore been a great potential tool to overcome problems related with the field culture for such species (Hidaka &Omara, 1989). The genetic improvements of this perennial woody plant often take many years using traditional plant-breeding methods (Kayim and Koe, 2006). Hence, plant tissue culture techniques can be applied as a helpful tool to reduce the time for improvement of citrus through somaclonal variation (Chandler *et al*., 1996). These studies were undertaken with the aim of controlling the techniques being able to lead in the genetic improvement of the selected citrus fruit species (Chakravarty *et al*., 1999). Plant tissue culture is an efficient method of vegetative propagation of various perennial trees. Different protocols of callus induction and plant regeneration using various techniques and explants, including somatic embryogenesis and organogenesis have been reported for various citrus species (Al-Taha, 2009; Jajoo, 2010; Lombardo *et al.*, 2011). Establishment of an efficient callus induction protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaledaand Forkan, 2006; Altaf *et al*., 2009). Citrus embryo explants were most responsive to callus induction and proliferation (Alka, 2010). Thus, for biotechnological research on citrus, a reliable callus induction protocol using embryo is essential. The present study was undertaken with an objective to develop an efficient callus induction protocol which is a major prerequisite for in vitro plant regeneration system involving citrus rootstocks.

MATERIALS AND METHODS

A. Plant materials

In this study citrus rootstock leaves were obtained from the Fajr institute, Regional Center of Mazandaran, Iran. Five rootstocks, planted in citrus collection, were used: Sour orange (*Citrus aurantium*), Citromelo (Duncan grape fruit × Poncirus trifoliate), Citrange (*Citrus sinensis* × *Poncirus trifoliate*), Poncirus (*Poncirus trifoliate*), Volkameriana (*Citrus volkameriana*).

B. Explants sterilization

Leaves of each rootstock variety were collected. Under the laminar flow cabinet. Leaves were immersed in ethanol 70% for 10 minutes, then in sodium hypochlorite solution 5% for1 minute and finally washed three times by sterilized distilled water.

C. Hormonal compounds

The experiment was conducted as factorial in a completely randomized design (three factors) with three replications. The factors included five citrus rootstocks (Sour orange, Citromelo, Citrange, Poncirus, Volkameriana), three levels of 2, 4-dichloro-phenoxyacetic acid (2, 4-D) (1, 2, 3 mg/l) and three levels of Kinetin (Kin) (1, 3, 6 mg/l).

Explants were cultured on Murashige and Skoog (MS) basal medium (Murashige & Skoog,1962) with 5% sucrose and 0.8% agar with 0.5 gr Malt extract. The pH of MS medium was adjusted to 5.7 before autoclaving at 121°C for 20 min. Callus is initiated in the dark at 26 ± 1°C.

D. Statistical analysis

Analysis of variance (ANOVA) was carried out using SAS (SAS Institute, Cary, N.C.) and MSTATC (MSTATC, East Lansing, Mich.). Treatments were compared using the protected Duncan's multiple-range tests.

RESULTS AND DISCUSSION

Callus induction of citrus cultivar is very dependent on root stocks and growth hormones. Analysis of variance showed that rootstocks, Kin,2,4- D, interaction of rootstocks × Kin, interaction of rootstocks × 2,4-D,interaction of 2,4-D × Kin and interaction of Kin × 2,4-D × root stocks were significant. The results of the analysis of variance showed in the Table 1.

Table 1: Results of Analysis of variance (ANOVA) of different factor on callus induction of five citrus rootstocks.

Callus (%)	df	Source of variation
54.12**	4	rootstocks
1217.45*	2	Kin
594.16*	2	2,4-D
301.13*	8	Root stock × Kin
654.75*	8	Root stock × 2,4-D
268.94*	4	Kin × 2,4-D
687.81*	16	Root stock × Kin× 2,4-D
304.88	77	error
	134	total
	15.22	cv

*,** Significant at 5% and 1% levels, respectively.

In general, among the all the rootstocks, maximum callus induction belong to Citromeloby91.66% and the lowest percentage of callus induction (50%) obtained from sour orange (Fig. 1). All genotypes showed various degree of response on callus induction. Ramdan *et al* (2014) observed that citrus rootstock had a basal role on percentage of callus induction. The success of in vitro culture depends mainly on the growth conditions of the source material and the genotypes of donor plants (Borjian and Arak, 2013). Fig. 2. showed the effect of different concentrations of Kinetin on callus induction. The highest percentage of callus induction belong to 6 mg/l Kin (82.31%) and the lowest belong to 3 mg/l hormone Kin (72.61 percent). The results show an increasing trend in the percentage of callus induction using Kin. Sativa *et al* (2010) observed the Kin (2 mg/l) had a maximum callus induction (86.33%) on *C. jambhiri* in MS medium. Response of 2,4-D on callus induction by leaves as explants was studied. Fig. 3. showed the effect of different concentrations of 2, 4-Don callus induction. The highest percentage of callus induction belong to 3 mg/l 2, 4-D (81.25%) and the lowest belong to 1 mg/l 2, 4-D(74.39 %). The results also show an increasing trend in the percentage of callus induction using 2, 4-D (Fig. 3). Result of Shawkat and Mirza (2006) showed that optimal callus induction response was observed on Murashige and Skoog medium (MS), supplemented with 1.5 mg/l2,4-D for all types of explants, with stem explants showing the highest response (92%).

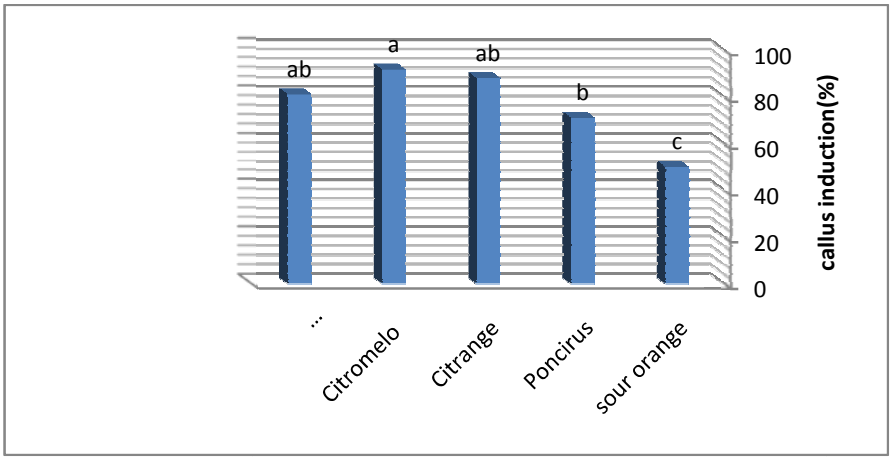

Fig. 1. Callus induction from leaf explant of five citrus rootstocks.

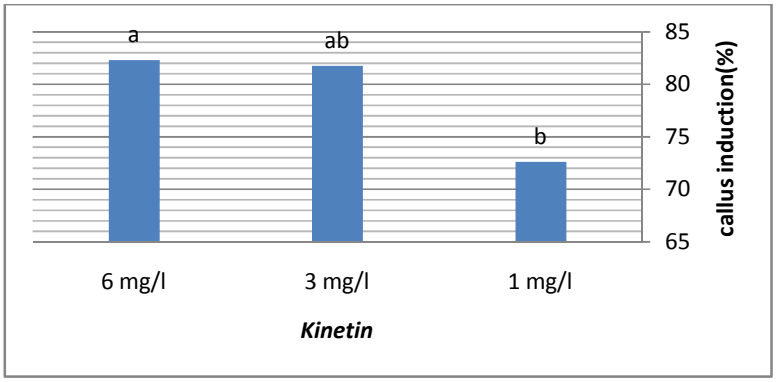

Fig. 2. Effect of different concentration of Kin on callus induction of leaf explant of different citrus genotypes.

Azim *et al* (2011) used 2, 4-dichlorophenoxy acetic acid (2, 4-D) on MS basal medium. These results showed that 2, 4-D (2 mg/l) produced highest (68%) callus induction. Ramdan *et al* (2014) observed the 2,4-D (2 mg/l) had a maximum callus induction (100%) on citrus root stock in MS medium.

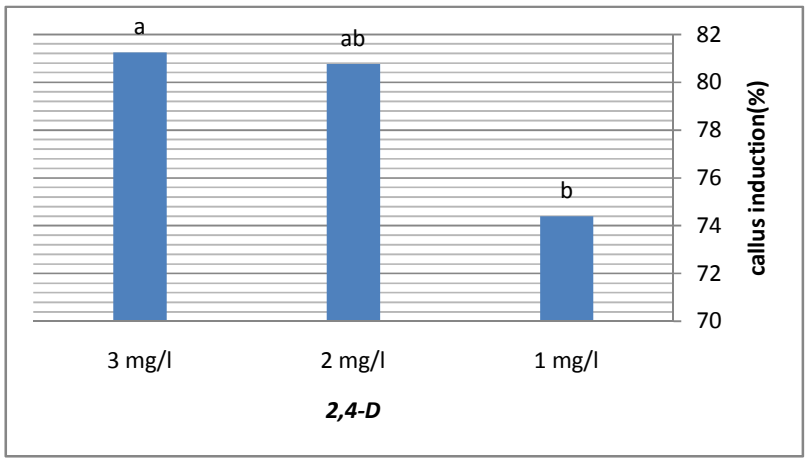

Fig. 3. Effect of different concentration of 2,4-D on callus induction of leaf explant of different citrus genotypes

The interaction effect between different rootstocks and different concentration of Kinetin on callus induction was shown in Fig. 4. The optimal callus induction response was observed on MS medium, supplemented with 6 mg/l kin for Citromelo (94.44%) and the lowest belong to 3 mg/l Kin in Sour orange (40%) (Fig. 4).

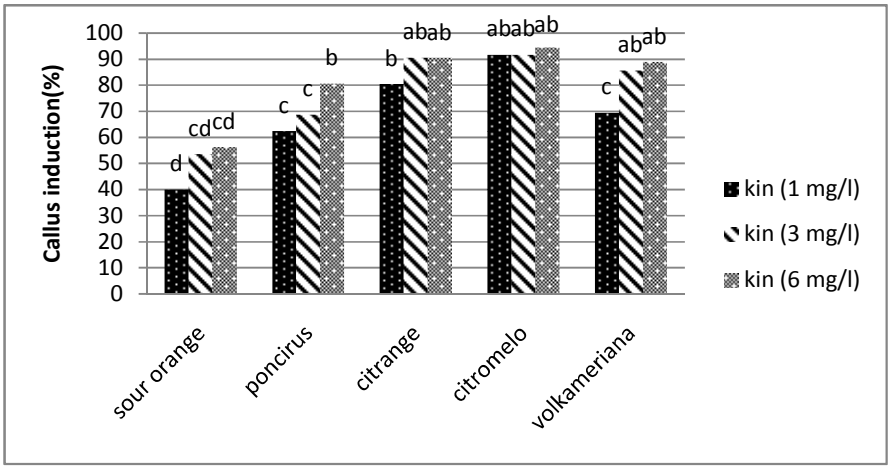

Fig. 4. Callus induction from leaf explant of different genotypes of citrus in MS medium supplemented with different levels of kinetin.

The interaction effect between root stocks and 2,4-D on callus induction in leaf explants also was shown in Fig. 5. The highest callus induction was observed in 3 mg/l 2, 4-Din Citromelo (94.44%) and the lowest callus induction was obtain in 1mg/l 2, 4-D on sour orange(35.71 %) (Fig. 5). Ramdan *et al* (2014) used 2,4-D and BAP for callus induction. In their study the highest frequency of the callus induction rate (100% and 83%) was observed with two combinations of 2, 4-D/BAP: 1 / 0.5 and 2/1 (mg/l). Medium containing only BAP (1mg/l) resulted in the formation of large numbers of roots. Also, the callus was induced on MT medium containing only 2, 4-D (1 mg/l) was brown in color and low quality compared to that produced on MT media containing 2, 4-D/BAP.

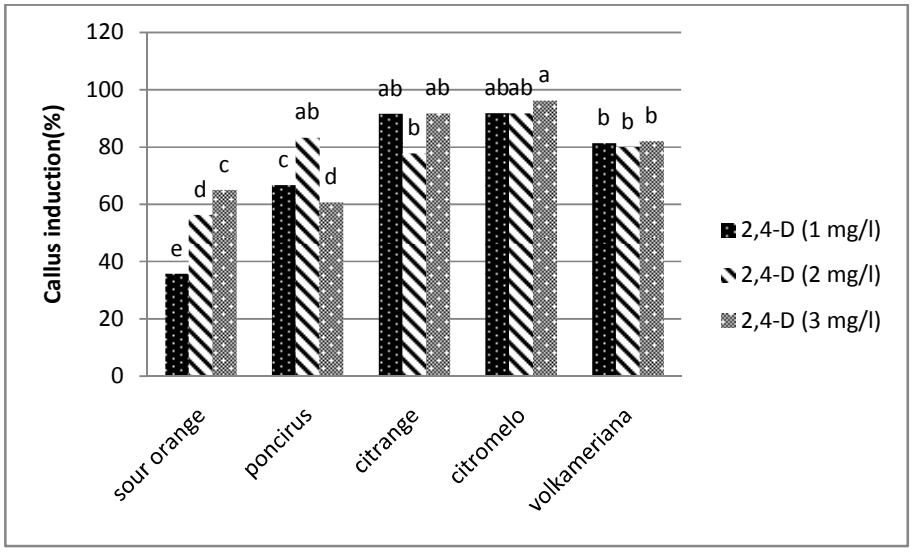

Fig. 5. Callus induction from leaf explant of different genotypes of citrus in MS medium supplemented with different levels of 2,4-D.

Fig. 6. showed the interaction effect of Kinetin and 2,4-D on callus induction frequency of leaf explants. The combination of 6 mg/l Kin with 3 mg/l of 2,4-D produced the highest callus induction (91.66%). The lowest percentage of callus induction was observed in 1mg/l Kin with 2 mg/l of 2,4-D (62.23 %) (Fig. 6).

Sativa *el al* (2010) reported that maximum callus induction (96%) was observed with 2, 4-D (1 mg/L) and from root segments, it was 48.66% on MS medium supplemented with 2, 4-D (2 mg/L). Table 2 showed the interaction of rootstocks × concentration of Kinetin × concentration of 2,4-D on callus induction in leaf explants.

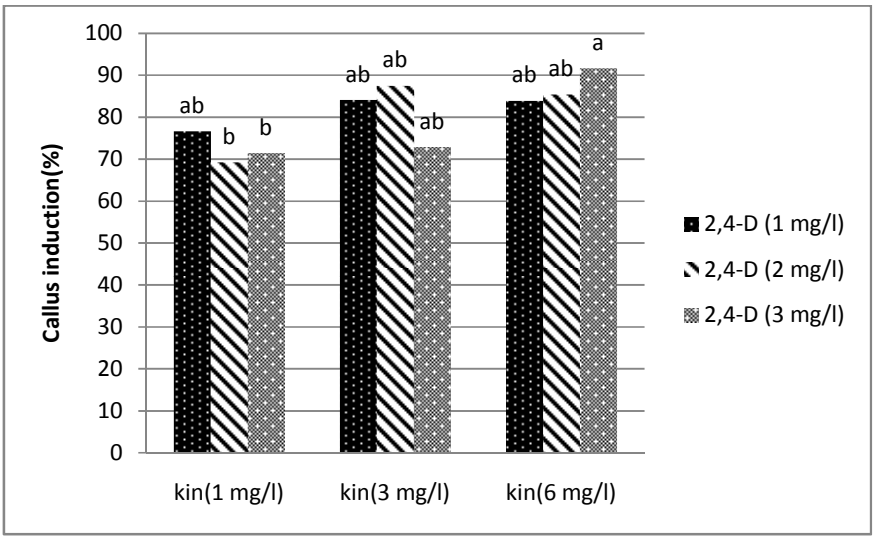

Fig. 6. Callus induction from leaf explant of MS medium supplemented with different levels of 2,4-D and Kinetin.

Table 2: Callus induction percentage of five citrus rootstocks according the combinations of 2,4-D and Kin.

Rootstock	2,4-D	Kin	Callus induction (%)
Sour orange	1 mg/l	1 mg/l	66.67 ±4.5c
Sour orange	2 mg/l	1 mg/l	8.33 ±62.84e
Sour orange	3 mg/l	1 mg/l	50 ±21.17d
Sour orange	1 mg/l	3 mg/l	0±71.17 f
Sour orange	2 mg/l	3 mg/l	66.67 ±4.5c
Sour orange	3 mg/l	3 mg/l	8.33±62.84 e
Sour orange	1 mg/l	6 mg/l	41.67 ±62.84d
Sour orange	2 mg/l	6 mg/l	0 ±71.17f
Sour orange	3 mg/l	6 mg/l	25 ±46.17e
Poncirus	1 mg/l	1 mg/l	58.33±12.84 c
Poncirus	2 mg/l	1 mg/l	75±3.83bc
Poncirus	3 mg/l	1 mg/l	33.33±37.84 d
Poncirus	1 mg/l	3 mg/l	66.77 ±4.4c
Poncirus	2 mg/l	3 mg/l	100±28.83 a
Poncirus	3 mg/l	3 mg/l	16.67 ±54.5e
Poncirus	1 mg/l	6 mg/l	75 ±3.83bc
Poncirus	2 mg/l	6 mg/l	75 ±3.83bc
Poncirus	3 mg/l	6 mg/l	91.67 ±20.5ab
Citrange	1 mg/l	1 mg/l	50 ±21.17d
Citrange	2 mg/l	1 mg/l	50 ±21.17d
Citrange	3 mg/l	1 mg/l	91.67±20.5ab
Citrange	1 mg/l	3 mg/l	91.67 ±20.5ab
Citrange	2 mg/l	3 mg/l	91.67 ±20.5ab
Citrange	3 mg/l	3 mg/l	91.67 ±20.5ab
Citrange	1 mg/l	6 mg/l	91.67 ±20.5ab
Citrange	2 mg/l	6 mg/l	91.67 ±20.5ab
Citrange	3 mg/l	6 mg/l	94.4 ±23.23ab
Citromelo	1 mg/l	1 mg/l	91.67 ±20.5ab
Citromelo	2 mg/l	1 mg/l	91.67 ±20.5ab
Citromelo	3 mg/l	1 mg/l	91.67 ±20.5ab
Citromelo	1 mg/l	3 mg/l	91.67 ±20.5ab
Citromelo	2 mg/l	3 mg/l	91.67 ±20.5ab
Citromelo	3 mg/l	3 mg/l	91.67 ±20.5ab
Citromelo	1 mg/l	6 mg/l	91.67 ±20.5ab
Citromelo	2 mg/l	6 mg/l	91.67 ±20.5ab
Citromelo	3 mg/l	6 mg/l	100±28.83 a
Volkamer	1 mg/l	1 mg/l	66.77 ±4.4c
Volkamer	2 mg/l	1 mg/l	75 ±3.83bc
Volkamer	3 mg/l	1 mg/l	83.33±12.16 b
Volkamer	1 mg/l	3 mg/l	83.33 ±12.16b
Volkamer	2 mg/l	3 mg/l	91.67 ±20.5ab
Volkamer	3 mg/l	3 mg/l	83.33 ±12.16b
Volkamer	1 mg/l	6 mg/l	91.67 ±20.5ab
Volkamer	2 mg/l	6 mg/l	91.67 ±20.5ab
Volkamer	3 mg/l	6 mg/l	100±28.83 a

In each column, any two means having a common letter are not significantly at p=0.05 based on Duncan's multiple range test.

The concentration of 6 mg/l Kin with 3 mg/l of 2,4-D in Citromelo and Volkameriana (100%), and 3 mg/l Kin with 2 mg/l 2,4-D (100 %)on Poncirus, showed the highest callus induction (Table 2). The basal medium supplemented with 2,4-D (2 mg/l) and kinetin (1.5 mg/l) has been reported to callus initiation of Malta (*Citrus sinensis*) (Azim *et al.,* 2011). Ramdan *et al* (2014) observed the 2,4-D (2 mg/l) had a maximum callus induction (100%) on citrus root stockin MS medium. They reported that citrus rootstock had a basal role on percentage of callus induction.

The success of in vitro culture depends mainly on the growth conditions of the source material (Caswell *et al.,* 2000; Delporte *et al.,* 2001), medium composition and culture conditions (Saharan *et al.,* 2004), and the genotypes of donor plants. Among those factors, the genotype appears to be important factor influencing the efficiency of in vitro culture (Borjian and Arak, 2013). The results also indicate that callus induction ability is greatly influenced by the concentration of plant hormones and is in agreement with those reported in citrus genotypes (Ramdan *et al.,* 2014; Azim *et al.,* 2011; Sativa *et al.,* 2011).

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