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CARROT (*DAUCUS CAROTA* L.) *IN VITRO* REGENERATION

Keramat RABIEI [1], Aleksey POLYAKOV [2], Mahmood KHODAMBASHI [1], Olga SHARAFOVA [2], Elena KALASHNIKOVA [3], Saadollah HOOSHMAND[1], Mansour OMIDI[4]

[1] Shahrekord University, Shahrekord, Iran,
[2] All Russian Research Institute of Vegetable Crops RAAS, Vereja, Ramensky district, Moscow region, Russia
[3] Russian State Agrarian University – MSXA named after K.A. Timiriazev
[4] Tehran University, Iran

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Summary

Experiments were carried out for optimization of conditions for morphogenic callus production and regeneration of carrot (*Daucus carota* L.) using four cultivars cultivated in Iran. Hypocotyl segments were put on media supplemented with 0.2 mg·L^{-1} 2,4-D for callus initiation and proliferation. Small portions of callus (25 mg) were cut and transferred to new media with different concentrations of 2,4-D including 0.2, 0.5 and 1 mg·L^{-1}. MS (Murashige & Skoog 1962) and MSm (Masuda *et al*. 1981) media were used in the experiments to identify the differences between these two media according to callus, embryoid and plantlet production.

Obtained results showed that low level of 2,4-D (0.2 mg·L^{-1}) was more effective in morphogenic callus production although higher amounts of it (0.5 mg·L^{-1} and 1 mg·L^{-1}) caused more callus growth. Kinetin in concentration of 0.1 mg·L^{-1} was more effective for regeneration than BAP used in concentration 1 mg·L^{-1} especially according to embryoid production. MSm medium was more useful than MS for callus production and regeneration of carrot plantlets. Among tested cultivars Nantes Improved had more capability to produce viable plantlets.

key words: 2,4-D, BAP, kinetin, morphogenesis, callus, embryoid, regenerant

INTRODUCTION

Callus production and regeneration are affected by genotype, type of explants, type and concentration of hormones applied in medium. Many attempts have been made to establish reproducible regeneration systems for carrot (*Daucus carota* L.) (Tukavin & Shmikova 2000; Kalashnikova 2003; Polyakov 2005; Polyakov & Chikrizova 2009).

Auxins are widely used to induce callus production. The most frequently used auxin to initiate callus cultures is 2,4-D. For breeding

Corresponding author:
e-mail: k_rabiei@yahoo.com; vita100plus@yahoo.com

purposes it is important to produce embryogenic callus. The process of somatic embryogenesis is often initiated in media containing high level of auxins, especially 2,4-D, but embryos usually do not develop further until the auxin concentration will be reduced (Edwin *et al*. 2008). Sharp *et al*. (1980) proposed that auxin induces an embryogenic determination in a portion of cells in callus or suspension cultures but at the same time causes of further development of them into embryos. It was suggested that division of the pro-embryogenic cells and their development into embryos are only resumed at lower auxin concentrations (Edwin *et al*. 2008).

The effect of cytokinins is most noticeable in tissue cultures where they are used, often together with auxins, to stimulate cell division and control morphogenesis. Synthetic cytokinins most commonly used in micropropagation: are kinetin and benzylaminopurine (BAP). Low concentration of cytokinin (typically 0.5-2.5 μM) is often added to media for the induction of embryogenic callus, especially in broad-leafed plants (Edwin *et al*. 2008). A requirement for a particular cytokinin is sometimes noted for the induction of embryogenesis (Fujimura & Komamine 1979).

This investigation was carried out to determine the effect of different concentrations of 2,4-D on embryogenic callus production and also to reveal the efficiency of BAP and kinetin on morphogenesis and embryogenesis of carrot plant.

MATERIALS AND METHODS

The investigations were carried out in the Department of Biotechnology of All Russian Research Institute of Vegetable Crops RAAS in 2008-2009. Coated seeds of four carrot cultivars those are cultivated in Iran including, Monarch, Nantes Improved, Tam Tam and Vilmorn were used to produce hypocotyl segments. In the experiment seeds of carrot were surface-sterilized by soaking in 70% (v/v) ethanol for 30 seconds and in 1% sodium hypochlorite solution for 10 minutes and were washed three times in sterile water for 5 minutes. Then seeds were placed on MS (Murashgue & Skoog 1962) medium containing sucrose 30 $g \cdot L^{-1}$, plant agar 5 $g \cdot L^{-1}$ and incubated under 25±1°C. The medium was autoclaved two times during 30 minutes (15 min + 15 min) under the temperature 121°C after the pH was adjusted to 5.6. Cultures were maintained under the culture room condition of light intensity (1000 $\mu mol \cdot m^{-2} \cdot sec^{-1}$), temperature of 25±1°C and 70-80% relative humidity with a 16/8 h light/dark photoperiod. After 2 weeks hypocotyl segments of seedlings (between 4-6 mm in length) were cut and placed on MS medium containing 0.2 $mg \cdot L^{-1}$ 2,4-D to initiate callus production. To estimate the effect of 2,4-D concentration on callus growth, calli were divided into pieces about 25mg and transferred on new MS and MSm media supplemented with three concentrations of 2,4-D (0.1, 0.5 and 1 $mg \cdot L^{-1}$). Ten pieces of callus per petri dish were used and three dishes were repeated for each experiment. The characteristics of callus including morpho- and embryogenesis (counted as a number of morpho- or embryogenic calli devided on a number of cultivated calli expressed in %) were determined and

recorded after one month of cultivation. Analysis of variance for callus weight was done in the form of factorial experiment based on randomized complete block design using SAS software version 9. The means were compared using Duncan's Multiple Range Test at probability level P=0.05.

To investigate the effect of BAP and kinetin on morphogenesis, calli were placed in MS and MSm media containing kinetin in concentration 0.2 $mg \cdot L^{-1}$ and BAP in concentration 1 $mg \cdot L^{-1}$ and number of embryoids and shoots were counted. Similar to the first experiment ten pieces of callus per petri dish were used and three dishes were repeated for each experiment. The estimation of morpho-, embryogenesis and the corresponding graphs were done using Excel 2007.

Regenerated plantlets and embryoids were removed from culture media and put on filter paper in glass tube containing MS liquid medium including sucrose 15 $mg \cdot L^{-1}$. After one month of cultivation well established plantlets having developed root system were removed from the culture tube and washed in running tap water. Then they were transferred to plastic pots containing oven-sterilized soil. For plantlet adaptation, a high relative humidity was maintained under transparent plastic cover for 3 weeks in a shady place and temperature 22 ± 2ºC. In the last week of acclimatization to allow plantlets directly contact with free air, the cover was pulled out several times per day and the plantlets were sprayed with water. Then the adapted plants were transferred to green house.

RESULTS AND DISCUSION

Morphogenic callus production. Analysis of variance for callus weight showed that the means for media, hormones and cultivars were significantly different but there were not major differences among interactions (Table 1). The comparison of means (Table 2) indicated that the mean of callus weight in MSm medium (42.8 mg) was higher than that of MS medium (39.6 mg). On MSm medium not only the initiation and proliferation of callus were better than that on MS medium, but also the percent of morphogenic callus production was higher for each cultivar (Table 3). The main difference between MS and MSm medium is the existence of casein hydrolysed (500 $mg \cdot L^{-1}$) in MSm. Casein hydrolysate can be a source of calcium, phosphate, several microelements, vitamins and most importantly a mixture of up to 18 amino acids. Casein hydrolysate is very effective for growth improvement in suspension culture, callus culture, shoot culture and embryogenesis (Edwin *et al*. 2008).

Table 1- Analyze of variance for callus weight

Source of variation	DF	MS
Block	2	94.5*
Medium	1	172.05**
2,4-D concentration	2	558.6**
Cultivar	3	186.5**
Medium × 2,4-D concentration	2	16.6^{ns}
Media × Cultivar	3	27^{ns}
2,4-D concentration × Cultivar	6	3.69^{ns}
Medium × 2,4-D concentration × Cultivar	6	2.5^{ns}

* Significant in the level of 0.05; ** Significant in the level of 0.01
ns: non-significant

Table 2. Mean comparison of callus weight (mg) for medium, concentration of 2,4-D and cultivar

Medium	Waight of callus (mean)	2,4-D concentration	2,4-D mean	Cultivar name	Cultivar mean
MS	39.6 a*	0.2	37.1 a	Monarch	39.4 a
MSm	42.8 b	0.5	40.0 b	Nantes Improved	37.7 a
		1	46.5 c	Tam Tam	43.5 b
				Vilmorn	44.4 b

* Numbers followed by the same letter are not significantly different

The rate of callus growth is also intensively affected by the concentration of 2,4-D supplied in the medium. Generally, in our experiment high level of 2,4-D led to the production of more callus mass (46.5 mg), but according to the results that are presented in Table 3 the morphogenic callus production was lower in that conditions (40%). The mean of callus weight in the medium that contained 0.2 $mg \cdot L^{-1}$ 2,4-D (37.1 mg) was the lowest among other concentrations but the rate of morphogenic callus was the highest (72%). There was similar situation for the amount of morphogenic callus production within each cultivar. Explants of Nantes Improved which were placed on MSm medium containing 0.2 $mg \cdot L^{-1}$ of 2,4-D produced the highest amount of morphogenic callus (90%) (Table 3). It was more than that of explants which were grown in similar medium fortified with 1 $mg \cdot L^{-1}$ 2,4-D (50%). The amount of callus proliferation also was depended on a cultivar. For example cultivars Vilmorn and Nantes Improved have produced the most and the least amount of callus mass respectively (44.4 mg and 37.7 mg), but the amount of morphogenic callus was higher in Nantes Improved (64%) in comparison with Vilmorn (46%). Differences between cultivars in callus production of some other crops have also been reported (Oggema *et al*. 2007; Silvertand *et al*. 1996).

Table 3. Morphogenic callus production by carrot cultivars in depends on media and 2,4-D concentration

Genotype	Medium	Concentration of 2,4-D	percent of morphogenic callus
Monarch	MS	0.2	70 ± 8.4
		0.5	43 ± 9.0
		1.0	40 ±8.9
	MSm	0.2	83 ±6.8
		0.5	50 ±9.1
		1.0	47 ±9.1
Nantes Improved	MS	0.2	77 ±7.6
		0.5	60 ±8.9
		1.0	43 ±9.0
	MSm	0.2	90 ±5.5
		0.5	60 ±8.9
		1.0	50 ±9.1
Tam Tam	MS	0.2	63 ±8.8
		0.5	50 ±9.1
		1.0	34 ±8.7
	MSm	0.2	73 ±8.1
		0.5	53 ±9.1
		1.0	40 ±8.9
Vilmorn	MS	0.2	47 ±9.1
		0.5	40 ±8.9
		1.0	30 ±8.4
	MSm	0.2	67 ±8.6
		0.5	50 ±9.1
		1.0	40 ±8.9

Effect of 2,4-D in morphogenic callus production has been investigated by other scientists. Callus cultures of *Arabidopsis thaliana* can be initiated and maintained on a medium containing 2,4-D, but their morphogenic capacity will be lost progressively as they were maintained longer on that medium and ultimately after 6-8 months of regeneration it was completely lost (Edwin *et al*. 2008). Fujimura and Komamine (1979), De Vries *et al*. (1988) and Ribnicky (1996) reported that in *Daucus carota* embryogenic cells can be obtained from hypocotyl explants in the presence of 2,4-D.

An experiment was carried out by Dinghou (1994) using different concentrations of 2,4-D to test the effect of this substance on morphogenic callus production in rice. When the medium did not contain 2,4-D response of the explants was not observed. When the concentration of 2,4-D in the media was 1 or 2 $mg \cdot L^{-1}$ the friable non embryogenic callus was formed and when the concentration of 2,4-D was 0.2 and 0.5 $mg \cdot L^{-1}$ adventitious bud and callus formation was observed.

Oggema *et al.* (2007) also reported that the level of 2,4-D in the culture medium significantly affected

the frequency, type and quality of calli that were formed. They identified that the optimum concentration of 2,4-D was more effective when it was at low levels. Radhakrishnan *et al*. (2001) also reported that cells grown under high concentration of 2,4-D showed the herbicidal effects and therefore slowing down the callus induction process.

Regeneration. The obtained results showed that MSm medium was more effective than MS in morphogenesis and embryogenesis of carrot. In almost all of the cases percent of morphogenic callus grown on MSm was higher than that of MS medium (Table 4). The highest amount of morphogenic calli (82.8%) were obtained when calli of cultivar Nantes Improved were transferred from MSm medium containing 2,4-D (0.2 $mg \cdot L^{-1}$) to MSm medium containing 0.1 $mg \cdot L^{-1}$ kinetin, whereas its amount on MS medium with the same hormone concentration was 65%. Differences were more obvious in comparing number of shoots per callus and percent of embryogenic callus of these two variants. Numbers of shoots produced per callus were 3.17 and 2.53 on MSm and MS media respectively. Also percent of embryogenic callus for these two media were respectively 73.3 and 53.3. There were similar results for other variants in all the other cultivars. It can be concluded that MSm media has direct effect on increasing morphogenic and embryogenic potential of carrot plant due to presence of casein hydrolysate in the medium (Table 4).

Addition of casein hydrolysate to MS medium was found to be essential for shoot formation from callus (Chand & Roy 1981). Suspension culture of wild carrot cells in a medium containing casein hydrolysate as the sole nitrogen source produced somatic embryos (Anderson 1976). Although there have been many reports of embryogenesis being promoted by the addition of casein hydrolysate, in many cases embryogenic callus and/or embryo formation did not occur without the presence of this amino acid source (Radojevic 1988; Nuti Ronchi *et al*. 1984; Gupta *et al*. 1987; Osifo 1988).

The positive effect of using lower concentrations of 2,4-D in early stages of callus production on morphogenesis and regeneration of explants in latter stages is obviously visible (Table 4). In almost all of the cultivars and media percent of morphogenic callus, embryogenic callus and number of shoots per callus were in the highest amount when 2,4-D in concentration 0.2 $mg \cdot L^{-1}$ was used for callus production and kinetin in concentration 0.1 $mg \cdot L^{-1}$ was used for regeneration. In general, high concentration of 2,4-D in the medium decreased regeneration ability. In addition, BAP was less effective than kinetin in embryogenesis. The lowest amount of morphogenesis (16.7%) was observed in cultivar Vilmorn when 2,4-D (1 $mg \cdot L^{-1}$) and BAP (1 $mg \cdot L^{-1}$) were used in the medium. Using kinetin instead of BAP led to production of 53.3% morphogenic callus and increased embryogenesis form 3.3% to 30%. The minimum number of shoots per callus (0.1) was produced in cultivar Tam Tam when calli that were grown in MS medium containing 2,4-D (1 $mg \cdot L^{-1}$) transferred to the MS medium containing BAP.

Table 4. The effect of cultivar, medium, 2,4-D, BAP and Kinetin on morphogenesis and embryogenesis of carrot

Geno-type	Medium	2.4-D concentra-tion	Cytokinin	Morphogen-esis (%)	Number of shoots per callus	Embryogen-esis (%)	Number of regenerated plantlets
Monarch	Ms	0.2	BAP	30.0 ± 8.4	0.53	6.70 ± 4.6	2
			Kin	55.0 ± 9.1	2.03	44.3 ± 9.1	8
		0.5	BAP	23.3 ± 7.7	0.57	3.30 ± 3.3	2
			Kin	45.6 ± 9.1	1.10	30.0 ± 8.4	7
		1	BAP	18.3 ± 6.2	0.30	0	1
			Kin	36.7 ± 8.8	0.90	22.4 ± 7.6	3
	Msm	0.2	BAP	33.0 ± 8.6	0.53	6.70 ± 4.6	3
			Kin	69.0 ± 8.4	1.47	56.6 ± 9.0	8
		0.5	BAP	28.0± 8.2	0.77	6.70 ± 4.6	3
			Kin	55.5 ± 9.1	1.33	40.0 ± 8.9	6
		1	BAP	26.7 ± 8.1	0.57	3.30 ± 3.3	2
			Kin	50.0 ± 9.1	1.10	30.0 ± 8.4	6
Nantes Improved	Ms	0.2	BAP	46.0 ± 9.1	0.80	6.70 ± 4.6	5
			Kin	65.0 ± 8.7	2.53	53.3 ± 9.1	10
		0.5	BAP	43.2 ± 9.0	0.73	10.0 ± 5.5	5
			Kin	52.0 ± 9.1	1.47	43.3 ± 9.0	7
		1	BAP	40.3 ± 9.0	0.60	3.30 ± 3.3	3
			Kin	45.4 ± 9.1	1.03	36.7 ± 8.8	6
	Msm	0.2	BAP	56.7 ± 9.0	1.23	10.0 ± 5.5	8
			Kin	82.8 ± 6.9	3.17	73.3 ± 8.1	14
		0.5	BAP	45.5 ± 9.1	1.07	6.70 ± 4.6	7
			Kin	71.3 ± 8.3	1.83	62.6 ± 8.8	11
		1	BAP	39.3 ± 8.9	0.70	10.0 ± 5.5	5
			Kin	60.0 ± 8.9	1.87	46.7 ± 9.1	10
Tam Tam	Ms	0.2	BAP	36.7 ± 8.8	0.67	6.70 ± 4.6	3
			Kin	49.4 ± 9.1	1.27	38.8 ± 8.9	8
		0.5	BAP	20.0 ± 7.3	0.50	0	2
			Kin	43.3 ± 9.0	0.97	30.0 ± 8.4	7
		1	BAP	6.70 ± 4.6	0.10	0	0
			Kin	40.1 ± 8.9	1.00	26.7 ± 8.1	6
	Msm	0.2	BAP	40.2 ± 9.0	0.97	6.70 ± 4.6	7
			Kin	70.0 ± 8.4	2.10	56.7 ± 9.0	12
		0.5	BAP	32.2 ± 8.5	0.57	6.70 ± 4.6	3
			Kin	56.6 ± 9.0	1.53	43.3 ± 9.0	8
		1	BAP	23.3 ± 7.7	0.37	3.30 ± 3.3	2
			Kin	36.7 ± 8.8	0.60	23.3 ± 7.7	3
Vilmorn	Ms	0.2	BAP	42.5 ± 9.0	0.30	6.70 ± 4.6	1
			Kin	62.3 ± 8.8	1.90	36.7 ± 8.8	11
		0.5	BAP	35.0 ± 8.7	0.27	3.30 ± 3.3	2
			Kin	46.7 ± 9.1	1.00	28.2 ± 8.2	9
		1	BAP	34.0 ± 8.6	0.23	3.30 ± 3.3	1
			Kin	40.0 ± 8.9	1.00	23.3 ± 7.7	7
	Msm	0.2	BAP	47.0 ± 9.1	0.73	10.0 ± 5.5	6
			Kin	71.3 ± 8.3	2.33	53.3 ± 9.1	12
		0.5	BAP	40.3 ± 9.0	0.33	6.60 ± 4.6	1
			Kin	62.3 ± 8.8	1.43	44.4 ± 9.1	9
		1	BAP	16.7 ± 6.8	0.23	3.30 ± 3.3	1
			Kin	53.3 ± 9.1	1.23	30.0 ± 8.4	8

However, the maximum number of organs per callus (3.17 for cultivar Nantes Improved) was obtained under cultivation of calli on the medium with 2,4-D used in concentration 0.1 $mg \cdot L^{-1}$ and then on the medium with kinetin - 0.2 $mg \cdot L^{-1}$.

Using 2,4-D in low concentration for callus production and induction of embryogenesis and kinetin for plant regeneration of carrot and other crops have been demonstrated in many reports. Silvertand *et al*. (1996) reported that in leek (*Allium ampeloprassum* L.) high 2,4-D levels gave a watery and soft callus while low 2,4-D gave compact embryogenic callus. Komamine *et al*. (1990) has described that in carrot 2,4-D in the concentration of 0.05 μM was required to be present for 6 days for inducing competent single cells to form somatic embryos. Beyond this duration auxin was inhibitory. Satoh *et al*. (1986) used 1 week old hypocotyls of carrot to produce callus in the medium containing 1 $mg \cdot L^{-1}$ 2,4-D and then transferred to new medium for regeneration. The formation of somatic embryos was strongly and moderately inhibited by the addition of 1 μM and 0.1 μM 2,4-D respectively, but not by 10 nM 2,4-D.

Type of cytokinin and its effect on morphogenesis basically depend on crop species. Halperin and Wetherell (1964) noted that 0.2 $mg \cdot L^{-1}$ kinetin could be used in various media for stimulating embryogenesis of carrot callus. Shary *et al*. (2006) reported that in *Melia azedarach* BAP was more suitable for organogenesis than kinetin which induced only non-organogenic calli. This is similar to results obtained by Nirmalakumari *et al*. (1993) and Drew (1996) in neem (*Azadirachta indica* Juss.) where kinetin alone induced non-morphogenic callus. There are reports that cytokinins can sometimes induce or promote root growth (Fries 1960) or adventitious root formation in the absence of auxins (Nemeth 1979). In nearly all cases only low rates of cytokinin have been effective. For example, shoots of sugar beet were rooted on MS medium containing 0.5 $mg \cdot L^{-1}$ kinetin without auxin (Konwar & Coutts 1990).

In our experiments 271 green, viable and adapted to *in vivo* conditions plantlets were obtained (Table 4). Among them plantlets of cultivars Monarch, Nantes Improved, Tam Tam and Vilmorn consisted of 19.8%, 34.5%, 23.5% and 22.2% respectively. So it is possible to recommend Nantes Improved as a suitable cultivar to succeed in further tissue culture experiments. Seventy four percent of plantlets were regenerated from embryoids suggesting the use of 2,4-D and kinetin to obtain sufficient number of regenerated plantlets through embryoid in carrot tissue culture experiment.

CONCLUSIONS

It was concluded that using 2,4-D in concentration 0.2 $mg \cdot L^{-1}$ was the most effective for initiation and proliferation of callus in carrot tissue culture. Higher levels of 2,4-D increas the speed of callus growth but decrease the potential of morphogenesis.

BAP used in concentration 1 $mg \cdot L^{-1}$ indueces direct morphogenesis but kinetin used in concentration 0.1 $mg \cdot L^{-1}$ induces production of embryoids and regeneration of plantlets from embryoids.

The most convenient system of carrot *in vitro* regeneration is the application of 2,4-D in concentration 0.2 $mg \cdot L^{-1}$ for callus initiation and using kinetin in concentration 0.1 $mg \cdot L^{-1}$ at the next stage to induce embryogenesis and to grow viable plantlets.

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REGENERACJA MARCHWI (*DAUCUS CAROTA* L.) *IN VITRO*

Streszczenie

Prowadzono doświadczenia mające na celu optymalizację warunków do wytwarzania morfogenicznego kalusa i regeneracji marchwi (*Daucus carota* L.), używając czterech odmian uprawianych w Iranie. Segmenty hypokotyli wykładano na pożywki z dodatkiem 0,2 mg·L^{-1} 2,4-D do inicjacji i namnażania kalusa. Wycinano małe cząstki kalusa (25 mg) i przenoszono na nowe pożywki o różnych stężeniach 2,4-D, w tym 0,2, 0,5 i 1 mg·L^{-1}. Stosowano pożywki MS (Murashige i Skoog 1962) i MSm (Masuda i in. 1981) i określono ich wpływ na wytwarzanie kalusa, embrioidów i roślin.

Uzyskane wyniki wykazały, że niskie stężenie 2,4-D (0,2 mg·L^{-1}) było korzystniejsze do wytwarzania morfogenicznego kalusa, natomiast wyższe stężenia (0,5 mg·L^{-1} oraz 1 mg·L^{-1}) wpływały na lepszy rozwój kalusa. Kinetyna w stężeniu 0,1 mg·L^{-1} była bardziej skuteczna do regeneracji niż BAP w stężeniu 1 mg·L^{-1}, szczególnie pod względem wytwarzania embrioidów. Pożywka MSm była korzystniejsza niż pożywka MS do wytwarzania kalusa i regeneracji roślin marchwi. Wśród badanych odmian odm. 'Nantes Improved' miała większą zdolność do wytwarzania zdolnych do przetrwania roślin.