

Effect of Salicylic Acid on Somatic Embryogenesis and Chlorogenic Acid Levels of Carrot (*Daucus carota* cv. Nantes) Explants

S. S. Hosseini

Department of Horticulture, Pardis Faculty of Agriculture, Gorgan University of Agricultural Sciences and Natural Resources (GUASNR), Golestan, Gorgan, I.R. Iran.

K. Mashayekhi

Department of Horticulture, Pardis Faculty of Agriculture, Gorgan University of Agricultural Sciences and Natural Resources (GUASNR), Golestan, Gorgan, I.R. Iran.

M. Alizadeh*

Department of Horticulture, Pardis Faculty of Agriculture, Gorgan University of Agricultural Sciences and Natural Resources (GUASNR), Golestan, Gorgan, I.R. Iran.

P. Ebrahimi

Gonbad Institute of Higher Education, P.O.Box.163, Gonbad, Golestan, Iran.

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*Corresponding author's email: alizadehpub@gmail.com

Many factors may influence the efficiency of somatic embryogenesis. This capability may be differ with regard to media compositions, genotype, tissue, organ ontology and the stage of differentiation. The effects of five salicylic acid concentrations (0, 25, 50, 75 and 100 μM) on different stages of carrot somatic embryogenesis were studied using petiole and root secondary phloem explants as starting materials. The salicylic acid treatments were applied in two culture media; B5 and NL supplemented with 0.5 mg l^{-1} 2,4-Dichlorophenoxy acetic acid (2,4-D) and 1.0 mg l^{-1} Indole-3-acetic acid (IAA), respectively. The chlorogenic acid (CGA) levels produced by the explants during embryogenesis were monitored using high performance liquid chromatography (HPLC) technique. The results proved that petioles are superior explants over root secondary phloems with regard to somatic embryogenesis. The B5 medium also exhibited induction of greater number of embryos over NL medium. The results of the present study unequivocally suggest that, irrespective of the type of explants and media culture, SA increments beyond than 75 μM negatively affect carrot somatic embryogenesis. A considerable elevation in CGA production during embryogenesis following SA treatments was also found. Chlorogenic acid produced by cultures was coincided with the SA treatments almost as the same manner that it affects somatic embryogenesis process. Salicylic acid at the rate of 100 μM induced highest level of CGA production and as result least number of embryos was formed.

Abstract

Keywords: Carrot, Chlorogenic acid, High performance liquid gas chromatography, Salicylic acid, Somatic embryogenesis.

INTRODUCTION

Somatic embryogenesis (a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell) is a multi-step regeneration process starting with formation of pro-embryogenic masses, followed by somatic embryo formation, maturation and regeneration. It is an alternative to traditional vegetative propagation methods as it offers a rapid large-scale propagation system (Sobri *et al.*, 2006). Acquiring embryogenesis potential has been reported to be not analogous among different plants or even their various cells, but it is an innate competence that only in special circumstances, *i.e.* induction conditions, can be emerged (Bonet *et al.*, 1998). This capability may differ with regard to genotype, tissue, organ ontology and the stage of differentiation etc. (Hosseini, 2009). Many factors may influence the efficiency of somatic embryogenesis. The influence of exogenous growth regulators and the accumulation of metabolites during cell culture and somatic embryogenesis and possible role of such compounds in plant improvement via somatic embryogenesis has been examined only in a few plants for instance, date palm (El Bellaj and El Hadrami 1998), rice (Zhou *et al.*, 2004) common bean (Luthria and Pastor-Corrales 2006) and cotton (Kouakou *et al.*, 2007).

Salicylic acid (SA) or 2-hydroxybenzoic acid belongs to a diverse group of phenolic compounds which encompasses an aromatic ring as well as one hydroxyl group. Salicylate generally found in numerous plant species and owing to its implication in most of the biological events has recently been grouped as a new plant growth regulator (Raskin, 1992a, b). Salicylic acid interferes in many biological events such as plant stomata closure, adventitious root initiation and thermogenesis during pollination. It can also be capable of furnishing resistance to pathogens and the biosynthesis of pathogenesis-related (PR) proteins and some kinds of phytoalexins. In addition, SA has a control over regulation of other plant growth substances as it inhibits ethylene production (Quiroz-Figueroa, 2001; Hosseini *et al.*, 2009).

There are numerous studies conducted on the effects of media compositions, mainly plant growth regulators on somatic embryogenesis. Salicylic acid as a new plant growth regulator (Raskin, 1992b) and its role in somatic embryogenesis has been targeted by many researches in different woody and herbaceous crops such as carrot (Roustan *et al.*, 1990; Nissen, 1994), coffee (Quiroz-Figueroa *et al.*, 2002), geranium (Hutchinson and Saxena, 1996), alfalfa (Meijer and Brown, 1988) and *Astragalus* (Jian-Ping *et al.*, 2001). Roustan *et al.*, (1989) reported that salicylic acid (50 μM) induces somatic embryogenesis in carrot hypocotyls whereas Hutchinson and Saxena (1996) accomplished embryogenesis in *Geranium* using lower concentrations of salicylic acid (20 μM). Working with carrot petioles, it was found that higher levels of salicylic acid (more than 100 μM) not only did not encourage somatic embryogenesis but also inhibited the same (Nissen, 1994). Corroboratory findings were already reported in alfalfa as well (Meijer and Brown, 1988).

Phenolic compounds constitute a wide range of plant substances which all possess an aromatic ring bearing one or more hydroxyl groups (Harborne, 1998). They are considered as detrimental compounds during *in vitro* culture, since their exudation and oxidation negatively affect the explants due to browning and necrosis (Benson 2000, Martin and Madassery, 2005). However, there are some reports on positive effects of phenolic compounds on *in vitro* morphogenic processes such as root formation and elongation, shoot proliferation, organogenesis, androgenesis and even somatic embryogenesis (Reis *et al.*, 2008).

A major class of phenolic compounds are hydroxycinnamic acids, which are found in almost every plant. Phenolic compounds, their antioxidant properties and distribution in carrots were investigated by Zhang *et al.*, (2004). They reported that carrots contained mainly hydroxycinnamic acids and derivatives. Among them chlorogenic acid (CGA) was a major hydroxycinnamic acid, representing from 42.2% to 61.8% of total phenolic compounds detected in different carrot tissues.

Reis *et al.*, (2008) observed that exogenous phenolic compounds added to the embryogenic

induction medium of Feijoa explants affect both the somatic embryogenesis induction and the further somatic embryo germination. Furthermore, their microscopic analysis showed a strong relationship between somatic embryo development and phenolic-rich cells. There are some other works representing that phenolic compounds are often positively associated with somatic embryo formation. Working with coffee, it was found that embryogenic calli developed only after browning of the initial explant (Neuenschwander and Baumann 1992). The browning and/or explants necrosis process, which is normally deleterious during in vitro culture, does not damage somatic embryo formation in their experiment. In the other hand, a more detailed analysis of the literature shows that differentiation of somatic embryos in some plants like cocoa is coincident with a decrease in phenolic content (Ndoumou *et al.*, 1997).

The present study was also conducted to examine somatic embryogenesis of two different carrot explants (petiole and root secondary phloem) cultured in two culture media and to explore the effects of elevated salicylic acid levels on CGA production and overall somatic embryogenesis.

MATERIALS AND METHODS

The present investigation was carried out in the Plant Tissue Culture Laboratory, Horticulture Department, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

Explant Selection, Preparation and Culture

Two types of explants (petiole / root secondary phloem) were exploited for culture establishment procured from carrot (*Daucus carota* cv. Nantes). Initially, the seeds/roots were washed thoroughly with normal tap water (30 min.) and surface sterilized with ethanol (70% v/v for 40 sec.) followed by sodium hypochlorite solution (5% v/v plus two drops of tween-20 for 15 min.). Sterile petioles were obtained following in vitro seed germination on full strength, solid, hormone free B5 medium (Gamborg *et al.*, 1968). The sterilized, clean roots were transversely cut up employing a hand mirotome. Disk-like, uniform explants were obtained by the help of a Trokar instrument. The media utilized for culture establishment were liquid B5 and NL (Neumann, 1966) containing 2,4-D (0.5 mg l⁻¹) and IAA (1.0 mg l⁻¹), respectively.

Salicylic Acid Treatments, Growth Conditions and Sub-culture Intervals

Five salicylic acid concentrations (0, 25, 50, 75 and 100 µM) in four replications were applied. The treatments in B5 medium were applied both in induction and realization phases of embryogenesis while in NL medium the same treatments were applied only in realization phase (in NL medium IAA was used as auxin source and there is no distinct phase for induction and realization as B5 medium). The cultured secondary phloem explants in Erlenmeyer were placed on a horizontal shaker (120 rpm) and the petiole explants were cultured in distinctive "nipple" culture flasks and arranged on an auxophyton apparatus (to rotate containers around a horizontal axis at the rate of 2 rpm). All the inoculated containers were incubated at 27 ± 2°C with continuous light generated by cool white fluorescent tubes (2000 lux). Petiole and secondary phloem explants growing on B5 medium were sub-cultured 3 and 5 weeks after inoculation respectively. Fresh, hormone-free B5 medium was used for this purpose. The petiole / phloem explants were examined for embryo development 3 and 5 weeks after sub-culture, respectively. The explants cultured on NL media were also examined for embryo development 5 (petiole) and 7 (phloem) weeks after inoculation. The embryos were observed under stereoscope connected to a monitoring system (20x and 40x magnification).

Estimation of Chlorogenic Acid

Plant Material Extraction

Following embryo observations by formerly above mentioned monitoring system the

samples were oven dried (50°C) and finely powdered from which 1 g was taken and transferred to a glass vial. Extraction was done using a reflux method comprising a simple water circulating system and a magnetic heater using Acetone (30 ml) as solvent. This was done for 1 hour followed by centrifugation (4000 rpm for 5 min.) The supernatant was isolated and debris was dissolved in 30 ml acetone and extraction procedure was again repeated for 1 hour. The final solution was evaporated using a rotary evaporator adjusted to 50°C. Prior to HPLC analysis each sample was dissolved in methanol (1 ml) by the help of an ultrasonic water bath. All extracts were filtered through 0.22 mm filters (Sigma, USA) and aliquots (25 ml) were analyzed by HPLC.

Chlorogenic acid standard was HPLC-grade purity procured from (Sigma, USA). HPLC was performed with a Merck Hitachi (MH) system (Merck Hitachi, Japan) comprising a quaternary pump (MH, L-7100), a vacuum degasser (Merck L-7614), a UV detector (Merck L-7400), and a 20- μ L sample injector (injector 2041 series, USA). Compounds were separated on a 250 mm \times 4.6 mm, C-18 column (Merck, Germany). Detection was carried out at 270 nm. The mobile phase comprised a mixture of acetonitril (10 ml), acetic acid (1 ml) and deionized water (98 ml). Acetonitrile and water were of HPLC-grade purity. Acetic acid was of analytical grade. A calibration curve also was constructed using the integrator values obtained from the quantification of standard solutions.

Experimental Design And Data Analysis

The present experiment was conducted as complete randomized design in factorial arrangement (explant \times treatment \times medium) with four replications. The percentage data were transformed using root square method ($\sqrt{\% + 0.5}$) prior to analysis. The results were analyzed using SAS software and the mean values were compared by LSD test in $p < 0.01$ probability.

RESULTS AND DISCUSSION

Somatic Embryogenesis

The procedures for carrot somatic embryogenesis using B5 and NL media were already standardized in our laboratory (Hosseini, 2009) and based on that sub-experiments including effects of different salicylic acid treatments were performed. The analyzed data for somatic embryogenesis is shown in Table 1. A significant difference between two media culture was recorded with regard to all stages of embryogenesis. The B5 medium showed higher potential for embryogenesis and more number of embryos were recorded as compared to NL medium. Apart from the media compositions, the synthetic auxin (2,4-D) supplemented to B5 medium can be considered as a source of variation and appearance of higher numbers of somatic embryos. Encouraging role of synthetic auxins, particularly 2,4-D in plant somatic embryogenesis is well known and many reports have revealed that adding 2,4-D to the medium will alter the somatic cells differentiation. The results of the present study for influence of natural / synthetic auxins on embryogenesis are analogous to records reported for banana (Strosse et al., 2006) and guava (Rai et al., 2007) embryogenesis.

Besides normal somatic embryos (Fig.1b), some embryoides, so called neomorph embryos were also developed (Table 1). These are actually under-developed embryos with abnormal morphological stature (Fig. 1a). The higher number of neomorphs was recorded in explants prepared from secondary phloem. Also, more number of neomorphs was recorded in B5 as compared to NL medium. Generation of neomorphs was also found to be affected by SA treatments. Though it was not significantly different with control, but apparently the highest level of neomorph embryos was found in explants treated with SA 100 μ M. John et al., (1995) reported that these types of embryos are induced during embryogenesis but they are not released to the culture medium as other normal ones. Developing these embryos and their maturation are not only associated to endogenous auxins but also highly correlated with the presence of higher concentration of abscisic

acid in the medium.

In addition to embryogenesis, root formation also occurred in some explants (Fig. 1c). Two B5 and NL media were significantly different with regard to adventitious root formation (Table 1). Auxins can be considered as a source of root induction in explants as IAA, a natural and photosensitive auxin was incorporated in NL medium, rooting is induced but IAA is gradually vanished due to photooxidation and as a result roots are emerged more rapidly.

Influence of Salicylic Acid Treatments

The results clearly demonstrated the significant effects of salicylic acid supplementation on different stages of somatic embryogenesis (Table 2). Apart from highest level of SA (100 μ M), the numbers of embryos were considerably increased following SA addition to both media. Different embryos observed in B5 medium supplemented with SA (50 μ M) is shown in Figure 1. In fact, low concentrations of SA stimulated rather than inhibited embryo formation. However, the results of the present study unequivocally suggest that, irrespective of the type of explants and media culture, SA increments beyond than 75 μ M negatively affect carrot somatic embryogenesis. Somatic embryogenesis was already induced from suspension cultures (derived from leaf callus) of *Plumbago rosea* L. an important medicinal plant (Komaraiah *et al.*, 2004). Furthermore, they observed that acetylsalicylic acid (ASA) alone induced embryogenesis but indole-3-acetic acid (IAA) failed to elicit a similar response. While considering the salient points of the present study, it can be stated that SA at the rate of 50 and 75 μ M are the most excellent levels for carrot somatic embryogenesis. In a preliminary study (data not shown) we observed that addition of 0.5 mM and more SA can completely inhibit carrot somatic embryo formation in either B5 or NL media.

Chlorogenic Acid Production

Generally in carrot, somatic embryogenesis is occurred in two distinct phases i.e. induction and emergence phases (Kamada and Harada 1979; Fridborg *et al.*, 1978). It means, embryogenic cells, which have embryonic competence, are induced when explants are cultured on medium containing 2,4-D (induction phase). Somatic embryos are formed following transfer of induced cells to the medium devoid of 2,4-D (emergence phase). However, when embryogenic cells are cultured at high cell density, somatic embryogenesis is strongly inhibited, even with the use of 2,4-D-free medium. The efficiency of somatic embryo formation can be improved by adding activated charcoal, which absorbs some inhibitory factors. Various phenolic compounds are accumulated in culture medium when charcoal is not present. For this reason, phenolic compounds have long been thought to inhibit somatic embryogenesis.

Previous studies revealed that carrots contained mainly hydroxycinnamic acids and derivatives that among them CGA was found to be a major hydroxycinnamic acid (Zhang *et al.*, 2004). Therefore we estimated various CGA levels during carrot embryogenesis as affected by salicylic acid treatments. Table 2 shows mean values of CGA levels produced by two carrot explants in B5 and NL media supplemented with different salicylic acid concentrations. There was not any significant difference between two media with regard to CGA evolution; however two carrot explants produced different levels of chlorogenic acids and phelom comprised more quantity of this phenolic compound as compared to petiole. Zhang *et al.*, (2004) also found that Phenolic content in different carrot tissues decreased from peel, phloem to xylem. So, it can be stated that CGA content is also tissue specifically produced. Furthermore, our salicylic acid treatments considerably raised the CGA production (Table 2). The results of the HPLC analysis obviously authenticated a considerable elevation in CGA production during embryogenesis following SA treatments (Table 3). It is clear that, following treatment with 25, 50 and 75 μ M SA, the CGA was also produced but in lower concentrations than 100 μ M. The results presented by Reis *et al.*, (2008) indicated that exogenous phenolic compounds added to the embryogenic

induction medium of Feijoa affect both the somatic embryogenesis induction and the further somatic embryo germination. They found that caffeic acid (140–560 μM) significantly increased somatic embryogenesis induction compared with the control. In the other hand, Ndoumou *et al.*, (1997) demonstrated the unhelpful effect of phenolic compounds on somatic embryogenesis of cocoa.

In conclusion, it can be stated that carrot petioles are superior explants over root secondary phloems and somatic embryogenesis may be performed efficiently using the former explants cultured on B5 medium. Salicylic acid enhances somatic embryogenesis in a dose-dependent manner up to 75 μM and beyond that inhibits the same. Chlorogenic acid produced by cultures was coincided with the SA treatments almost as the same manner that it affects somatic embryogenesis process. Salicylic acid at the rate of 100 μM induced highest level of CGA production and as result least number of embryos was formed.

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Tables

Table 1. Mean values for number of carrot somatic embryos as affected by explant, medium and salicylic acid treatments.

Source of variation		Somatic embryogenesis						Total embryos	Number of roots
		Globular	Heart-shaped	Torpedo	Cotyledonary	Neomorph embryos			
Medium	B5	7.94 ^a	7.02 ^a	6.49 ^a	6.15 ^a	5.80 ^a	14.08 ^a	4.20 ^b	
	NL	7.48 ^a	5.97 ^b	5.14 ^b	4.90 ^b	4.39 ^b	12.07 ^b	5.49 ^a	
	LSD	0.55	0.44	0.51	0.51	0.46	0.60	0.40	
Explant	Petiole	8.33 ^a	7.08 ^a	6.32 ^a	6.51 ^a	5.77 ^a	14.40 ^a	5.21 ^a	
	Phloem	7.23 ^b	6.25 ^b	5.75 ^b	4.95 ^b	4.88 ^b	12.41 ^b	4.04 ^b	
	LSD	0.47	0.41	0.48	0.47	0.44	0.56	0.38	
SA treatments (μM)	0	6.72 ^c	4.75 ^c	4.39 ^c	4.43 ^c	6.39 ^a	10.55 ^c	5.33 ^b	
	25	7.90 ^b	6.94 ^b	5.95 ^b	6.04 ^b	5.29 ^b	13.64 ^b	6.21 ^a	
	50	9.49 ^a	8.57 ^a	7.84 ^a	7.50 ^a	4.13 ^c	16.93 ^a	3.08 ^c	
	75	9.33 ^a	8.75 ^a	8.07 ^a	4.43 ^a	4.25 ^c	16.86 ^a	3.02 ^c	
	100	5.46 ^d	4.30 ^d	3.92 ^d	3.63 ^d	6.58 ^a	9.05 ^d	5.50 ^b	
	LSD	0.81	0.66	0.76	0.75	0.69	0.89	0.61	

Table 2. Mean values of chlorogenic acid produced by two carrot explants in B5 and NL media supplemented with different salicylic acid concentrations.

Source of variation	Chlorogenic acid (%)	LSD
Medium	B5	6.475 ^a
	NL	7.184 ^a
Explant	Petiole	2.227 ^b
	Root secondary phloem	11.196 ^a
Salicylic acid treatments (μM)	0	3.142 ^d
	25	3.190 ^d
	50	5.206 ^c
	75	7.528 ^b
	100	14.492 ^a

Figures

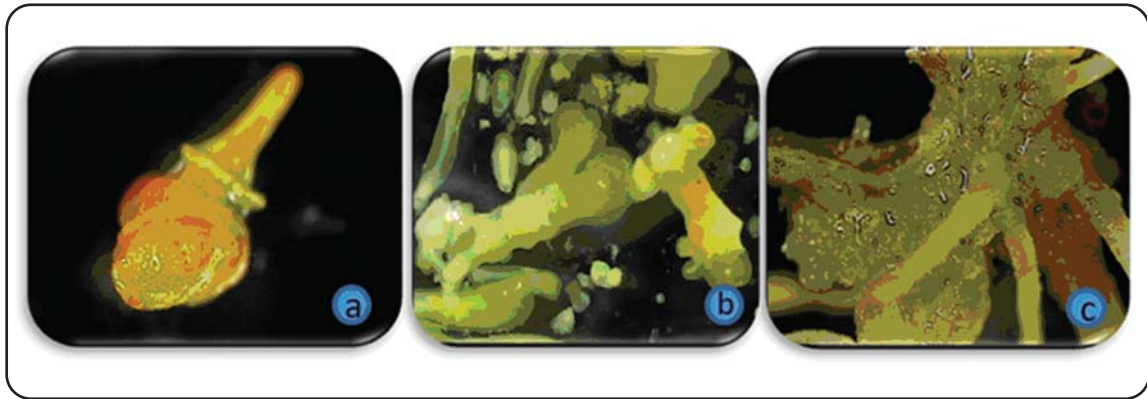


Fig 1. Under-developed or neomorph embryo (a) and normal embryos (b); Callus and roots produced from petiole explants cultured on NL medium supplemented with 25 μ M salicylic acid(c).

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