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Embryogenesis in medicinal plant Syrian Rue (*Peganum harmala* L.)

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Abstract
Peganum harmala L. is a shrub perennial plant of *Zigophyllaceae.* It is used for treatment of parkinson in folk medicine and has antitumor and antioxidant activity. This species is usually propagated by seeds but the span of seed viability is short. On the other hand, since this is a wild plant and is not cultivated, it is exposed to extinction due to overuse. In order to solve this problem and optimize secondary metabolite production in this plant, the first step is *in-vitro* optimization of callus induction and shoot regeneration. To achieve this goal, leaf, hypocotyl and embryo axis were cultured on MS medium containing different concentrations of 2,4-D (0, 0.25 and 0.5 mg L^{-1}) in combination with BA (0, 0.5, 1 and 2 mg L^{-1}). The results of analysis of variance showed that the main effects of hormones and explants and some interactions were significant on callus induction and shoot regeneration. Maximum callus induction and shoot regeneration was obtained in the medium supplemented with 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BA for the leaf and hypocotyl explants, respectively.

Keywords: Callus induction; *Peganum harmala* L.; Plant regeneration; 2,4-D

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Introduction

Syrian rue, *Peganum harmala* L., is a perennial shrub plant of *Zigophyllaceae. P. harmala* is distributed and used as a medicinal plant in semi-arid areas of the world (Frison *et al.* 2008; Goel *et al.* 2009; Ababou *et al.* 2013). This plant grows as a perennial shrub (30-100 cm height) with short creeping roots, white flowers and round seed capsules carrying more than 50 black seeds (Smita *et al.* 2012; Ababou *et al.* 2013). Seeds and other parts of *P. harmala* have long been used for medicinal purposes and as a fungicide as well as herbicide due to the presence of harmine (Berlin *et al.* 1993). It has been reported that this plant has anti-tumor, antispam, anti-HIV, antioxidant, antimicrobial, antifungal effects as well as immune system stimulatory and blood sugar decreasing properties (Mahmoudian *et al.* 2002; Panhwar and Abro 2007; Sodaeizadeh *et al.* 2009; Asgarpanah and Ramezanloo 2012). Furthermore, this plant has been effective in the treatment of dermatitis (El-Rifaie 1980) and cancer (Adams 1983).

Syrian rue has some important chemical compounds including indole alkaloids. This species is a rich source of beta-carboline alkaloids. Production of these alkaloids has been studied in callus and suspension culture and the cell culture in this plant has shown good potential in biochemical conversions (Zhu *et al.* 2000).

Tissue culture of medicinal plants is usually used for mass propagation purposes, conservation and production of active compounds for herbal and pharmaceutical industries (Zatimeh *et al.* 2017). Cytokinins are systemically used in the tissue culture media to induce shoot proliferation due to their ability to direct the dividing cells to

differentiate into shoots (Taiz and Zeiger 2002). BA has been used in many studies due to being cheap and effective on shoot regeneration (Arafeh *et al.* 2003; Tahtamouni 2017). Establishing a reliable method for plant regeneration and application of genetic engineering techniques to increase the level of alkaloid production is necessary. There are many attempts for micropropagation of Syrian rue using tissue culture including culture of apical meristem and cotyledonary node (Goel *et al.* 1983; Khawar *et al.* 2005), cotyledon (Gulati and Jaiwal 1990; Saini and Jaiwal 2000), leaf and hypocotyl (Ehsanpour and Saadat 2002; Abd El-Rahman *et al.* 2008). Zatimeh *et al.* (2017) also used cytokinins of BA and TDZ for shoot multiplication in node explants. There are limited reports of direct shoot regeneration in Syrian rue. In the present study, the effect of 2,4-D and BA on callus induction and shoot regeneration on different types of Syrian rue explants were investigated.

Materials and Methods

The mature seeds of Syrian rue were collected from an agricultural research center in Khorasan Razavi Province, Iran. The seeds were surface sterilized in 1.5% (w/v) sodium hypochlorite solution for 15 min and rinsed three times with sterile distilled water. To prepare sterile plant material, seeds were then cultured on basal MS medium (Murashige and Skoog 1962) and incubated in a growth chamber for one month. Embryo axis, hypocotyl and leaf explants were used in this research. To prepare the embryo axis, the end of some seeds was cut with a scalpel and the embryo was extruded with pressure on the middle of the seed and the axis section was used as explant. Leaf and hypocotyl segments were also isolated from sterile plants and used as explants. Different concentrations of 2,4-D (0, 0.25 and 0.5 mg L^{-1}) and BA (0, 0.5, 1 and 2 mg L^{-1}) were used for callus induction and shoot regeneration. The pH of media was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The medium was solidified with 8% (w/v) agar (Sigma). The explants were cultured in sterile dishes (7×12 mm) each containing 25 ml of culture medium, sealed with parafilm and maintained at 25 ± 2 °C under 16-hour photoperiod (30 µ moles m^{-2} s^{-1}). This research was arranged as a factorial experiment using completely randomized design with three replications. After 4 to 6 weeks, the number of explants producing callus and regenerated plants were counted from each replication. Data analysis was performed using SAS (SAS Institute 1990) and MSTATC (Freed and Eisensmith 1990) software. Means were compared by Duncan's multiple range test at 0.05 probability**.**

Results

The callus initiation and somatic embryogenesis occurred one and two weeks after culture of explants on MS medium containing different hormone treatments, respectively (Fig. 1). Somatic embryos were maturated and regenerated in the same medium after the consumption of hormone content and decreasing the level of auxin in the medium. The results of analysis of variance showed that the main effects of hormones (2,4-D, BA) and explants and 2,4-D × BA interaction were significant in terms of callus induction and shoot regeneration (Table 1). Explant × BA interaction

Table 1. Analysis variance for the main effects and interactions of hormones 2,4-D, BA and explants on callus induction and shoot regeneration in medicinal plant *Peganum harmala.*

Source of variation	df	Mean squares	
		Callus	Regeneration
2,4-D	2	19.18**	4.73**
BA	3	1.96**	0.44*
Explant	2	1.93**	2.57**
2,4-D × BA	6	9.50**	2.73**
Explant × 2,4-D	4	0.08^{ns}	0.07^{ns}
Explant × BA	6	0.54**	0.16^{ns}
Explant × 2,4-D × BA	12	0.12^{ns}	0.14^{ns}
Error	72	0.16	0.21

ns, * and **: not significant, and significant at 5% and 1% probability levels, respectively.

was also significant in relation to callus induction. Callus induction and shoot regeneration were not observed on MS basal medium without hormones. The level of callus induction and shoot regeneration enhanced by increasing the concentration of 2,4-D to 0.5 mg L^{-1}. The highest frequency of callus induction and shoot regeneration was achieved at 1 mg L^{-1} BA, however, callus induction and shoot regeneration decreased at higher concentration and had suppressing effect. As can be seen in Table 2, the explants of leaf and hypocotyl had the highest callus induction and regeneration with no significant difference. The mean comparison of treatment combinations showed that the highest frequency of embryonic callus and regeneration simultaneously were observed on the treatment containing 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BA in leaf and hypocotyl explants. This also was the best treatment for the measured traits in each type of explants, separately.

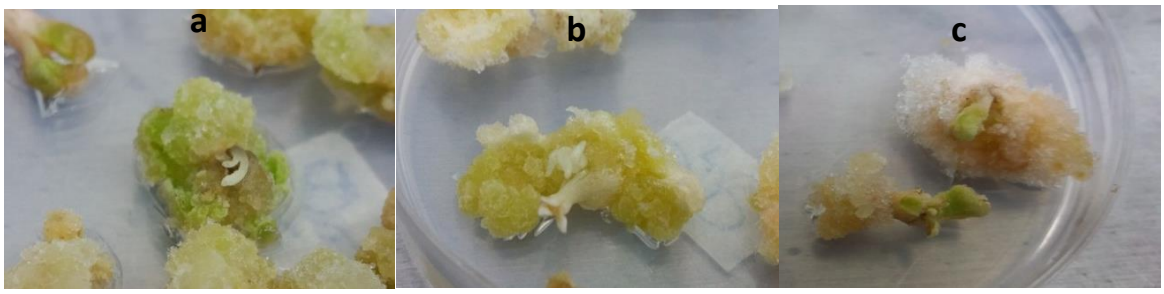

Figure 1. Callus initiation and somatic embryogenesis on the leaf (a), hypocotyl (b) and embryo axis (c) explants in *Peganum harmala.*

Table 2. Mean comparison of effects of main factors on the number of explants with callus induction and regeneration.

Trait	Explant			BA (mg L^{-1})				2,4-D (mg L^{-1})		
	Hypocotyl	Embryo	Leaf	0	0.5	1	2	0	0.25	0.5
Callus	2.19^{a}	1.86^{b}	2.31^{a}	1.93^{b}	2.33^{a}	2.37^{a}	1.85^{b}	1.28^{b}	2.53^{a}	2.56^{a}
Regeneration	0.75^{a}	0.42^{b}	0.94^{a}	0.67^{ab}	0.67^{ab}	0.89^{a}	0.59^{b}	0.36^{b}	0.67^{a}	1.08^{a}

Different letters within the same factor in each row represent significant differences by Duncan's multiple range test ($p \leq 0.05$).

Table 3. Mean comparison of interactions between explant type and different concentrations of hormones on callus induction and regeneration of medicinal plant *Peganum harmala*

BA (mg L^{-1})	Explant	2,4-D (mg L^{-1})	Average number of explants producing callus	Average number of explants with regeneration
0	Leaf	0	0.00^{g}	0.00^{f}
		0.25	3.00^{a}	1.33^{bcd}
		0.5	3.00^{a}	1.00^{cde}
	Hypocotyl	0	0.00^{g}	0.00^{f}
		0.25	3.00^{a}	1.00^{cde}
		0.5	3.00^{a}	1.00^{cde}
	Embryo	0	0.00^{g}	0.00^{f}
		0.25	2.67^{ab}	0.67^{def}
		0.5	2.67^{ab}	1.00^{cde}
0.5	Leaf	0	1.00^{ef}	0.33^{ef}
		0.25	3.00^{a}	1.00^{cde}
		0.5	3.00^{a}	1.33^{bcd}
	Hypocotyl	0	1.00^{ef}	0.33^{ef}
		0.25	3.00^{a}	0.67^{def}
		0.5	3.00^{a}	1.33^{bcd}
	Embryo	0	1.00^{ef}	0.00^{f}
		0.25	3.00^{a}	0.33^{ef}
		0.5	3.00^{a}	0.67^{def}
1	Leaf	0	1.67^{cde}	0.33^{ef}
		0.25	3.00^{a}	0.67^{def}
		0.5	3.00^{a}	2.33^{a}
	Hypocotyl	0	1.67^{cde}	0.33^{ef}
		0.25	2.67^{ab}	0.67^{def}
		0.5	3.00^{a}	2.00^{ab}
	Embryo	0	1.00^{ef}	0.00^{f}
		0.25	2.33^{abc}	0.33^{ef}
		0.5	3.00^{a}	1.33^{bcd}
2	Leaf	0	3.00^{a}	1.67^{abc}
		0.25	2.00^{bcd}	0.67^{def}
		0.5	2.00^{bcd}	0.67^{def}
	Hypocotyl	0	2.67^{ab}	0.67^{def}
		0.25	2.00^{bcd}	0.67^{def}
		0.5	1.33^{def}	0.33^{ef}
	Embryo	0	2.33^{abc}	0.67^{def}
		0.25	0.67^{fg}	0.00^{f}
		0.5	0.67^{fg}	0.00^{f}

Different letters in the same column represent significant differences by Duncan's multiple range test ($p \leq 0.05$).

Discussion

Plant tissue culture is *in vitro* cultivation of plant cell or tissue under aseptic and controlled environmental conditions, in liquid or on semi-solid well-defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant. This technique affords alternative solutions to problems arising due to the current rate of extinction and decimation of flora and ecosystem. The whole process requires a well-equipped culture laboratory and nutrient medium (Kalia 2009). Recently developed *in vitro* propagation techniques offer high rate multiplication alternatives for plants of horticultural, economic and medicinal importance (Deb and Pongener 2012), as well as medium- to

long-term conservation of valuable germplasm by means of slow growth storage and cryopreservation (Previati *et al.* 2008). *In vitro* propagation methods are essential components of plant genetic resource management and are becoming increasingly important for the conservation of rare and endangered plant species (Sidhu 2010). Callus culture is one of the techniques of tissue culture in which a differentiated tissue is removed to produce a mass of undifferentiated cells called the callus *in vitro* (Rahimmalek and Goli 2013). This is considered to be the most efficient method for crop improvement by the production of somaclonal and gametoclonal variants. This technology has vast potential to produce superior quality plants and allows the isolation of useful variants from well-adapted high-yielding genotypes with better disease resistance and stress tolerance (Brown and Thorpe 1995). Certain types of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the occurrence of somaclonal variability (George 1993), which can lead to the development of commercially important improved varieties (Lee and Chen 2014). There are many studies confirming the positive role of auxin in combination with cytokinin on plant regeneration in *Santolina canescens* Lagasca (Casado *et al.* 2002), *Bupleurum fruticosum* (Feraternale *et al.* 2002), *Peganum harmala* (Saini and Jaiwal 2000) and *Acacia tortilis* (Sané *et al.* 2001). A combination of two or more different types of growth regulators is usually required for successful *in vitro* shoot proliferation of plants, with the cytokinin-auxin interaction considered to be the most effective for regulating plant growth (Ozudogru *et al.* 2011).

Somatic embryogenesis phenomenon is based on two steps, the first include the induction of embryogenic competence (referred to as embryogenic masses or clumps), in the presence of high concentration of auxin. The second step involves the development of embryogenic cell into embryos in the absence or in presence of lower concentration of auxin. 2,4-D has an important role in inducing somaclonal variation that could be due to the genetic variation in explants, the time number of subculture, culture conditions and using mutagenesis (Collin and Edwards 1998). Also, there are various studies showing the role of 2,4-D on the induction of embryonic callus (McKerise and Brown 1996; Fehér *et al.* 2002; Mohammadi-Nasab *et al.* 2011). It has been reported that there is a high correlation between somatic embryogenesis and variation in pH gradient caused by stress or 2,4-D. pH of cytoplasm and vacuole increases during embryogenesis. It is suspected that 2,4-D acts as a stress agent at high concentration (Fehér *et al.* 2002). Mohammadi-Nasab *et al.* (2011) reported that significant increase was observed in the number of embryos by enhancing the concentration of 2,4-D to 10 mg L^{-1} in *Medicago sativa* L. Saini *et al.* (2000) observed that the frequency of regeneration and the length of regenerated shoots decreased by enhancing the concentration of BAP on cotyledonary nodes and hypocotyl explants showing the importance of cytokinin concentration effect on shoot formation. In the present study, the highest regeneration occurred on the treatment of 1

mg L^{-1} BA that was in accordance with the results of Goel *et al.* (2009). Zatimeh *et al.* (2017) reported that using BA had the higher stimulatory effect to Kin and TDZ on shoot multiplication in Syrian Rue. Among various cytokinins, BA is widely used as cheapest and the most effective cytokinin for shoot regeneration (Chaudhary *et al.* 2007; Sonia *et al*. 2007; Sadeghian *et al*. 2014; Zamanifar *et al.* 2015). In the present study, we used the explant of embryo axis for the first time and various concentrations of BA to induce callus and shoot regeneration in medicinal plant Syrian Rue. In this research, a protocol for regeneration was presented that can be used for propagation and transformation of this important medicinal plant preparing the possibility of fast multiplication of selected clones and increase in the production of secondary metabolites using genetic engineering methods.

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بهینه سازی کشت بافت گیاه دارویی اسپند

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چکیده

گونه اسپند با نام علمی *Peganum harmala* گیاهی علفی و چند ساله از تیره Zigophyllaceae می‌باشد. این گونه در طب سنتی برای درمان پارکینگسون مورد استفاده قرار گرفته و دارای اثرات ضد تومور و فعالیت آنتی‌اکسیدانی است. اسپند به طور معمول به وسیله بذر تکثیر می‌شود، ولی قدرت زنده‌مانی بذر کوتاه است. از سوی دیگر، از آن جایی‌که این گیاه به صورت وحشی می‌روید و کشت نمی‌شود، به دلیل استفاده بی‌رویه در معرض خطر انقراض قرار دارد. برای رفع این مشکل و تولید بهینه متابولیت‌های ثانویه این گیاه، اولین گام بهینه‌سازی القای کالوس و باززایی اندام‌های هوایی در محیط درون شیشه می‌باشد. به این منظور ریزنمونه‌های برگ، هیپوکوتیل و محور جنینی در محیط کشت MS حاوی غلظت‌های مختلف از هورمون‌های D-۲،۴ (۰، ۰/۲۵ و ۰/۵ میلی‌گرم در لیتر) و BA (۰، ۰/۵، ۱ و ۲ میلی‌گرم در لیتر) کشت داده شدند. نتایج نشان داد که بیشترین مقدار القای کالوس و باززایی به طور هم‌زمان، در تیمار هورمونی ۰/۵ میلی‌گرم در لیتر D-۲،۴ و ۱ میلی‌گرم در لیتر BA در ریزنمونه برگ و بعد از آن هیپوکوتیل رخ داده است.

واژه‌های کلیدی: اسپند؛ باززایی؛ کالوس؛ محیط کشت MS؛ D-۲،۴