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Original Article

Somatic Embryogenesis and Plant Regeneration in Evening Primrose (*Oenothera biennis* L.)

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Abstract

Callus induction responses and regeneration through callus-mediated SE were studied from evening primrose (*Oenothera biennis* L.) as an important medicinal plant of Onagraceae mainly known for its gamma-linoleic acid (GLA) content. The effects of cytokinins, 6-Benzylaminopurine (BAP) and N-(2-furfurylamino)1-H-purine-6-amine [Kinetin (Kin)] and their concentrations (0, 0.5, 1.0 and 1.5 mg/l) in combinations with 2,4-dichlorophenoxyacetic acid (2, 4-D) concentrations (0.25, 0.75 mg/l) on callus induction and SE for three explant types (petiole, leaf and epical bud) of "Shiraz" variety were separately evaluated. In all types of explants, the highest callus fresh weights belonged to 1 mg/l BAP or Kin in combinations with 2, 4-D (either 0.25 mg/l or 0.75 mg/l). The maximum fresh weight of callus was obtained from leaf explants plated on culture medium containing of 0.25 mg/l 2, 4-D and 1 mg/l Kin. The maximum The maximum number of embryos was achieved from leaf explants related to 0.75 mg/l 2, 4-D and 1 mg/l Kin. plantlets were successfully raised from *in vitro* developed embryos. Efficient plant regeneration via SE may provide a reliable system for studying the molecular mechanism of SE and a route for the genetic transformation of evening primrose.

Keywords: Evening primrose, Plant growth regulators, Callus induction, Embryo, Somatic embryogenesis

Introduction

Evening primrose (*Oenothera biennis* L.) is one of the 145 species of the family Onagraceae (Oenotheraceae) [1]. This species is a biennial herb native to Eastern North America that has become naturalized on all countries except Antarctica [2]. It is considered to have beneficial health effects, largely due to its gamma-linoleic acid (GLA) content and is grown as an oil seed crop [1] used to make medicine, nutrients, and health products [3,4]. The rapid increase in demand for evening primrose oil (containing GLA) production has caused this species to be considered as a commercial agricultural crop. Based on this opinion, some current unsuitable traits relevant to this species, such as indeterminate inflorescence, high seed shattering during ripeness and a long life cycle (biennial plant) are highly required to be improved [5]. This necessity could appropriately be responded by plant breeding and biotechnology. Somatic embryogenesis (SE) as a powerful biotechnology knowledge is one of the well-known modes by which study of plant cell differentiation becomes possible *in vitro* [6]. It is of tremendous importance due to its vast practical applications such as micropropagation specially by means of artificial seeds [7,8], crop improvement via cell selection, genetic transformation, somatic hybrid and polyploid plant production [9], germplasm preservation, virus elimination, and *in vitro* metabolite production [10]. The SE is a process by which somatic cells differentiate into somatic embryos [11], either directly from the explants or indirectly (after an intervening callus phase) [10]. It is a valuable technique in medicinal plant improvement programs, such as propagation and

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genetic transformation [12]. Ghasemnezhad *et al*., 2011 reported plant regeneration via callus-mediated SE in *O.biennis* (variety not cited) from hypocotyle explants [13]. Faramarzi *et al*., 2013 reported indirect SE for cultivars VNK and NC-1 from petiole, leaf and epical bud explants [14]. In the present study, the aim was to establish plant regeneration via SE in *O. biennis* for local variety "Shiraz" (important for its destination in crop breeding program), using plant growth regulators (PGRs), BAP and Kin in combination with 2, 4-D.

Materials and Methods

The study was carried out at the plant tissue culture laboratory of Department of Plant Breeding and Biotechnology situated in Urmia University.

Plant Material

Seeds of evening primrose variety "Shiraz", the natural population of Shiraz, Iran provided from Pakanbazr Company, Esfahan, Iran.

Media Preparation

In all of the experiments a medium comprised of MS salts [15] and B5 vitamins [16], containing 3% sucrose (Scharlau) and 0.8% agar (Duchefa) was used as a compound basal medium. The pH of the media was adjusted to 5.7 prior to inclusion of agar and autoclaved for 20 min at 121 ºC. Appropriate concentrations of PGRs, 2, 4-dichlorophenoxyacetic acid (2, 4-D), 6-Benzylaminopurine (BAP), and N-(2-furfurylamino)1-H-purine-6-amine [Kinetin (Kin)] were added to the media based on related treatments in experiments. Cultures were incubated at 25±2°C 16h light / 8h dark and 2000 Lux light intensity.

Explant preparation

After complete washing, the seeds were surface sterilized by immersion in 70% (v/v) ethanol for 1 min and 5% (v/v) sodium hypochlorite for 3 min with gentle shaking followed by 3 rinses each time 5 min in sterilized water. The surface sterilized seeds were transferred to 6 cm diameter glass jars containing above-mentioned compound basal medium supplemented with 1 mg/l BAP, pH 5.7 and solidified with 0.8% agar. Seeds were germinated and 2-4 cm arisen seedlings used for explant preparation following one-month incubation at 25±2 ºC.

Experiments

In order to study the effects of PGRs on callus induction, SE and plantlet regeneration an experiment was conducted. As shown in Table 1, explants were prepared from 30-day-old *in vitro* growing seedlings (2-4 cm length) and cultured in induction callus medium (MS salts, B5 vitamins and 3% sucrose supplemented with appropriate types and concentrations of PGRs). The calli were initiated after 20 days which allowed subcultures to be done in the same media at 2 weeks itervals. Weighing of calli was performed at the end of second subculture. After the third subculture, the formed embryos were counted and transferred to the same media for the regeneration of plantlets.

Table 1 Schedule of cultures conducted to evaluate the effects of PGRs on callus induction, SE and plantlet regeneration

Scheduling of Cultures	Explant culture (Leaf, Petiole, Epical bud)	Callus culture	Callus sub-culture 1	Callus sub-culture 2	Embryo culture
Timing	Day: 0-20	Day: 20-35	Day: 35-50	Day: 50-65	Day: 65-100
Developmental stages	Callus induction	Callus proliferation	Callus proliferation	Embryo formation	Plantlet formation
Recording of Results	-	-	Callus weighing	Embryo counting	Determining of regenerated plantlets %

Media used: MS salts, B5 vitamins, and 3% Sucrose, supplemented with 2, 4-D (0.25 and 0.75 mg/l) and BAP (0.0, 0.5, 1.0, and 1.5 mg/l), or with 2, 4-D (0.25 and 0.75 mg/l) and Kin (0.0, 0.5, 1.0, and 1.5 mg/l).

Experimental Design, Data Collection and Statistical Analysis

Two experiments, were separatety conducted each in a factorial arrangement based on a completely randomized design; one to evaluate the effects of BAP+2, 4-D and the other one to investigate the effects of Kin+2, 4-D on the callus induction and SE. In either experiment, each treatment had 4 replications consisting 90 mm×15 mm plastic petri plates containing 12 explants. Statistical analysis was performed using SAS software version 9.1.

Treatment means were compared using Tuckey's studentized range (HSD) test at α=0.05.

Results and Discussion

Two of numerous factors which affect SE in plants: explant type and PGRs (type and concentration) were studied.

Callus Induction

Explants of three types (petiole, leaf and apical bud) were cultured on basal compound medium supplemented with different concentrations of 2, 4-D (0.25 and 0.75 mg/l) and cytokinins (Kin and BAP) (0, 0.5, 1 and 1.5 mg/l). Calli were initiated from all types of explants and their fresh weights determined (Tables 2 and 5). There were no responses in control media lacked any cytokinin. The same responses were observed in all explant types in which 1 mg/l cytokinin (BAP or Kin) in combination with 2, 4-D (either 0.25 mg/l or 0.75 mg/l) led to high yields; lower and upper concentrations (0.5 or 1.5 mg/l) of cytokinins (BAP or Kin) led to low yields (Table 2). Compared with BAP, Kin appeared to be more effective in callus yield; in all explant types, maximum callus fresh weight belonged to Kin+2, 4-D (Table 2).

Somatic Embryogenesis

Somatic embryos related to all explant types were formed by the second subculture on the same media, basal compound medium supplemented with different concentrations of 2, 4-D (0.25 and 0.75 mg/l) and cytokinins (Kin and BAP) (0, 0.5, 1 and 1.5 mg/l). Figure 1 shows SE resulted from leaf calli. Mean number of embryos produced for each explant type is summarized in Tables 3 and 5. There were no responses in control media lacked any cytokinin. Similar responses were observed in all explant types in which 1 mg/l cytokinin (Kin or BAP) in combination with 2, 4-D led to high yields (Table 3). Compared with BAP, Kin appeared to be more effective in callus yield; in all explant types, the highest embryos produced belonged to Kin+2, 4-D (Table 3).

Comparison of explant types based on embryo production revealed high potential of leaf explants (Tables 3 and 5).

Abdi *et al*., 2007 reported indirect somatic regeneration from leaf and petiole explants in *Valeriana officinalis* [17]. In their research [17], combinations of different concentrations of Kin with different concentrations of auxin types (2, 4-D, NAA and picloram) were used for callus induction from which 5mg/l Kin+1mg/l 2, 4-D was the best media combination (maximum callus production) in both leaf and petiole explants. Ebrahimzadeh *et al*., 2007 reported high callus induction from leaf explants of *Anthurium andreannum* using Kin+auxins in comparison with BAP+auxins [18].

Table 2 Effect of medium containing different levels of BAP+2, 4-D and Kin+2, 4-D on callus fresh weight

Cytokinin	2, 4-D	Cytokinin	Mean fresh weight of callus		
			petiole	Leaf	Apical bud
Kin	0.25	0	0i	0j	0j
		0.5	0.4125d	1.1575e	0.94fg
		1	1.0175a	3.1775a	2.145a
		1.5	0.57c	1.795b	1.595c
	0.75	0	0i	0j	0j
		0.5	0.41d	0.41i	0.7025hi
		1	0.72b	0.72h	1.85e
		1.5	0.45dc	0.755i	1.1925e
BAP	0.25	0	0i	0j	0j
		0.5	0.3025ef	0.895fg	0.94fg
		1	0.7115b	1.545c	1.415d
		1.5	0.4675dc	1.315d	1.047i
	0.75	0	0i	0j	0j
		0.5	0.2675f	0.7975gh	0.57i
		1	0.675b	1.2525df	1.0425f
		1.5	0.3775ed	0.99f	0.73h

Means followed by different letters in each column are significantly different at the 5% level of probability according to tukey's test

Hoori *et al*., 2007 also in their work in two *Medicago* species cited the Kin+2, 4-D as the best PGRs combination for cullus induction [19]. In the work presented here, callus induction and SE were occurred from leaf, petiole and epical buds in each of two combined PGRs (Kin+2, 4-D and BAP+2, 4-D) in which leaf explants appeared to be the most responsive both for the cullus induction (3.17 gram related to 1mg/l Kin+0.25 mg/l 2, 4-D) and the number of formed embryos (11.5 related to 1 mg/l Kin +0.75 mg/l 2, 4-D). The differences with respect to callus fresh weight resulted from explants, may most probably be explained by the presence of endogenous growth regulators inside the veins of leaf explants [20, 21]. Anzidie *et al*., 2000 reported that presence of both auxin and cytokinin is required for SE [22] which is in agreement with our results. In this study high 2, 4-D concentration (0.75 mg/l) had a higher embryo yield than those of low 2, 4-D concentration (0.25 mg/l). This is in agreement with findings by Ghasemnezhad *et al*., 2011 who have shown that increasing in number of embryo produced in evening primrose is related to increasing with 2, 4-D concentration [13]. Comparison of explant types revealed the high potential of somatic embryo production for the leaf explants (Tables 3 and 5). This is not in agreement with findings by Faramazi *et al*., 2013 in which petiole explants showed the most productive one [14]. The differences with respect to explant responses might be due to different genotypes used [23-28].

Plantlet Formation

The torpedo-shaped bipolar somatic embryos following culturing on compound basal medium supplemented with different concentrations of 2, 4-D (0.25 and 0.75 mg/l) and cytokinins (Kin or BAP) (0.5, 1 and 1.5 mg/l) were germinated and converted into plantlets. Tables 4 and 5 indicate the mean numbers of regenerated plantlets for all explant types. There were no responses in control media lacked any cytokinin and 2, 4-D. High yield responses were observed in all explant types in which 1 mg/l cytokinin (Kin or BAP) in combination with 2, 4-D used (Table 4). Compared with BAP, Kin appeared to be more effective in plantlets regeneration; in all three explant types, maximum regenerated plantlets belonged to Kin+2, 4-D (Table 4). Comparison of explant types based on plantlet regeneration revealed high potential of leaf explants (Tables 4 and 5). Maximum frequency of plantlet formation (9.75) was observed at 1 mg/l Kin in combination with 0.25 mg/l 2, 4-D for leaf explants (Table 4). At low (0.5 mg/l) and high (1.5 mg/l) concentrations of Kin less frequencies of germination and plant formation were observed (Table 4).

Acclimatization of Plantlets

Somatic embryos were germinated and converted to plantlets. The plantlets were rooted *in vitro* and successfully acclimatized under the greenhouse conditions and developed into mature plants. A survival of 95% was achieved when the plantlets were transferred to plastic pots containing sterile peat: perlite: soil (1:1:2) mixture.

Table 3 Effect of medium containing different levels of BAP+2, 4-D and Kin+2, 4-D on mean number of embryos

Cytokinin	2, 4-D	Cytokinin	Mean number of embryo		
			Petiole	Leaf	Apical Bud
Kin	0.25	0	0h	0h	0g
		0.5	2.8125ed	3.75f	3.5e
		1	4.5b	7.25c	5.5c
		1.5	1.5fg	2.5g	2f
	0.75	0	0h	0h	0g
		0.5	3.937bc	6.15d	4.75cd
		1	6.812a	11.5a	9.5a
		1.5	2.063ef	4.5d	3.5e
BAP	0.25	0	0h	0h	0g
		0.5	0.875h	2.5g	1.5f
		1	3.5cd	4.5ef	4.25c
		1.5	1.062g	1h	1.25f
	0.75	0	0h	0h	0g
		0.5	1.5fg	5.5ed	3.25e
		1	4.812b	8.5b	7.5b
		1.5	2.5e	3.75f	1cf

Means followed by different letters in each column are significantly different at the 5% level of probability according to tukey's test

Table 4 Effect of medium containing different levels of BAP+2, 4-D and Kin+2, 4-D on mean number of regenerated plantlets

Cytokinin	2, 4-D	Cytokinin	Mean number of regenerated plantlets		
			Petiole	Leaf	Apical Bud
Kin	0.25	0	0h	0i	0g
		0.5	3d	5.75d	4.25d
		1	5.25a	9.75a	8.5a
		1.5	2.25ef	4.25fg	2.5e
	0.75	0	0i	0h	0g
		0.5	2f	3.5ef	3e
		1	3.5bc	6.75c	5.25c
		1.5	1g	2g	1.125f
BAP	0.25	0	0h	0i	0g
		0.5	1g	4.3775e	2.25f
		1	3.75b	9.25b	7.25b
		1.5	2f	3.5ef	1.0625f
	0.75	0	0h	0i	0g
		0.5	0h	0i	0g
		1	2.75ed	2.75fg	4.25d
		1.5	1g	1h	1f

Means followed by different letters in each column are significantly different at the 5% level of probability according to tukey's test

Table 5 Comparison of explant types based on maximum mean of traits (fresh weight of callus, number of embryos and number of regenerated plantlets)

PGRs and maximum means	Mean fresh weight of callus (gram)			Mean number of embryos			Mean number of regenerated plantlets		
	petiole	Leaf	Apical bud	petiole	Leaf	Apical Bud	petiole	Leaf	Apical Bud
2, 4-D	0.25	0.25	0.25	0.75	0.75	0.75	0.25	0.25	0.25
Kin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
BAP	-	-	-	-	-	-	-	-	-
(MAX)	1.01	3.17	2.14	6.81	11.5	9.5	5.25	9.75	8.5

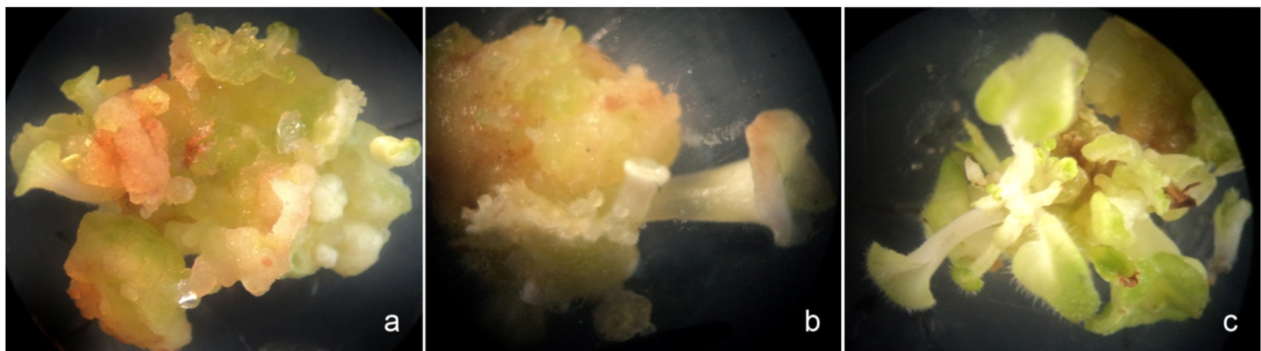

Fig. 1 Stages of initiation and development of callus-mediated SE in *Oenothera biennis* L. on MS salts, B5 vitamins and 3% sucrose supplemented with BAP (1 mg/l) and 2, 4-D (0.75 mg/l) at 25±2 ºC: (a) Embryogenic callus and different stages of SE; (b) Embryogenic callus showing numerous globular-staged and two torpedo-staged embryos; (c) Culture showing matured green somatic embryos.

Conclusion

Seedling derived explants were able to produce somatic embryos which germinated and converted into plantlets. This is an efficient plant regeneration protocol which holds great promise for *Oenothera biennis* L.

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