Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2015 Vol. 5 (4) October-December, pp.36-42/Hafez et al.
Research Article

MICROPROPAGATION AND REGENERATION POTENTIALITY OF EVENING PRIMROSE (*OENOTHERA BIENNIS* L.)

Reza Faramarzi Hafez, *Mortaza Ghadimzadeh, Amir Fayaz Moghaddam and Morad Jafari
Department of Plant Breeding and Biotechnology, Urmia University, Urmia, Iran
**Author for Correspondence*

ABSTRACT
Evening primrose (*Oenothera biennis* L.) is a biennial and herbaceous plant and is one of the most important medicinal species in the world. In order to study the potential of micropropagation and direct regeneration (without callus intervened process), two types of explants, petiole, and apical bud were used. Explants were inoculated onto Murashige and Skoog (MS) salts+B5 vitamins supplemented with 6-benzylaminopurine (BAP), and N-(2-furfurylamino)1-H-purine-6-amine [Kinetin (Kn)] at different concentrations (0.0, 0.25. 0.75, and 1.25 mg/l). Results showed that there were significant ($p \leq 0.5$) differences among two cytokinins (BAP and Kn) and among two explants (epical bud and petiole) for the number of induced shoots and regenerated plantlets per explants. Number of shoots per explants and regenerated plantlets were strongly influenced by cytokinins (types and concentrations) and explant types. The highest multiplication rate was achieved via apical bud explants on Kn supplemented media. For the root induction, Naphthalene acetic acid (NAA) at 0.75 mg/l gave the highest percent of rooted plantlets. Regenerated plantlets were successfully weaned in air-conditioned culture room. This protocol is useful for large scale and true-to-type production of genotypes in *O. biennis*.

***Keywords*:** *Evening Primrose, Oenothera biennis, Micropropagation, Plant Growth Regulators, Regeneration*

INTRODUCTION
Oenothera (Evening primrose, Sundrops) is a genus belonging to the family Onagraceae (Oenotheraceae) (Eid and Sleem, 2007) consists of 145 species of annual, biennial and perennial herbs classified into 18 sections (Greiner and Kohl, 2014). *Oenothera biennis* L. (Evening primrose) is a biennial herb, native to North America and found in parts of Asia and Europe (NTP, 2009) grows on nutrient-poor, coarse sand or clay soils, on roadsides and disturbed grounds (Leo, 2013). Although flowers, leaves, roots, even all parts of the plant, are edible (NTP, 2009), and could be used as ornamental plants, it is primarily a minor oilseed crop and is grown for the production of essential fatty acids (gama-linoleic acid) (Greiner and Kohl, 2014) used to make medicine, nutrients, and health products (Deng *et al.*, 2001). *O. biennis* is grown commercially in more than 30 countries (NTP, 2009) and conventionally propagated through the seeds. However, seed propagation of this plant has agronomic problems such as establishment difficulties (Hampton and Phetpradap, 1992) caused by poor and heterogeneous germination (Ghasemnezhad *et al.*, 2011).
Therefore, an alternative method for efficient propagation is needed for this economically important species. In addition to indirect planting (Transplanting method), micropropagation (an important technique for the rapid multiplication of crops) is currently considered as an efficient alternate method and proposed as a solution for solving this problem in Iran (Ghasemnezhad *et al.*, 2011; Faramarzi *et al.*, 2013). To be successful in micropropropagation, an appropriate true-to-type and direct *in-vitro* plant regeneration is needed. There are only a few reports of direct and genetically constant regeneration of *Oenothera biennis* or even among *Oenothera* genus. Majority of these reports belong to callus-mediated propagation (Ghasemnezhad *et al.*, 2011) or organogenesis through inducing of adventitious shoots (Mendosa *et al.*, 2001; Taniguchi *et al.*, 2002). Faramarzi *et al.*, (2013) reported direct shoot bud formation from preexisting buds in *O.biennis*. The present investigation outlines comparison of this reproducible *in vitro* method (apical bud culture), with a *de novo* bud formation through petiole culture for large scale production of true-to-type material of used genotypes. Efficiencies of these two

regeneration routes in relation to types and concentrations of plant growth regulators (PGRs) were also compared.

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 (seed germinatin, regeneration and *in vitro* rooting) MS medium comprised of MS (Murashige and Skoog, 1962) salts and B5 (Gamborg *et al.*, 1968) vitamins containing 3% sucrose (Scharlau) and 0.8% agar (Duchefa) was used as compound basal medim. The pH of the media was adjusted to 5.7 prior to inclusion of agar and autoclaved for 20 min at 121ºC. Appropriate concentrations of 2,4-D, BAP and Kn were added to the medium based on related treatment in each experiment.

Cultures Conditions

Cultures were kept in appropriate controlled environments as shown in Table 1.

Table 1: Appropriate controlled environments used in experiments

Experiment	Temperature (ºC)	Relative humidity (%)	Photoperiod (h)		Light Intensity (Lux)	Source
			Light	Dark		
Seed germination	25±2	optimum	--	24	--	--
Shoot induction and Regeneration	25±2	optimum	16	8	2000	*
In vitro rooing	28±2	optimum	16	8	2000	--
Acclimatization	28±2	50-60	16	8	4000	*

*: *Cool daylight fluorescent tubes*

Explants Preparation

Seeds were washed thoroughly in running tap water for 20 min and 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 times rinses each time 5 min in sterilized water. The surface sterilized seeds were transferred to 90 mm×15mm plastic petri plates containing above-mentioned compound basal medium and incubated at 25±2ºC (Table 1). Apical buds and petioles prepared from 30-day-old *in vitro*-growing seedlings (2-4 cm length) and used as experimental materials.

Regeneration Experiment

To investigate the effects of plant growth regulators and type of explants in regeneration, an experiment was carried out. In this experiment, explants (epical bud and petiole), were cultured in 90 mm×15mm plastic petri plates containing compound basal medium supplemented with different concentrations of cytokinins (BAP: 0.0, 0.25, 0.75, 1.25 mg/l and Kn: 0.0, 0.25, 0.75, 1.25 mg/l). Compound basal medium without exogenous supply of growth regulators was used as control. After establishment of cultures, two sub cultures with passage time of two weeks were done on the same media. Differentiated shoots (1.5-2 cm in length) then were excised and individually transferred to the 6 cm diameter glass jars containing the same media to achieve suitable height via elongation.

In- vitro Rooting Experiment

For the root induction under *in vitro* condition, shoots (3-5 cm in length) of each epical bud and petiole explants after 35 days, were exised from cultures and transferred to the 6 cm diameter glass jars containing half strengh of compound basal medium supplemented with different concentrations of auxins (NAA: 0.0, 0.75 and 1.5 mg/l and 2,4-D: 0.0, 0.75 and 1.5 mg/l).

Acclimatization

Three weeks after root induction, *in vitro* regenerated plantlets were carefully removed from the culture jars and the roots gently cleaned with running tap water to remove agar. The plantlets were then transferred to 9 cm diameter plastic pots filled with a mixture of autoclaved peat, perlite and soil (1:1:2, v/v) moistened with nutrient solution (half strength liquid MS salt) and kept inside containers and covered with plastics to attain high relative humidity. Plastic covered containers were then transferred in an air-conditioned culture room maintained at a temperature of 25±2ºC under a 16 hours photoperiod with a light intensity of 2000 Lux provided by cool daylight florescent tubes. Within a period of four weeks in order to gradually lowering the RH, plastics of containers were removed gradually and plants were fertilized with the same nutrient solution every three days.

Experimental Design, Data Collection and Statistical Analysis

Two independent experiments (regeneration and rooting), were conducted each in a factorial arrangement based on a completely randomized design; one to evaluate the effects of grouth regulators and type of explnts in the regeneratin and the other to investigate the effects of grouth regulators in the *in vitro* rooting of plantlets. For regeneration experiment each treatment had 4 replications consisting 90 mm×15 mm plastic petri plates containing 10 explants. Rooting experiment had also 4 replications consisting of culture jars each containing 5 plantlets. Statistical analysis was performed using SAS software version 9.1. Treatment means were compared using Tuckey's studentized range (HSD) test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Seedling Preparation

MS medium supplemented with 1mg/l BAP which appeared to be high frequency of seed germination according to our preliminary seed germination test (data not shown), was employed to achieve *in vitro* growing seedlings of evening primrose. Using this medium, Sterile seeds germinated after 14-21 days at 97%.

Shoot Induction

Analysis of variance showed significant ($p \leq 0.5$) differences among cytokinin types, cytokinin concentrations and their interaction for the number of induced shoots per explants (Table 2). Mean number of formed shoots and regenerated plantlets are shown in Table 3. Mean values comparison of formed shoots revealed that shoots were induced only when one type cytokinin either BAP or Kn was used and there were no responses in control media lacked BAP or Kn. Mean number of induced shoots were different for the two investigated cytokinins. Of the two cytokinins evaluated, Kn was more effective ($p \leq 0.5$) in shoot proliferation than BAP; the highest mean number was 8 and 9.93 for 0.75 mg/l BAP and 0.75 mg/l Kn respectively (Table 3). Mean number of induced shoots also were different for two investigated explants. In each concentration of either cytokinin, comparison of shoots arisen from explants revealed high efficiency of epical bud than of petiole explant; the highest mean number for epical bud and petiole explants using 0.75 mg/l BAP were 8.0 and 4.32 and using 0.75 mg/l Kn were 9.93 and 5.93 respectively (Table 3). Mehra *et al.*, (1998), using cotyledon and apical bud in miropropagation of *O. hookeri*, found maximum shoot proliferation in the latter case. This is in accordance with our result in which apical bud produced more shoots than of petiole explant.

Regeneration

Excision and transfer of shoots into fresh medium with the same formulation resulted in shoot elongation and regeneration of plantlets. Analysis of variance showed significant ($p \leq 0.5$) differences among two cytokinins (BAP and Kn), cytokinin concentrations, and their interaction for the number of regenerated plantlets per explants (Table 2). Kn appeared to be more effective than BAP; for either explant the maximum mean number of regenerated plantlets (9.56 for apical bud and 5.2 for petiole explant) belonged to Kn (Table 3). Comparison of three concentrations (0.25, 0.75, and 1.25) of either cytokinins (BAP and Kn) and either explants, revealed that 0.75 mg/l was more effective on regeneration than both lower and higher concentratins (0.25 and 1.25 mg/l) (Table 3). Similarly, mean number of produced plantlets were different for two investigated explants. In each concentration of either cytokinin,

comparison of plantlets derived from explants revealed that bud explant had higher efficiency of plant regeneration than those with petiole explant; the highest mean number of regenerated plantlets for epical bud and petiole explants using 0.75 mg/l BAP were 7.43 and 3.8 and using 0.75 mg/l Kn were 9.56 and 5.2 respectively (Table 3).

Table 2: Variance analysis of number of induced shoots and regenerated plants from epical bud and petiole explants in different concentrations of BAP and Kn

Source of variation	df	MS of number of shoots		MS of number of plantlets	
		Apical bud	Petiole	Apical bud	Petiole
Cytokinins a	1	8*	5.69*	14.44*	13.26*
Cytokinin concentrations b	3	111.43*	57.67*	100.75	109*
a × b	3	1.75*	1.4*	2.23*	5.69*
Error	24	0.31	0.14	0.24	0.13
CV%		13.63	14.5	11.62	12.57

**: Significant at 5%*

Table 3: Means of induced shoots and regenerated plants from epical bud and petiole explants in different concentrations of BAP and Kn

Cytokinin	Cytokinin concentration (mg/l)	Mean number of shoots		Mean number of plantlets	
		Apical bud	Petiole	Apical bud	Petiole
Kn	0.0 (Control)	0^{e}	0^{e}	0^{f}	0^{e}
	0.25	5.43^{c}	4^{b}	5^{c}	3.2^{b}
	0.75	9.93^{a}	5.93^{a}	9.56^{a}	5.2^{a}
	1.25	3.5^{d}	4.13^{b}	$4.68d^{c}$	4.1^{b}
BAP	0.0 (Control)	0^{e}	0^{b}	0^{f}	0^{f}
	0.25	5^{c}	2.78^{c}	3.25^{e}	2.1^{c}
	0.75	8^{b}	4.32^{b}	7.43^{b}	3.8^{b}
	1.25	1.5^{e}	1.81^{d}	$3.56d^{c}$	1.1^{d}

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

The effect of BAP and Kn in shoot induction of some medicinal plants has been reported (Balachandran *et al.*, 1990). Chen *et al.*, (2000), in comparison of the effects of two cytokinins on shoot induction and regeneration, reported that Kn had a more significant effect than BAP. This report is in agreement with our results in evening primrose. Visser *et al.*, (1992) reported that auxin in the presence of cytokinin, leads to callus induction, while using cytokinin alone increase the amount of direct regeneration. In this study media also supplemented with only cytokinin and without any auxin resulted in shoots without callus formation. This fits with findings by Patnaik and Debata (1996), Chen *et al.*, (2000), Sivanesan and Jeong (2007) and Bhalla *et al.*, (2009), related in induction and formation of shoots.

Rooting of Regenerated Shoots

Analysis of variance showed significant ($p \leq 0.5$) differences among rooting treatments (0.75 mg/l 2,4-D, 1.5 mg/l 2,4-D, 0.75 mg/l NAA and 1.5 mg/l NAA) in number of rooted plantlets (Table 4). The first rooting induction occurred 12 days after exposure of plantlets to root induction media and healthy roots were produced within 3 weeks after auxin pretreatments in all treatments (Figure 1.B). Half-strength MS medium without addition of any growth regulator (control) failed to induce root formation even after 4 weeks (Table 5). The lack of rooting in control cultures is indicative of no spontaneous rooting occurred. Root formation was observed on all investigated auxins (NAA and 2,4-D) and their concentrations (0.75 and 1.5 mg/l). Between two auxins tried, NAA had pronounced effect on *in vitro* rooting than 2,4-D; compared with 2,4-D (55% and 30%), NAA (100% and 80%) showed best rooting performance in 0.75mg/l and 1.25mg/l respectively (Table 5). This is in agreement with findings by Owens and Debra

(1991), Nhut (1998), Enayati and Dabir Ashrafi (2003), and Uranbey (2005) who reported NAA as an efficient auxin in *in vitro* rooting of regenerated shoots. Although increasing auxin concentration in the absence of cytokinin leads in decreasing shoots and increasing roots (Sears and Deckard, 1982), but excess of it inhibit rooting (Bong *et al.*, 1996). This is in accordance with our results in which maximum and minimum rooting performances were obtained in auxin (NAA or 2,4-D) concentrations of 0.75 and 1.5 mg/l respectively (Table 5). In each of two auxins the concentration 0.75 mg/l showed more effective and number of rooted plantlets was decreased when induction rooting media contained 1.5 mg/l auxin.

Acclimatization of Plantlets

In vitro rooted plantlets were successfully acclimatized under the greenhouse conditions (Figure 1.C). A survival of 95% was achieved when the rooted shoots were transferred to pots containing peat, perlite and soil in the proportion of 1:1:2.

Table 4: Variance analysis of influence of different levels of 2,4-D and NAA on rooting

Sourse of variation	df	MS of rooting
Treatment	4	15.7*
Error	15	1.75
CV%		12.88

**: Significant at 5%*

Table 5: Effect of different levels of 2,4-D and NAA on rooting

Type and concentration of auxins (mg/l)	Mean number of rooted plantlets	Rooting response (%)
Control 0.0	0^{e}	0
NAA 0.75	5^{a}	100
NAA 1.5	4^{b}	80
2,4-D 0.75	2.75^{c}	55
2,4-D 1.5	1.5^{d}	30

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

Figure 1: Non-callus-mediated regeneration of *O. biennis* via Apical bud explant. A: Shoot development, B: *In-vitro* rooted plantlet, C: Hardened plant

Conclusion

This study describes a simple protocol which could be employed for rapid true-to -type multiplication and concervation of *O. biennis* via apical bud explant.

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