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In Vitro Direct Shoot Regeneration of Plantlet in Medicinal Plant of *Oenothera biennes*

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Abstract

Evening primrose (*Oenotherabiennis* L.) is a two-year herbaceous plant and one of the important species of medicinal plants in the world. Oil in the plant contains essential fatty acids that the body cannot produce it. In order to investigate explants callusing and regeneration of evening primrose, two separate experiments were conducted as factorial based on a completely randomized design with three replications in tissue culture laboratory of Payame Noor University of Mashhad during 2015. In the first experiment, treatments included naphthalene acetic acid (NAA) at four levels (0, 0.5, 1, 2 mg/L), 6-Benzylaminopurin (BAP) at five levels (0, 0.25, 0.5, 1, 2 mg/L) and three explants (hypocotyls, embryo and leaf). In the second experiment, treatments included BAP at four levels (0, 0.5, 1, 2 mg/L), 2.4-Dichlorophenoxyacetic acid (2.4.D) at four levels (0, 0.5, 1, 2 mg/L) and three explants (hypocotyls, embryo and leaf). In the first experiment, the highest number of callus and regeneration explants was recorded at 0 mg/L and 2 mg/L of BAP respectively. As the concentration of BAP was increased from 0 to 1 mg/L, regeneration increased. Also with increasing concentrations of NAA, callus number was increased to 1 mg/L and continued to decrease in 2 mg /L. The most regeneration in embryo's explant was obtained at 2 mg/L of BAP and 1mg/L of NAA concentration. The most direct regeneration was observed in leaf explants at 0.25 mg/L of BAP concentration. In the second experiment with increasing of BAP level, relative frequency of callus decreased to 1 mg/L continued increasing to 2 mg/L, while regeneration frequency was increased.Callus induction was seen in all explants with different concentration of BAP and 2, 4-D. The most callus induction was seen in leaf and hypocotyl explants, at media containing 0.5 mg/L BAP in combination with 0.5 mg/L of 2, 4-D (3.33).

Keywords

Evening Primrose, Medicinal Plant, Tissue Culture, Somatic Embryogenesis

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1. Introduction

Evening primrose (*Oenothera biennis* L.) is a biennial plant which is cultivated as an annual crop [3, 9]. It is an oil seed crop which due to the presence of gamma linolenic acid (GLA) an unsaturated fatty acid has noticeable pharmaceutical and nutritional values [20, 23]. Rheumatic arthritis, breast pain, skin disorders like eczema, and alcohol disorders are positively influenced by evening primrose seed oil [12, 15]. The increasing market for this plant has given producer the opportunity to replace food crops by non-food crops [7]. Despite the presence of higher levels of GLA in seeds of plants such as black currant (*Ribes nigrum*), borage (*Borago officinalis*) and the oil (produced by some species of the fungus *Mucor* genus [16], evening primrose oil appears to have the most biologically active form of GLA [4, 25, 33, 36]. Special composition of fatty acids in triacylglycerol molecules makes the GLA of evening primrose oil easily accessible to hydrolysis by pancreatic lipase in the small intestine [28]. Although evening primrose has a good potential to become a commercial agricultural crop for the

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production of GLA, but some disadvantages, such as indeterminate inflorescence, high seed shattering during ripening and heterogeneous germination could result in significant impediments. Despite all attempts to decrease the seed-shattering characteristic, it is still a major problem in the production of evening primrose [34]. Although agronomical technique such as consideration of optimum harvesting time and appropriate harvest method increase seed yield by reducing seed shattering [9], breeding new cultivars with determinate growth behavior and low shattering is a strategy to overcome disadvantages of this plant. Since traditional breeding methods take a long time, biotechnological techniques such as tissue culture via somatic embryogenesis accelerate the breeding process. In fact, few researches have been carried out in this matter [9, 17, 24, 31, 26]. The aim of the present research was to investigate an appropriate protocol for induction of callus and regeneration of evening primrose with best combination hormones concentrations to get the largest number of regeneration[10].

2. Materials and Methods

In order to investigate explants callusing and regeneration of evening primrose, two separate experiments were conducted as factorial based on a completely randomized design with three replications in tissue culture laboratory of Payame Noor University of Mashhad during 2015. In this experiment, for the first time the embryo explants were used for regeneration of evening primrose. In the first experiment, treatments included 6-Benzylaminopurin (BAP) at five levels (0, 0.25, 0.5, 1, 2, $mg.L^{-1}$) in combination with naphthalene acetic acid (NAA) at four levels (0, 0.5, 1, 2, $mg.L^{-1}$) and three explants (hypocotyls, embryo and leaf). In the second experiment treatments were BAP (0, 0.5, 1, 2, mg/L) in combination with 2,4-Dichlorophenoxyacetic acid (2,4-D) at four levels (0, 0.5, 1, 2, $mg.L^{-1}$) and three explants (hypocotyls, embryo and leaf). Explants were prepared from sterilized seeds germinated in ½MS medium. Full ripened seeds were treated with ethanol (70% v/v) for 30 s. Surface sterilization was carried out by immersing in sodium hypochlorite (3% v/v) containing 2 drops of Tween 80, for 20 min. Sterilized seeds were subsequently germinated in ½MS at a pH of 5.8. For callus formation, depending on the size of seedling, 2 to 4 weeks after germination, explants (1 cm in length) were prepared from different parts of the plant, and were transferred to MS medium containing different levels of 2,4-D and BAP and NAA .In the first experiment, the culture medium containing growth regulators MS with different concentrations BAP (0, 0.25, 0.5, 1, 2) mg/L in combination with NAA (0, 0.5, 1, 2, mg/L) and in the second one BAP (0, 0.5, 1, 2, mg/L) was used in combination with 2,4-D (0, 0.5, 1, 2, mg/L) and leaf explant, hypocotyl and embryos were cultured for this purpose. Any space petri was considered as one repetition. Factorial experiment in a completely randomized designed with three replication was used. Statistical analysis was carried out using MSTAC software and the least significant difference of mean values at 1% probability (LSD) was computed.

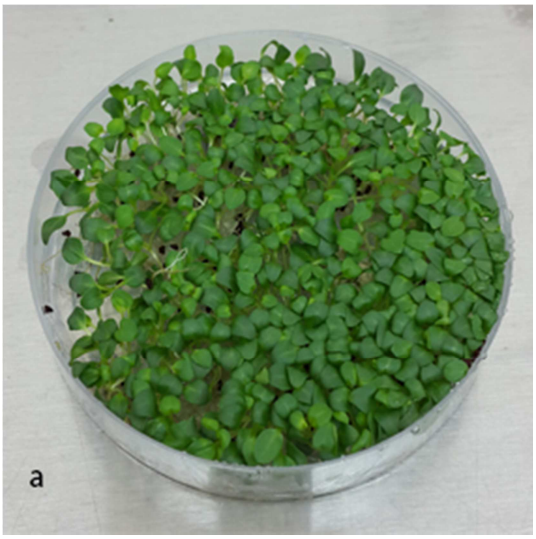

Figure 1-a). Seedling, 4 weeks after germination.

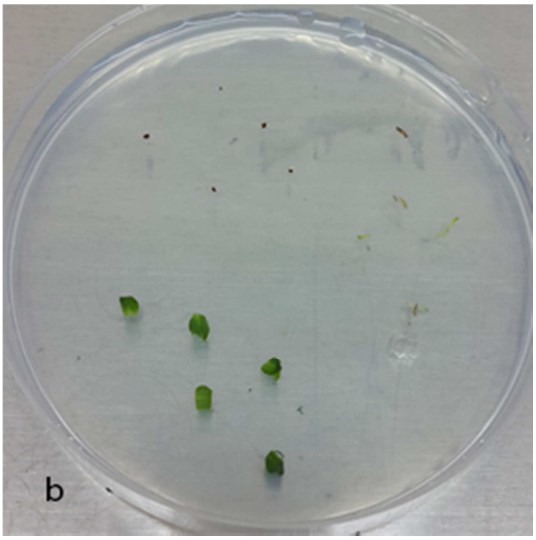

Figure 2-b). Explants were transferred to MS medium containing different levels of 2, 4-D and BAP and NAA.

3. Results

3.1. First Experiment

The effect of various combinations and concentrations of hormones (BAP and NAA) in evening primrose explants was investigated. All treatments were significant at the 1% level and significance was observed for callus formation and regeneration (Table 1). Highest callus number and regeneration was obtained at 0 mg/L and 2 mg/L

concentration of BAP respectively (Figure 3 and 4). The medium containing 1 mg/L NAA produced the maximum number of callus per explant (figure 5) and the most regeneration was obtained at 0.5 mg/L concentrations of NAA and no significant difference at 0 mg/L and 1mg/L (figure 6). The study on the effect of explants on callus showed that leaf, hypocotyl and embryo were the best explant respectively.

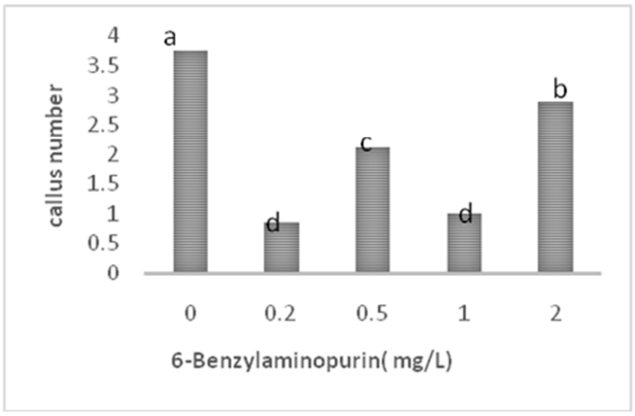

Figure 3. The effect of different levelsof BAP on callus number.

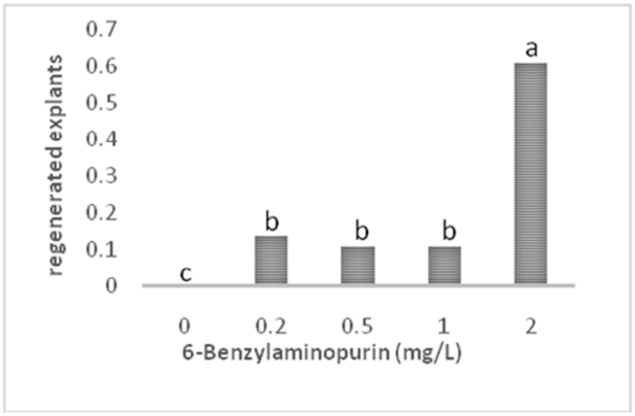

Figure 4. Effect of different levels of BAP onregeneration.

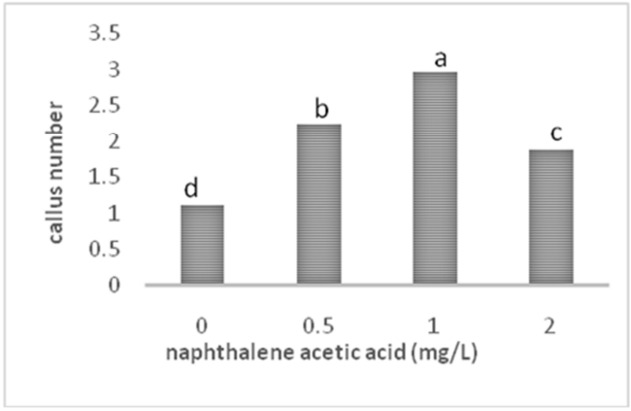

Figure 5. The effect of different levelsof NAA on callus number.

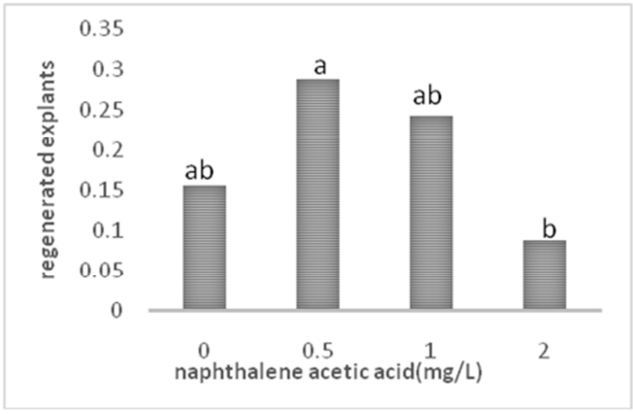

Figure 6. Effect of different levels of NAA onregeneration.

Table 1. Analysis for variance for callus induction and regeneration for BAP and NAA and explant.

	ms		
regeneration	**callus**	**df**	**sours**
**2.056	**54.785	4	BAP
**0.361	**29.400	3	NAA
**1.093	**22.627	12	BAP*NAA
**2.956	**30.072	2	EXPLANT
**1.831	**6.253	8	BAP*EXPLANT
**0.756	**0.717	6	NAA* EXPLANT
**0.890	**0.832	24	BAP*NAA*EXPLANT
0.940	0.167	120	ERROR
		179	TOTAL

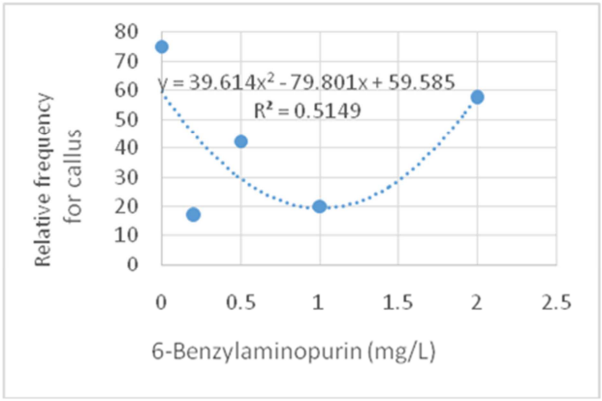

Figure 7. The relative frequency of callus for BAP.

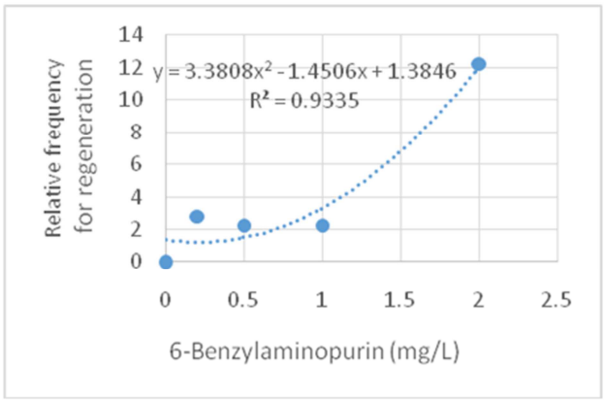

Figure 8. The relative frequency of regeneration for BAP.

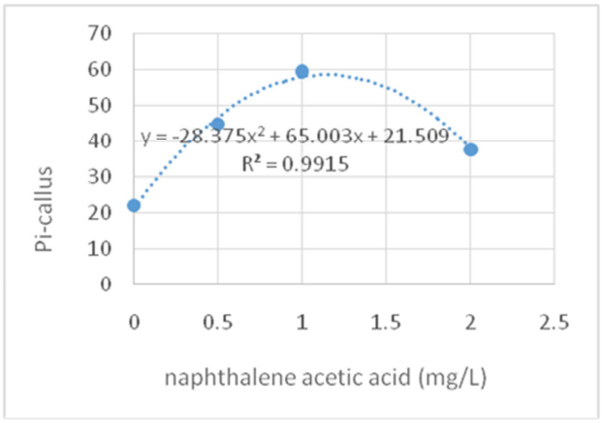

Figure 9. The relative frequency of callus for NAA.

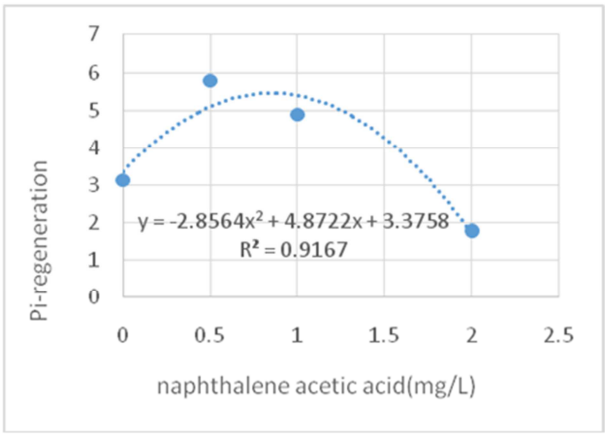

Figure 10. The relative frequency of regeneration for NAA.

In terms of regeneration, embryos was the best explant because of younger tissue. There was a significant correlation between BAP levels and relative frequency of callus. Relative frequency of callus decreased by increasing the BAP level to 1 mg/L, while increased in callus showed a significant down ward trend but increased with increasing frequency BAP (figure 7). BAP had significant effect on relative frequency of regeneration, and correlation coefficient was high, frequency regeneratin increased with increasing concentration of BAP level (figure 8). When the concentration of NAA increased from 0 to 1 mg/L, both relative frequency of callus and regeneration increased, while addition of NAA more than 1 mg/L reduced relative frequency of callus and regeneration.Correlation coefficient was high and showed direct relationship between various level of NAA and relative frequency of callus and regeneration, (R^2=0.99) (figure 9, 10).

The interaction effect of explant with BAP and NAA for the callus induction and regeneration were statistically significant. The most number of callus (5) was obtained in medium with NAA alone at 0.5, 1 and 2 mg/L concentration in all explants and medium containing 2 mg/L of BAP in combination with 1 mg/L of NAA in all explants. (Table 2).

The interaction effect of explant with BAP and NAA for the induction of regeneration resulted, the most regeneration was seen in media containing 2 mg/L of BAP and 1 mg/L NAA in embryo explant (3.66). In leaf explants, regeneration was obtained only in media containing BAP alone at 0.25 mg/L concentration (1.66), while hypocotyl explants did not produced regeneration in any media.

Table 2. The intraction effect of explant,BAP,NAA for the callus induction.

NAA concentration ($mg.l^{-1}$)				BAP concentration ($mg.l^{-1}$)	explant
2.00	**1.00**	**0.50**	**0.00**		
5.00^{a}	5.00^{a}	5.00^{a}	0.00^{i}	0.00	
2.67^{ef}	3.33^{de}	1.66^{g}	2.66^{ef}	0.25	
2.67^{ef}	2.33^{f}	$^{bcd}4.00$	3.00^{e}	0.50	leaf
0.00^{i}	3.33^{de}	2.00^{fg}	0.00^{i}	1.00	
0.00^{i}	$^{a}5.00$	$^{ab}4.66$	$^{cde}3.67$	2.00	
5.00^{a}	5.00^{a}	5.00^{a}	0.00^{i}	0.00	
0.00^{i}	0.00^{i}	0.00^{i}	0.00^{i}	0.25	
$^{bcd}4.00$	1.33^{gh}	1.66^{g}	2.33^{f}	0.50	hypocotyl
0.00^{i}	1.33^{gh}	1.99^{g}	0.00^{i}	1.00	
0.00^{i}	5.00^{a}	3.00^{e}	3.33^{de}	2.00	
5.00^{a}	5.00^{a}	5.00^{a}	0.00^{i}	0.00	
0.00^{i}	0.00^{i}	0.00^{i}	0.00^{i}	0.25	
0.00^{i}	0.00^{i}	0.00^{i}	0.00^{i}	0.50	embryo
0.00^{i}	1.00^{h}	1.33^{gh}	0.00^{i}	1.00	
0.00^{i}	5.00^{a}	3.33^{de}	1.66^{g}	2.00	

3.2. Second Experiment

The effect of different concentrations of BAP and 2, 4-D hormones on callus induction was statistically significant ($P<0.01$), while there was no regeneration in all treatments (Table 4). BAP had significant effect on relative frequency of callus. When the concentration of BAP increased from 0 to 1 mg/L, relative frequency of callus increased, while addition of BAP more than 1 mg/L reduced callus. The correlation coefficient between 2, 4-D and relative frequency of callus was not significant (R^2=0.3785). The most relative frequency of callus was obtained at concentration of 2mg/L 2, 4-D (Figure 12). The interaction effect of explant, BAP, and 2, 4-D, on callus induction was statically significant. Callus induction was seen in all explants with different concentration of BAP and 2, 4-D. The most callus induction was seen in leaf and hypocotyl explants, at media containing 0.5 mg/L BAP in combination with 0.5 mg/L of 2, 4-D (3.33), while there was no regeneration in any explants.

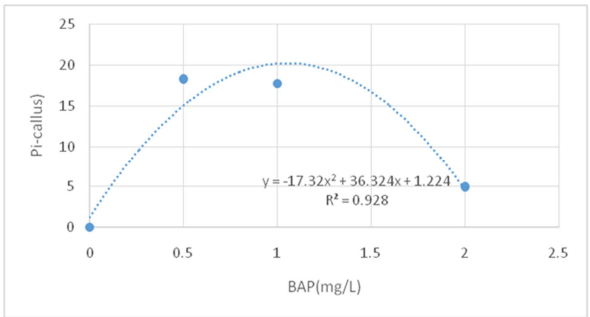

Figure 11. The relative frequency of callus for BAP.

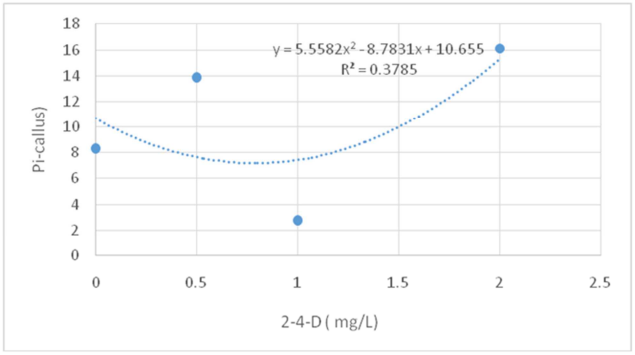

Figure 12. The relative frequency of callus for 2,4-D.

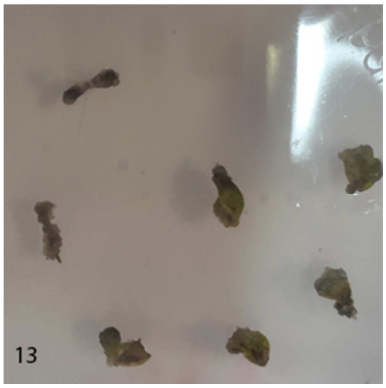

Figure 13. Callus of evening-primrose (the hypocotyl and leaf three weeks after culturing).

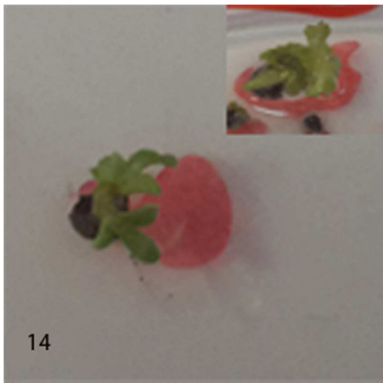

Figure 14. Different stages of somatic embryogenesis of embryo of evening-primrose, 4weeks.

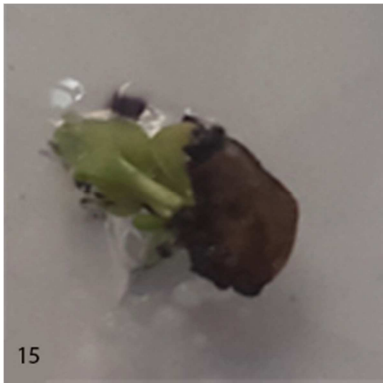

Figure 15. Regeneration of laef.

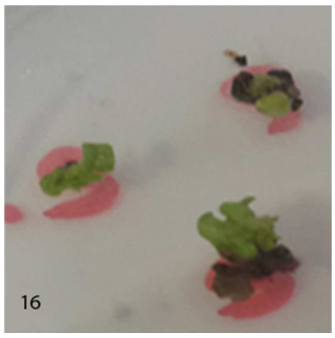

Figure 16. Different stages of somatic embryogenesis of embryo of evening-primrose.

Table 3. In traction effect of explant,BAP,NAA for the induction of regeneration.

NAA (mg.l^{-1})				BAP (mg.l^{-1})	explant
2.00	**1.00**	**0.50**	**0.00**		
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00	
0.00^{f}	0.00^{f}	0.00^{f}	^{bc}1.66	0.25	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.50	leaf
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	1.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	2.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.25	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.50	hypocotyl
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	1.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	2.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.00	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.25	
0.00^{f}	0.00^{f}	0.00^{f}	0.00^{f}	0.50	embryo
0.00^{f}	0.00^{f}	^{cd}1.33	0.00^{f}	1.00	
0.00^{f}	^{a}3.66	^{b}2.00	^{e}0.66	2.00	

Table 4. Analysis for variance for callus induction and regeneration for BAP and 2, 4-D and explant.

	ms		
regeneration	**callus**	**df**	**sors**
-	**7.639	3	BAP
-	**3.210	3	2,4-D
-	**4.651	9	BAP*2,4-D
-	**0.424	2	EXPLANT
-	**0.729	6	BAP* EXPLANT
-	**1.914	6	2,4-D* EXPLANT
-	**2.010	18	BAP*2,4-D*EXPLANT
0	069/0	96	ERROR
-		143	TOTAL

Table 5. In traction effect of explant, BAP, 2, 4-D, for the callus induction.

2,4-D				BAP (mg.l^{-1})	explant
2.0	**1.0**	**0.5**	**0**		
0.00e	0.00e	0.00^{e}	0.00^{e}	0	
^{d}1.00	0.00^{e}	^{a}3.33	0.00^{e}	0.5	leaf
^{cd}1.33	0.00^{e}	0.00^{e}	^{b}2.30	1.0	
0.00^{e}	^{e}0.33	0.00^{e}	^{cd}1.33	2.0	
0.00^{e}	0.00^{e}	0.00e	0.00^{e}	0	
^{d}1.00	0.00^{e}	^{a}3.33	0.00^{e}	0.5	hypocotyl
^{d}1.00	0.00^{e}	0.00^{e}	1.30^{cd}	1.0	
0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	2.0	
0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0	
^{b}2.30	0.00^{e}	0.00^{e}	0.00^{e}	0.5	embryo
^{a}3.00	0.00^{e}	^{e}1.66	0.00^{e}	1.0	
0.00^{e}	^{cd}1.33	0.00	^{e}0.00	2.0	

4. Discussion

The study on the effect of explants on callus showed that leaf, hypocotyl and embryo were the best explant respectively.

In terms of regeneration, embryos were the best explants because of younger tissue.

As the most number of callus (five) was obtained in medium with NAA alone at different concentration, this hormone has important effect on callus conduction comparing with BAP hormone. Based on these results and results obtaining other researchers, callus induction and regeneration depended on kinds of explants and different concentration of hormones as well [8, 14].

Medium containing 2 mg/L of BAP in combination with 1 mg/L of NAA in all explants, produced more callus that shows that determined concentration of this hormone is needed for callus induction in all explants.

The most callus induction was seen in leaf and hypocotyl explants, at media containing 0.5 mg/L BAP in combination with 0.5 mg/L of 2, 4-D (3.33). The effect of different concentrations of 2, 4-D on somatic embryogenesis has been reported [29, 35]. In an experiment on cell culture of carrot, Ribnicky et al. [30] showed that, adding 2, 4-D to the medium strongly increased the free IAA and its ester conjugated form. Thus, it can be concluded that 2, 4-D induces the biosynthesis of IAA from tryptophan and related amino acids [19].

The results obtained by other researchers regarding tissue culture showed that BAP and NAA are the most effective regulators for propagation of leaves and roots. Regulators and hormones have interaction and can improve the effects of each others. The ratio of auxin to cytokinin is important.The auxin-cytokinin ratio used in culture media determines the degree of shoot and root formation in tissue culture. A high ratio of cytokinin to auxin favours shoot production, whereas a high auxin to cytokinin ratio favours root production. Intermediate levels of both hormones enhance callus formation. Therefore, with changing thisratio, the propagation ability and callusformation can be controlled in all explants [6, 18]. Somatic embryogenesis in evening primrose depended on stress affected cell cultures in addition of different concentration of hormones [21, 27, 29, 35, 37]. According to other experiments, optimum concentration of hormones for callus induction and regeneration is different based on type of plant, hormones used in culture medium, plant growth stage and type of explant [13, 22].

Salehi et al. [32], investigated the effect of different concentrations of 6-benzyl amino purine (BAP) (0, 2.2, 4.4, 8.8 µmol/L) and indole-3-acetic acid (IAA) (0, 0.5, 1.1, 2.2 µmol/L) and resulted that the maximum shoot regeneration frequency and the highest number of shoots obtained in BAP (4.4 µmol/L) in combination with IAA (0.5 µmol/L). The lowest shoot regeneration frequency was seen in BAP free treatment.

Other researchers showed that shoot tips were better than meristem segments of Telfairia occidentalis in terms of shoot induction [1]. Different studies confirm that high concentrations of auxins affected induction, formation rate and number of roots in some medicinal plants [2, 5, 11].

5. Conclusions

The results of the present study showed that evening primrose is a good plant for tissue culture. The potency of callus regeneration is good, and it seems that MS medium is the best. But with respect to somatic embryogenesis, it was found that the kind of medium and its composition play an important role.

It has been shown that although NAA directly stimulates the conversion of non-embryonic cells to embryonic ones, but in the most cases additional hormones like cytokinin or gibberellin are needed to reduce the undeveloped embryos. Thus, further studies focused on other kinds of auxin and the combination of cytokinin and gibberellin with auxin are recommended to optimize an easy protocol for evening primrose somatic embryogenesis.

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