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RESEARCH ARTICLE

Rapid and high throughput regeneration in fennel (*Foeniculum vulgare* Mill.) from embryo explants

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ABSTRACT: Callus induction and regeneration of fennel from embryo explants were stabilized in the presence of cefotaxime antibiotic and different plant growth regulators (PGRs). The experiments were conducted under a factorial experiment, based on a completely randomized design (CRD). Genotypes; Fasa, Meshkinshar and Hajiabad were applied under different concentration of cefotaxime (0 and 100 mg l^{-1}), NAA (0 and 0.2 mg l^{-1}), IAA (0 and 0.4 mg l^{-1}) and BAP (0, 0.5 and 1mg l^{-1}). Regeneration, proliferations and root induction were taken placed on studied media, after 35 days without sub-culturing. The highest rate of proliferation with 200 shoots per explant was observed on B5 medium, containing100 mg l^{-1} cefotaxime and 1.0 mg l^{-1} BAP. Callus induction and proliferations were observed in all media containing 100 mg l^{-1} cefotaxime that can be related to auxin like activity of cefotaxime in fennel tissue culture.

KEYWORDS: Fennel, Cefotaxime, PGRs, Embryo, Proliferation

INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.), a member of the umbelliferae family, native to Mediterranean region [18], is one of the oldest and traditionally most significant medicinal aromatic plants [25, 30, 45]. Fennel is a well-known culinary herb and vegetable [1, 4, 35]. Recent studies have documented the widespread use of this plant as a treatment due to its some features like its antispasmodic and hepatoprotective effects on both humans and animals [25, 45]. Increased demand and international consumption of fennel in food and pharmaceutical products call for our attention to this plant [15]. Despite of wide range of its use, there are various conditions that can limit its growth. Drought and salinity are major abiotic stresses, affecting growth, essential oil, and the total crop production of fennel [18, 30]. Besides, some biotic stresses containing larvae (*Systole albipennis*), several Lepidoptera, aphids, and fungi, can cause serious damage to fennel crop [22]. Conventional breeding methods have certain limitations for genetic improvement of this crop due to low efficiency and also they are time consuming [22, 37]. Gene transformation is a major area of interest within the field of plant breeding as it is more efficient and faster than traditional breeding strategies. Plant regeneration during gene transformation is the inevitable stage which is not well studied in fennel. Plant tissue culture technique with high frequency of plant regeneration and proliferation is now widely used for genetic manipulation and crop propagation [7, 13, 33, 47]. In the past three decades, there has been an increasing interest in developing a regeneration protocol from callus

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in fennel. Several studies have discovered that fennel has an innate tendency to micro-propagate, regenerate from callus and suspension cultures [21-23], and regenerate through somatic embryogenesis [4, 24]. However, some of these techniques have shown low success in regeneration, which is not useful for gene transformation. To date, little or no attention has been paid to the use of combination of hormones and antibiotics for fennel propagation and transformation. Recently, researchers have shown that some antibiotics, such as cefotaxime, can improve regeneration in some plants [29, 48]. Cefotaxime, a cephalosporin antibiotic with low toxicity on eukaryotes and effective at low doses, is the best selective agent in plant tissue culture and gene transformation [11, 40]. A number of studies have found that besides its function in the elimination of microbial contaminations and auxin-like activity [34], cefotaxime enhances plant regeneration through improving somatic embryogenesis [11, 32], microspore embryogenesis [2], protoplast culture [19], and stimulating shoot regeneration [9] alike. However, cefotaxime can have an inhibitory effect on plant regeneration [34] and reduces the production of somatic embryo in some plants [39, 42]. Despite of many reports on fennel regeneration, there are no experiment on the regeneration of our selected genotypes by using their embryo as explant and combining of plant hormones with cefotaxime. Therefore, this results will be very applied for tissue culture and metabolic manipulation of fennel.

MATERIALS AND METHODS

Plant material and explant preparation

Three fennel ecotypes were selected from the gene bank of College of Aburaihan, University of Tehran, with different growth habits including Fasa (early-maturity), Meshkinshahr (middle-maturity), and Haji Abad (late-maturity). These genotypes are superior genotypes in seed yield and the essential oil as they were selected from 50 different genotypes in previous studies [5]. In order to prepare explants, first, seeds were surface-sterilized for 20 s in 70% ethyl alcohol, then for 10 min in 2.5% sodium hypochlorite. Seeds were washed 3-4 times with sterile distilled water after each step. They were then soaked for 3-6 hours in sterile distilled water and embryos obtained by cutting the end and pressing the middle of the turgid seeds (Fig. 1A). cotyledons were removed from around the embryo explant [13, 26, 43].

PGRs and cefotaxime combinations for callus induction and plant regeneration

B5 medium [16] containing full strength of macro- and micro-elements, vitamins, and sucrose (30 g l^{-1}), including BAP (0, 0.5, 1.0 mg l^{-1}), NAA (0, 0.2 mg l^{-1}), IAA (0, 0.4 mg l^{-1}) and cefotaxime (0, 100 mg l^{-1}), were used in a factorial experiment for callus induction and regeneration of desired genotypes. We applied a wide levels for each factors but only reported those concentrations that produced regenerated plants in or experiments. Embryo explants were cultured in Petri dishes each containing 25 ml of the medium and incubated at 25 ±2 °C under 16/8-h light/dark conditions. Cefotaxime was sterilized by passing through sterile filter membrane (0.2 µm), and added into the media after they were autoclaved and cooled rarely.

Shoot development, rooting of plantlets and hardening

Both shoot elongation and rooting were accomplished on the same culture medium, with no sub culturing. After 2-3 weeks, a long thick-white root appeared. Indirect regeneration and proliferation were the main traits that were examined in this experiment. Plantlets with well-developed roots were transplanted into pots containing perlite and water covered with plastic lids. Then they were acclimatized gradually by slow removal of the lids over a week. Finally, after one month, Plantlets were transplanted in to pots containing perlite and water and then to soil for hardening (Fig. 1 F).

Data Recording and Statistical analysis

Data of callus frequency and shoot regeneration were calculated by dividing the number of calli and shoots by the original number of cultured explants, respectively. Statistical analysis was performed using SAS and Excel software. The data were analyzed by ANOVA, and treatment means were compared with Duncan's multiple-range test. Significant differences between treatments (different PGR and Cefotaxime concentration effect on regeneration and proliferation of embryo explants of different genotypes) were determined based on Duncan's multiple range test at $p<0.05$.

RESULTS

Analysis of variance for the effect of different PGRs, Cefotaxime concentration and genotypes on direct regeneration and proliferation of fennel embryo cultures showed that different PGRs and Cefotaxime concentration displayed significant effect on regeneration and proliferation of embryo explants from different genotypes. However, there was no difference between the genotypes in response to in vitro culture (Table 1). In the absence of cefotaxime we observed callus induction and regeneration after that (indirect regeneration) on three media, containing different levels of BAP and 0.2 mgl^{-1} NAA plus 0.4 mgl^{-1} IAA. The rest media without antibiotic had no visible of callus production and showed low rate of regeneration.

Shoot production was observed within 2-3 weeks after explant culture. Most of the explants on media containing cefotaxime, initiated callus and finally produced shoots (Table 2). Results showed that the combination of NAA and IAA had a significant effect on plant regeneration and BAP had an obvious effect on proliferation. In most of the media containing 100 mg l^{-1} cefotaxime, callus growth and shoot regeneration were obtained, however media free of cefotaxime showed low rate of regeneration and proliferation.

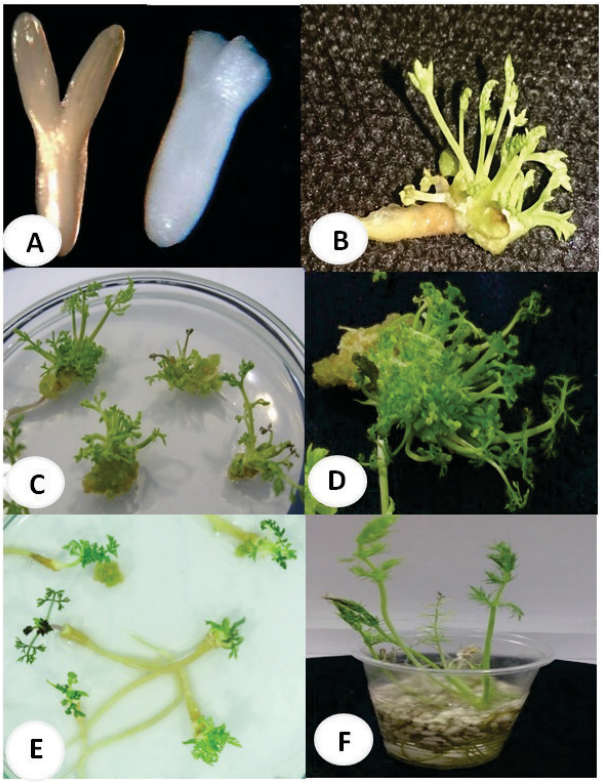

Figure 1. Fennel tissue culture, A) Preparation of explants. B) Embryo explant of fennel in the absence of cefotaximein 4.0 mgl^{-1}IAA and 0.2 mgl^{-1}NAA C. Embryo explant of fennel in the presence of cefotaxime (100 mg l^{-1}) and 1.0 mg l^{-1} BAP. D) Shoot elongation without any subculture in the presence of cefotaxime (100 mg l^{-1}) and 1.0 mg l^{-1} BAP. E) Root elongation of callus in fennel in the presence of cefotaxime (100 mg l^{-1}) in the same media with no sub-culturing. F) Regenerated plants in perlite in growth chamber for hardening

Table 1. Analysis of variance for the effect of different PGRs, Cefotaxime concentration and genotypes on direct regeneration and proliferation of fennel embryo culture

Source	DF	Mean Square	
		Indirect regeneration	Proliferation
V	2	2.8	15.24
BAP	2	1.5	38.65**
V*BAP	4	11.4**	2.28
NAA	1	304.4**	7.66
V*NAA	2	9.29*	4.14
BAP*NAA	2	30.29**	6.93
V*BAP*NAA	4	12.7**	7.62
IAA	1	165**	1.92
V*IAA	2	7.86*	30.72**
BAP*IAA	2	11.62**	65.89**
V*BAP*IAA	4	19.12**	4.45
NAA*IAA	1	79.9**	4.49
V*NAA*IAA	2	1.26	1.58
BAP*NAA*IAA	2	22.49**	4.22
V*BAP*NAA*IAA	4	6.63*	13.99
Cef	1	909.7**	579.14**
V*Cef	2	6.49*	39.59**
BAP*Cef	2	2.8	23.63*
V*BAP*Cef	4	17.3**	2.65
NAA*Cef	1	53.5**	1.91
V*NAA*Cef	2	18.4**	1.43
BAP*NAA*Cef	2	26.8**	18.64*
V*BAP*NAA*Cef	4	9.61**	8.72
IAA*Cef	1	225.1**	34.43*
V*IAA*Cef	2	1.2	6.06
BAP*IAA*Cef	2	4.5	43.8**
V*BAP*IAA*Cef	4	10.5**	1.72
NAA*IAA*Cef	1	250.2**	4.12
V*NAA*IAA*Cef	2	5.8	1.82
BAP*NAA*IAA*Cef	2	12**	7.44
V* BAP*NAA*IAA*Cef	4	3.2	10.17

Table 2. The effect of cefotaxime and PGRs on callus production and shoot regeneration in three different genotypes (continued)

Genotypes	Cefotaxim (mg/L)	BAP (mg/L)	NAA (mg/L)	IAA (mg/L)	PIR[a] ±SE	PP[b] ±SE
Fasa	0	0	0.2	0.4	85.71±14.28	26.66±6.66 [ab]
	0	0.5	0.2	0.4	94.44±5.55 [a]	31.06±14.39 [b]
	0	1	0.2	0.4	100±0 [a]	6.25±6.25 [b]
	0	0	0	0	0[c]	0 [c]
	0	0	0	0.4	0 [c]	0 [c]
	0	0	0.2	0	0 [c]	0 [c]
	0	0.5	0	0	0 [c]	0 [c]
	0	0.5	0	0.4	0 [c]	0 [c]
	0	0.5	0.2	0	0 [c]	0 [c]
	0	1	0	0	0 [c]	0 [c]
	0	1	0	0.4	50±50 [b]	50±50 [a]
	0	1	0.2	0	0[c]	0 [c]
	100	0	0.2	0.4	100±0 [a]	0 [c]
	100	0.5	0.2	0.4	92.85±7.14 [a]	25±25 [b]
	100	1	0.2	0.4	77.5±2.5 [a]	75±25 [a]
	100	0	0	0	100±0 [a]	50±50 [b]
	100	0	0	0.4	0	0 [c]
	100	0	0.2	0	100±0 [a]	25±25 [b]
	100	0.5	0	0	50±50 [b]	14.28±14.28 [b]
	100	0.5	0	0.4	100±0 [a]	50±0 [b]
	100	0.5	0.2	0	66.66±16.66 [a]	10±10 [b]
	100	1	0	0	0	0 [c]
	100	1	0	0.4	80.15±8.7 [a]	62.5±37.5 [a]
	100	1	0.2	0	75±25[a]	16.66±16.66 [b]
Meshkinshahr	0	0	0.2	0.4	100±0 [a]	0 [c]
	0	0.5	0.2	0.4	83.33±16.66 [a]	0 [c]
	0	1	0.2	0.4	100±0 [a]	0 [c]
	0	0	0	0	0 [c]	0 [c]
	0	0	0	0.4	0 [c]	0 [c]
	0	0	0.2	0	0 [c]	0 [c]
	0	0.5	0	0	0 [c]	0 [c]
	0	0.5	0	0.4	0 [c]	0 [c]
	0	0.5	0.2	0	0 [c]	0 [c]
	0	1	0	0	0 [c]	0 [c]
	0	1	0	0.4	0 [c]	0 [c]
	0	1	0.2	0	0 [c]	0 [c]
	100	0	0.2	0.4	100±0 [a]	0 [c]
	100	0.5	0.2	0.4	0 [c]	83.33±16.66 [a]
	100	1	0.2	0.4	81.94±6.94 [a]	77.08±10.41 [a]
	100	0	0	0	100±0 [a]	75±25 [a]
	100	0	0	0.4	100±0 [a]	0 [c]
	100	0	0.2	0	100±0 [a]	100±0[a]
	100	0.5	0	0	100±0 [a]	80±20 [a]
	100	0.5	0	0.4	90±10 [a]	41.66±8.330 [b]
	100	0.5	0.2	0	100 [a]	39.28±10.71[c]
	100	1	0	0	50±50 [b]	50±500 [b]
	100	1	0	0.4	85.71±14.28 [a]	51.66±31.66[b]
	100	1	0.2	0	100±0 [a]	75±250 [a]

Table 2. (Continued)

Genotypes	Cefotaxim (mg/L)	BAP (mg/L)	NAA (mg/L)	IAA (mg/L)	PIR[a] ±SE	PP[b] ±SE
	0	0	0.2	0.4	100±0[a]	0 [c]
	0	0.5	0.2	0.4	81.74±3.96[a]	0 [c]
	0	1	0.2	0.4	65±15[ab]	0 [c]
	0	0	0	0	0[c]	0 [c]
	0	0	0	0.4	0[c]	0 [c]
	0	0	0.2	0	0[c]	0 [c]
	0	0.5	0	0	0[c]	0 [c]
	0	0.5	0	0.4	0[c]	0 [c]
	0	0.5	0.2	0	0[c]	0 [c]
	0	1	0	0	0[c]	0 [c]
	0	1	0	0.4	0[c]	0 [c]
Hajiabad	0	1	0.2	0	0[c]	0 [c]
	100	0	0.2	0.4	100[a]	0 [c]
	100	0.5	0.2	0.4	62.5±12.5 [ab]	35.71±35.71[b]
	100	1	0.2	0.4	50±0[ab]	75±25[a]
	100	0	0	0	0 [c]	0[c]
	100	0	0	0.4	0 [c]	0 [c]
	100	0	0.2	0	100±0 [a]	81.25±18.75[a]
	100	0.5	0	0	100±0 [a]	90±10[a]
	100	0.5	0	0.4	100±0 [a]	82.85±2.85[a]
	100	0.5	0.2	0	100±0 [a]	50±50[ab]
	100	1	0	0	83.33±16.66 [a]	66.6±16.66[ab]
	100	1	0	0.4	85.71±14.28 [a]	30±30[b]
	100	1	0.2	0	100±0 [a]	16.66±16.66[b]

Assessment the effects of cefotaxime on the regeneration of fennel embryo showed that all media containing cefotaxime have convincing proliferation, however, the best medium. with an average of 200 regenerated shoots, was related to 1.0 mg l^{-1} BAP and 100 mg l^{-1} cefotaxime (Fig. 1D).

Cefotaxime was most effective when it is used in combination with other factors i.e. hormones, and has a positive effect on direct regeneration and proliferation (Table 1). In general, the average number of regenerated shoot per explant were 35 and 10, consequently in the presence and absence of cefotaxime, this was indicated their significant differences (Fig. 1C, 1B).

In media with or without cefotaxime, there was no significant difference in the rooting frequencies of regenerated shoots from embryo explants (Fig. 1E). In all conditions, thick-white and sometimes divergent root was observed. Furthermore, no albino shoots or undesirable phenotypes were detected in our research. Shoot elongation and rooting were successfully achieved for all of studied media with no sub-culturing.

DISCUSSION

The results demonstrated that in the absence of cefotaxime, regeneration and proliferation mostly can be take placed in media containing auxins (NAA, IAA) and the presence of antibiotic, had an important role in callus growth, shoot proliferation. The combination of 1.0 mg l^{-1} BAP and 100 mg l^{-1} cefotaxime was the best medium for proliferation (Table 1 and Fig. 1C, 1D).

Applying of some superior fennel ecotypes from a breeding program, choosing seed embryo as a young and fresh explant with proliferation potential, and using combination of hormones with cefotaxime are innovation for this study. Our results revealed that in fennel tissue culture, IAA with NAA, BAP and Cefotaxime are mostly important for callus induction, shoot proliferation and both traits consequently. High throughput proliferation in a short period and no need to sub culturing are important in micro-propagation and genetic manipulation [13].

The results of the present investigation showed that a suitable combination of auxins and cefotaxime is

important for organogenesis of fennel from embryo explants. In the absence of cefotaxime, auxins had the most important role on callus induction and it seems there is no interaction between cytokinin and organogenesis. In other words, in the absence of cefotaxime, callus production and organogenesis were observed in all concentrations of BAP with 0.4 mgl^{-1} IAA and 0.2 mg l^{-1} NAA. Martin (2004) reported that Auxins or its combination with cytokinins can induce organogenesis [27]. However, Manoir et al. (1985) found that the best combinations of auxins and cytokinin can lead to rapid clonal propagation in bitter and sweet fennels [12]. Anzidei et al. (2000) claimed that equal ratio of auxin and kinetin extremely stimulated shoot regeneration [4]. Several studies have also revealed that maximum shoot regeneration was obtained in the presence of high concentration of cytokinin and low auxin [3, 49]. According to previous studies, auxin hormones like NAA and IAA had positive effect on callus induction, somatic embryogenesis and proliferation [14, 31, 46, 50].

Results also indicate that in the presence of cefotaxime, in most of the media, callus production, organogenesis and proliferation were observed. Recently, in vitro studies have shown that cefotaxime had profound effect on callus growth, embryogenesis or plant regeneration in wheat [10, 48], *pearl millet* [17], barley [28], *Mentha piperita* [41], *Solanum chacoense [38]*, *Brassica napus* [2], sweet sorghum [10], *Centella asiatica* [36], and maize [11]. The best medium for proliferation was recognized the one containing 1 mg l^{-1} BAP and 100 mg l^{-1} cefotaxime, which was auxin-free. The most striking result to emerge from this data is that cefotaxime can play the role of plant growth regulator, such as auxin. According to previous studies on cefotaxime, it is realized that this antibiotic is likely to mimic plant growth regulators, especially auxin-like activity [9, 11, 36, 38].

In this investigation, rooting of shoots happened in all conditions (whether in the presence of both auxin and cytokinin or cefotaxime). Although root formation in fennel would happen in the media containing auxin, it would be extremely prevented in the presence of cytokinin [12]. This can be explained by the species and type of explant. Embryo explants have a high capacity for regeneration than other parts of the plant. This proves that for each species and explant, optimum combinations of specific hormones have to be identified. Tawfik and Noga (2002) reported that different explants show different responses to combinations of PGRs for callus induction and plant regeneration [44].

In this study, genotypes had different response to proliferation on media supplemented with 100 mg l^{-1} cefotaxime. Anzidei et al. (2000) found that the main effective factors for organogenesis were genotype and PGRs. Consequently, numerous studies showed that genotypic differences had great effect on plant regeneration [6, 8]. Due to various effects of cefotaxime on different species.

CONCLUSION

In this study we found that cefotaxime can have positive effect on callus production, shoot and root regeneration in fennel. Also, cutting part of embryo explant (cotyledon) and optimum concentration of PGRs and cefotaxime, can increase the efficiency of tissue culture.

In conclusion, we have introduced an effective protocol for fennel tissue culture from embryo explants that can be used in fennel embryo culture for breeding approaches, particle bombardment, and agrobacterium-mediated genetic transformation.

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باززایی سریع و با کارایی بالا در رازیانه از جداکشت جنین

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چکیده

به منظور دستیابی به کالوس زایی و چند شاخه زایی در رازیانه، اثرات مختلف آنتی بیوتیک سفاتوکسیم و هورمون‌های رشد بر روی جنین آن مورد آزمایش قرار گرفت. این آزمایش در قالب طرح فاکتوریل کاملا تصادفی انجام شد.در این آزمایش از ۳ ژنوتیپ فسا، مشکین شهر و حاجی آباد با دو غلظت ۰ و ۱۰۰ میلی‌گرم بر لیتر آنتی بیوتیک سفاتوکسیم، غلظت‌های ۰و ۰/۲ میلی‌گرم بر لیتر NAA، ۰ و ۰/۴ میلی‌گرم بر لیتر IAA و ۰، ۰/۵ و ۱ میلی‌گرم بر لیترBAP استفاده شد. اندام زایی، چندشاخه زایی و ریشه زایی بعد از حدود ۳۵ روز و بدون انجام واکشت در همان محیط اولیه مشاهده شد. بالاترین میزان چندشاخه زایی در محیط حاوی ۱۰۰ میلی گرم بر لیتر آنتی بیوتیک و ۱ میلی گرم بر لیتر BAP مشاهده گردید. بطور کلی بالاترین میزان کالوس‌زایی و چند شاخه‌زایی در حضور ۱۰۰ میلی‌گرم بر لیتر آنتی بیوتیک سفاتوکسیم بدون نظر گرفتن سایر هورمون‌ها مشاهده گردید که این خود نشان‌دهنده فعالیت شبه اکسینی آنتی‌بیوتیک سفاتوکسیم است.

کلید واژه: رازیانه، آنتی بیوتیک سفوتاکسیم، هورمون‌های رشد، جنین، چندشاخه‌زایی