The Effect of Plant Growth Regulators on Regeneration of Fennel (*Foeniculum vulgare* Mill.) Embryo

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Abstract

A rapid method was developed to achieve high callus growth and multiple shoot regeneration of fennel from embryo culture using different plant growth regulators (PGRs). The experiments were conducted under a factorial experiment, based on a completely randomized design (CRD). Here, we applied an Iranian superior fennel ecotype (called Fasa) and all the processes including callus induction and multiple shoot and root regeneration were evaluated after 35 days, on average, and on the same media, without any sub-culturing. The best rate of proliferation was related to the auxin-rich medium with 10-20 shoots per explant. Regenerated plants were phenotypically normal. This high throughput and rapid regeneration method, regarding the positive effect of cefotaxime as a necessary antibiotic in plant transformation, can be the best approach for fennel metabolic engineering.

Abbreviations: BAP—6-Benzylaminopurine; IAA—Indol-3-Acetic Acid; NAA—Naphthalene acetic acid; PGR—Plant Growth Regulator

***Keywords*: Plant Regeneration, Proliferation, Callus, Indirect Regeneration, Auxin**

Introduction

Fennel (*Foeniculum vulgare* Mill), a member of the umbelliferae family, native to Mediterranean region (Graifenberg *et al.* 1996), is one of the oldest and traditionally most important medicinal aromatic plant. (Anzidei *et al.* 1996; Mohamed and Abdu 2004). Recent studies have documented the widespread use of this plant as a treatment for both humans and animals (Mahfouz and Sharaf-Eldin, 2007).

Drought and salinity are major abiotic stresses, affecting growth, essential oil, and the total crop production of fennel (Graifenberg *et al.* 1996). Besides, some biotic stresses containing larvae (*systole albipennis*), several Lepidoptera, aphids, and fungi can cause serious damage to fennel crop (Hunault *et al.* 1989). Conventional breeding methods have certain limitations for genetic improvement of this crop due to low efficiency and also they are time consuming (Pandey *et al.* 2013). Plant tissue culture technique with high frequency of plant regeneration and proliferation is now widely used for genetic manipulation and crop propagation (Ebrahimi *et al.* 2003; Bennici *et al.* 2004).

In the past three decades, there has been an increasing interest in developing a regeneration protocol from callus in fennel. Several studies have discovered that fennel has an innate tendency to micro-propagate (Du Manoir *et al.* 1985; Hunault and Du Manoir 1992), regenerates from callus and suspension cultures (Hunault *et al.* 1989), and regenerates through somatic embryogenesis (Hunault and Maatar 1995; Anzidei *et al.* 2000). However, some of these techniques have shown low success in regeneration, which is not useful for gene transformation.

Despite a large number of successful experiments on fennel regeneration, there are no reports on this plant for rapid and efficient micro-propagation and regeneration capacity, and other methods had little effect on its proliferation. Therefore, here we introduce a new optimized method for fennel proliferation through embryo explants and PGRs. The aim of this research was to find the best combination of PGRs to achieve the best rate of multiple shoot regeneration.

Materials and methods

A superior genotype (Fasa) with early maturity growth habit (Bahmani *et al.* 2012) was selected for this study. In order to prepare explants, seeds were surface-sterilized for 20 seconds in 70% ethyl alcohol and then treated for 10 minutes with 2.5% sodium hypochlorite. Seeds were washed 4 times with sterile distilled water after each step. They were then soaked for 3-6 hours in sterile distilled water and embryos were obtainable by cutting and pressing the middle of the turgid seeds (Ebrahimi *et al.* 2003) (Fig.1a). In this study, the explants were prepared by cutting and removing part of the cotyledons from the top (Fig. 1b), as previously this method has been reported to increase callus formation (Tawfik and Noga, 2001).

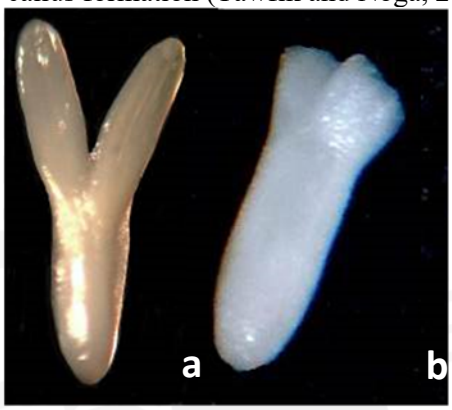

Fig. 1- Preparation of explants. **a:** Embryos detached from seed. **b** Explant with shoot meristem (parts of cotyledons from the top of the embryo are cut)

PGRs and cefotaxime combinations for callus induction and plant regeneration

B5 medium (Gamborg *et al.* 1968) containing full strength of macro- and micro-elements, vitamins, and sucrose (30 g l^{-1}), were used for callus induction. The different plant growth regulators (PGRs) including BAP (0, 0.5, 1.0 mg l^{-1}), NAA (0, 0.2 mg l^{-1}) and IAA (0, 0.4 mg l^{-1}) were added to each medium. Hence, a factorial experiment of hormonal combination containig BAP, NAA and IAA were used as follows: (1) 0 BAP+ 0 NAA+0 IAA, (2) 0.5 BAP+0 NAA+0 IAA, (3) 1 BAP+0 NAA+0 IAA … and (12) 1 BAP+0.2 NAA+0.4 IAA. All these culture media were adjusted to pH 5.7-5.8 and then solidified with 8% agar.

Shoot and root development of plantlets

Both shoot elongation and rooting were accomplished on the same culture medium, without sub-culturing.

Statistical analysis

Analysis of variance (ANOVA) was performed using SAS (Version 9.00). Means were compared using Duncan's multiple range test.

Results and Discussion

Results showed that only on four media, fennel embryos had callus induction and indirect regeneration. However, explants had direct regeneration on remaining media. Results showed that the combination of NAA and IAA had a significant effect on callus production and proliferation (Fig.2).

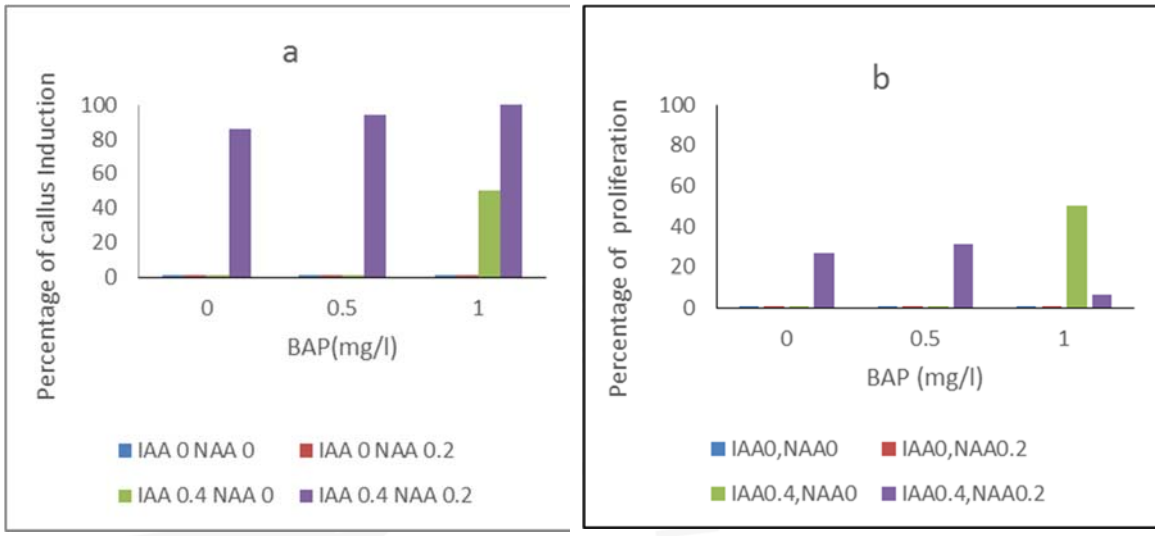

Fig.2 – (a) Effect of 3 horomones on callus induction of embryo explants (b) Effect of 3 horomones on proliferation of embryo explants

Fig. 3-(a) Embryo explant of fennel in 0 mgl $^{-1}$ BAP, 0 mgl^{-1} IAA and 0.2 mgl^{-1} NAA (b) Regenerated plants in perlite in growth chamber

Shoot elongation and rooting were successfully achieved on previously described media without sub-culturing or using new medium (Fig.3a). Shoots were strong enough and 100% of shoots rapidly produced roots on the same medium. After one month, plantlets were transplanted into pots containing perlite and water for hardening (Fig.3b).

The results demonstrated that auxin hormones (NAA, IAA) had significant effects on indirect regeneration and proliferation. This method revealed some advantages compared to former explants (hypocotyl, root, etc.) as former explants were too old to respond to PGRs, rapidly and effectively, but the embryo explant, which has been used for micro-propagation and genetic transformation, was younger and responded to the medium more quickly.

It seems that in all concentrations of BAP in combination with 0.4 mg.l $^{-1}$ IAA and 0.2 mg l^{-1} NAA, callus production and organogenesis were observed. Otherwise, direct shoot regeneration happened. Martin (2004) had also come to this conclusion that with using auxin hormones alone or in combination with cytokinin, inducing shoots depends on hormone concentration and type of explant. However, Manoir *et al.* (1985) found that the best combinations of auxins and cytokine in can lead to rapid clonal propagation in bitter and sweet fennels. Anzidei *et al.* (2000) claimed that equal ratio of auxin and kinetin extremely stimulated shoot regeneration. According to previous studies, auxin hormones like NAA and IAA had

positive effects on callus induction, somatic embryogenesis and proliferation. (Naing *et al.* 2013).

References

Anzidei, M., Bennici, A., Schiff, S., Tani, C. and Mori, B. 2000. Organogenesis and somatic embryogenesis in *Foeniculum vulgare*: histological observations of developing embryogenic callus. Plant Cell Tiss Org Cult; 61**:** 69-79.

Anzidei, M., Vivona, L., Schiff, S. and Bennici, A. 1996. In vitro culture of *fueniculum vulgare mill*: callus characteristics in relation to morphogenesis. Plant Cell tiss Org Cult; 45: 263-268.

Bahmani, K., Izadi-Darbandi, A., Ashraf afari, A., Sadat Noori, S.A. and Farajpour, M. 2012. Assessment of Genetic Diversity in Iranian Fennels Using ISSR Markers. J Agric Sci Can Cen Sci; Edu 4, doi:10.5539/jas.v4n9p79.

Bennici, A., Anzidei, M. and Vendramin, G.G. 2004. Genetic stability and uniformity of *Foeniculum vulgare Mill.* Regenerated plants through organogenesis and somatic embryogenesis. Plant Sci; 166:221-227.

Du Manoir, J., Desmarest, P. and Saussay, R. 1985. Invitro propagation of fennel *(Foeniculum vulgare miller*). Sci Hort; 27:15-19.

Ebrahimie, E., Habashi, A.A., Ghareyazie, B., Ghannadha, M. and Mohammadie, M. 2003.A rapid and efficient method for regeneration of plantlets from embryo explants of cumin (*Cuminum cyminum*). Plant Cell Tiss Org Cult; 75**:** 19-25.

Graifenberg, A., Botrini, L., Giustiniani, L. and Lipucci Di Paola, M. 1996. Salinity Affects Growth, Yield and Elemental Concentration of Fennel. Hortsci; 31(7):1131-1134.

Hunault. G., Desmarest, P. and Du Manoir, J. 1989. *Foeniculum vulgare* Miller: Cell culture, regeneration, and the production of anethole. Biotech Agric Forest; 7: 185–211.

Hunault, G. and Du Manoir, J. 1992. Micropropagation of fennel (*Foeniculum vulgare Miller*). Biotec Agric Forest; 19:199–217.

Hunault, G. &Maatar, A. 1995. Enhancement of somatic embryogenesis frequency by giberellic acid in fennel. Plant Cell Tiss Org Cult; 41: 171-176.

Mahfouz, S.A. and Sharaf-Eldin, M.A. 2007. Effect of mineral vs. biofertilizer on growth, yield, and essential oil content of fennel (*Foeniculum vulgare Mill.).* Int. Agrophys; 21: 361-366.

Martin, K.P. 2004. Efficacy of different growth regulators at different stages of somatic embryogenesis in *eryngium foetiduml*. - A rare medicinal plant. In Vitro Cell Dev Biol Plant; 40:459–463.

Mohamed, M.A.H. and Abdu, M. 2004. Growth and Oil Production of Fennel (*Foeniculum vulgare* Mill): Effect of Irrigation and Organic Fertilization. Biol Agri Hort; 22: 31-39.

Naing, A.H., Kim, C.K., Yun, B.J., Jin, J.Y. and Lim, K.B. 2013. Primary and secondary somatic embryogenesis in *Chrysanthemum. cv*. Plant Cell Tiss Org Cult; 112(3):361-368.

Pandey, S., Mishra, A., Kumar Patel, M. and Jha, B. 2013. An Efficient Method for Agrobacterium-Mediated Genetic Transformation and Plant Regeneration in Cumin (*Cuminum cyminum L.*). Appl Biochem Biotec; doi: 10.1007/s12010-013-0349-1.

Tawfik, A.A. and Noga, G. 2001. Adventitious shoot proliferation from hypocotyl and internodal stem explants of cumin. Plant Cell Tiss Org Cult; 66: 141–147.