



## A RAPID AND EFFICIENT METHOD FOR REGENERATION OF *Cuminum setifolium* (Boiss.) KOS.- POL (1916)

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### ABSTRACT

*Cuminum setifolium* (Boiss.) Kos.- Pol (1916) with common name of white cumin, a member of the Apiaceae family, is one of the most important medicinal plants. Generally, there is not information about *in vitro* culture of *Cuminum setifolium*. For the first time, tissue culture initiated and regenerated and fragmented embryo was the best explant for *Cuminum setifolium* regeneration. In this way, a great callus induction and regeneration only on the same medium without any subculturing was occurred, leading to reduce the tissue culture period, infection and chemical consumption. In this research, B5 and MS media containing different concentrations of plant growth regulators (PGRs), NAA and 2,4-D only or together with kinetin (Kin) and different explant were used. The experiment was conducted into completely randomized design with 10 treatments and at least 30 replicate per treatment. The highest callus induction was obtained from B5 medium supplemented with 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP. Regeneration occurred in some treatments without kinetin, showing that kinetin is not essential for *Cuminum setifolium* regeneration. The B5 medium without growth regulators and B5 medium supplemented with 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP were the best treatments for regeneration with 56.67 and 43.23 percentages, respectively.

**Keywords:** medicinal plants, *Psammogeton setifolius*, tissue culture, *Torilis setifolia*.

### INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high quality plant based medicine (Tripathi and Tripathi, 2003). Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha, 1994).

Umbelliferae (Apiaceae) is a plant family having about 200 genera and 2900 species in the world and about 100-117 genera and 450 species in Iran which distributed all around the country (Mozaffarian 1983). Cumin (*Cuminum cyminum* L.) with local name of green cumin and white cumin (*Cuminum setifolium* Boiss., Kos.- Pol (1916). Syn. *Psammogeton setifolius* (Boiss.) Boiss.(1872), *Torilis setifolia* Boiss.(1856) are the closest relative members of the Umbelliferae family. White cumin (*Cuminum setifolium*) growing wild on mountains of countries such as Iran, Turkmenistan, Afghanistan, Pakistan, Tien-Shan and central Asia (Mozaffarian 1983; Reching 1987). Recent studies suggest that green cumin (*Cuminum cyminum* L.) plant is one of the species subject to renewed interest as an important medicinal crop (Ebrahimie *et al.* 2003). Production of green cumin (*Cuminum cyminum* L.) is limited due to several biotic stresses of which kaphra beetle (*Trogoderma granarium*) and wilt disease are the most serious (Champawat and Pathak 1990; Agrawal 1996). Therefore, white cumin (*Cuminum setifolium*) as a wild type may be useful in future studies for improvement of green cumin (*Cuminum cyminum* L.) with cross pollination for obtaining hybrids between them as well as

genetic manipulation for finding stands with superior characteristics such as tolerance to disease, pests, drought and salinity, improved growth and development, increase of yield and other parameters used in the crop production.

With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen-free material. Micropropagation of various plants, species, including many medicinal plants, has been reported. Plant regeneration from shoot and stem meristems has yielded encouraging results in medicinal plants like *Chtharanthus roseus*, *Rehmannia glutinosa*, *Rauwolfia serpentina*, *Isoplexis canariensis* (Paek *et al.* 1995; Roy *et al.* 1994; Perez-Bermudez and Seitz, 2002).

Numerous factors are reported to influence the success of *in vitro* propagation of different medicinal plants (Tripathi and Tripathi, 2003). The effects of auxins and cytokinins on shoot multiplication of various medicinal plants have been reported. Benjamin *et al.* (1987) has shown that 6-Benzylaminopurine (BA), at high concentration, stimulates the development of *Atropa belladonna*. Lal and Ahuja (1996) observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0-5.0 mg l<sup>-1</sup>. Direct plantlet regeneration from male inflorescences of medicinal yam on medium supplemented with 13.94 μM kinetin has also reported (Borthakur and Singh, 2002). It has been observed that cytokinin is required, in optimal quantity, for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytokinin triggers the rate of shoot proliferation (Tripathi and Tripathi, 2003). Baran and Wakhlu (1988) has indicated that the production of multiple shoots is higher in *Plantago ovata* on a medium having 4-6 M kinetin along with 0.05 μM NAA. The nature and condition of explants has also been shown to have a significant

influence on the multiplication rate of *Clerodendrum colebrookianum* (Mao *et al.* 1995).

The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. With the stimulus of endogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced. There are many reports on the regeneration of various medicinal plants via callus culture. Satheesh Kumar and Bhavanandan (1988) have reported the regeneration of shoots from callus of *Plumbago rosea* using appropriate concentrations of auxins and cytokinins. Mantell and Hugo (1989) have also reported a high frequency of shoot, root, and microtuber production from *Dioscorea alata* depending on the culture medium used, the type of explant from which the calli originated, and the photoperiod.

Plant regeneration via somatic embryogenesis from single cells, has been demonstrated in many medicinal plant species. Arumugam and Bhojwani (1990) noted the development of somatic embryos from zygotic embryos of *Podophyllum hexandrum* on MS medium containing 2  $\mu$ M BA and 0.5  $\mu$ M IAA. Ghosh and Sen (1991) reported regeneration and somatic embryogenesis in *Asparagus cooperi* on MS medium having 1.0 mg l<sup>-1</sup> NAA and 1.0 mg l<sup>-1</sup> kinetin. Zhou *et al.* (1994) using a medium containing 2,4-Dichlorophenoxyacetic acid (2,4-D) and TDZ, have achieved the induction of somatic embryogenesis in cells from *Cayratia japonica*. Somatic embryogenesis and subsequent plant regeneration from callus derived from immature cotyledons of *Acacia catechu* has also been achieved on medium supplemented with 2.0 mg l<sup>-1</sup> kinetin, 2.0 mg l<sup>-1</sup> 2,4-D and 2.0 mg l<sup>-1</sup> NAA (Gastaldo and Caviglia, 1996). High frequency somatic embryogenesis and plant regeneration from suspension culture of *Acanthopanax koreanum* have been reported on a medium containing 4.5  $\mu$ M 2,4-D (Choi *et al.* 1997). Das *et al.* (1999) reported high frequency somatic embryogenesis in *Typhonium trilobatum* on medium containing 1.0 mg l<sup>-1</sup> kinetin and 0.25 mg l<sup>-1</sup> NAA. Chand and Sahrawat (2002) have reported the somatic embryogenesis of *Psoralea corylifolia* L. from root explants on medium supplemented with NAA and BA. Ebrahimi *et al.* (2003) used fragmented embryo explants for cumin (*Cuminum cyminum* L.) tissue culture leading to suitable regeneration in a short time and without any subculture. In this research the best treatments were B5 medium containing 0.2 mg l<sup>-1</sup> IAA and 1 mg l<sup>-1</sup> BAP or 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP.

Tissue culture protocols have been developed for several plants but there are many other species, which are over exploited in pharmaceutical industries and need conservation.

Generally Umbeliferaceae species are antimicrobial. This family breeding is slow, troublesome and endless through classic process (Hunault *et al.* 1989) and there is limited genetic diversity to breed this crop. Seed dormancy is one of these crop obstacles to be cultured widely. This dormancy is embryonic and only cold treatment can cause

the seed to germinate. Biotechnological manipulation of crop at cellular and molecular levels, is a new efficient way to breed secondary products for pharmaceuticals industry and overcome diseases. Thus an efficient micro propagation system with high regeneration frequency is required. The most essential goal of tissue culture is shortening the period of breeding and production of many plants without any pathogen. Simultaneous regeneration and callus induction help to achieve this aim.

The aim of this study was callus initiation and regeneration of *Cuminum setifolium* (Boiss.) Kos.- Pol (1916) for the first time.

## MATERIALS AND METHODS

*Cuminum setifolium* seeds and immature seeds were collected from wild pistachio forest (Sanganeh, Chahchaheh and Shoorlogh) located in NE Mashhad city at spring season and transferred to Khorasan agricultural and natural resources research center laboratory. Seeds were carefully surface sterilized in 2% (v/v) sodium hypochlorite for 20 minutes, and rinsed 3 times with distilled water and dried at room temperature. After omit of seeds hairs, they were cultured on aseptic moist filter paper in Petri dish and maintained at 4°C and darkness for 20 days for germination. This cold period is essential for embryo to grow and to be able to extrude from the seed. The end of the seeds was cut with scalpel and the immature embryo and embryo were extruded with pressure on the seed middle. In this research, different explant namely hypocotyls, cotyledon, immature embryo and embryo were used. The explants were cultured on B5 and MS basal medium containing 20 g l<sup>-1</sup> and 30 g l<sup>-1</sup> sucrose respectively, 8 g l<sup>-1</sup> agar as well as plant growth regulators with different concentrations of NAA, 2,4-D only or together with Kin (0,0.5,1mg l<sup>-1</sup>) with and without cold pretreatment. Cold pretreatment was 60 days at 4°C. Cultures were maintained at 25±2°C under a 16h photoperiod (2500 lux). In this research, regeneration and callus induction occurred on the same medium without any subculture. Eight weeks after embryo transferring, the callus and regeneration percentage and regenerated stems per explant were scored. The experiment was conducted into completely randomized design with 10 treatments and at least 30 replicate per treatment. Analysis of variance was carried out using SAS packages. Bonferroni's method (Maxwell and Delaney, 1990) was used to compare means.

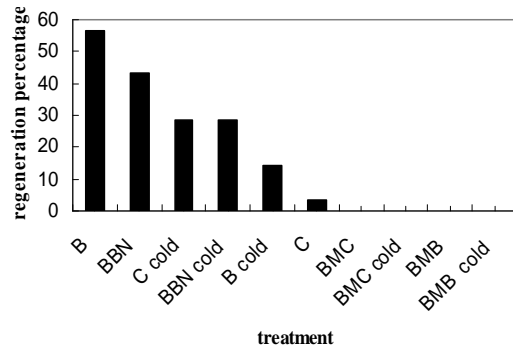
## RESULT

Callus induction and regeneration of *Cuminum setifolium* were observed on B5 medium, 20 days and 60 days after explants transferring, respectively. There was some callus induction on MS medium, but there was not any regeneration. There was some callus induction and regeneration by hypocotyls and immature embryo explant (Fig. 2, 3, 4, 5). There was significant different between treatments for regeneration and callus induction (Table I). Generally callus amount in treatments supplemented with auxin and cytokinin were more than treatments without cytokinin. The best treatment for callus induction was B5 medium containing 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup>

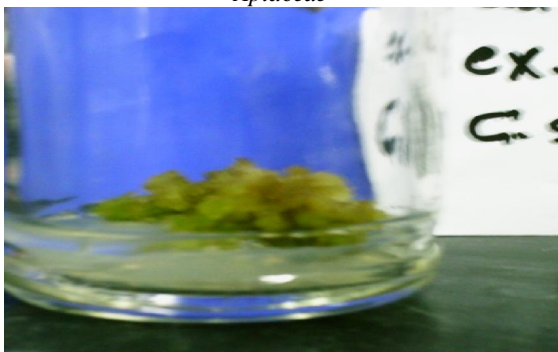
<sup>1</sup> BAP (Table I). There was also significant different between treatments for regeneration. The best treatments for regeneration were B5 medium without growth regulator (56.67%) and B5 medium supplemented with 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP (43.23%) (Table I and Fig. 1). The others treatments such as B5 medium contain 0.5 mg l<sup>-1</sup> Kin + 1 mg l<sup>-1</sup> 2,4,D; MS medium contain 1 mg l<sup>-1</sup> Kin + 1 mg l<sup>-1</sup> 2,4,D and MS medium contain 1 mg l<sup>-1</sup> Kin + 2 mg l<sup>-1</sup> 2,4,D although callus were initiated but were not perfect treatments for regeneration. In the B5 medium supplemented with 0.5 mg l<sup>-1</sup> Kin + 1 mg l<sup>-1</sup> 2,4,D

with cold pretreatment was initiated some callus. There were some regeneration on the B5 medium supplemented with 0.5 mg l<sup>-1</sup> Kin + 1 mg l<sup>-1</sup> 2,4,D without cold pretreatment (3.33%) and with cold pretreatment (28.57%). The best treatment for callus initiation (40%) and regeneration (43.23%) simultaneously was B5 medium containing 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP (Table I and Fig. 6, 7). The highest shoot number was also observed on B5 medium containing 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP (Table I).

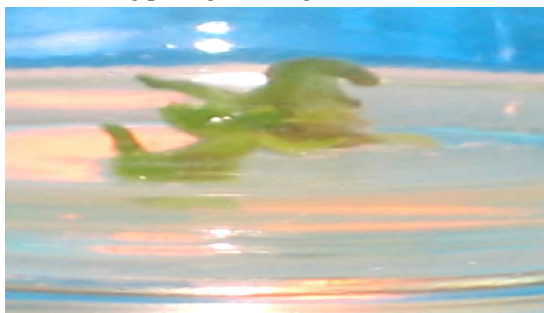
**Regeneration C.setifolium**



**FIG.1.** Comparison between different treatments for regeneration of *Cuminum setifolium* (Boiss.) Kos.- Pol, Apiaceae



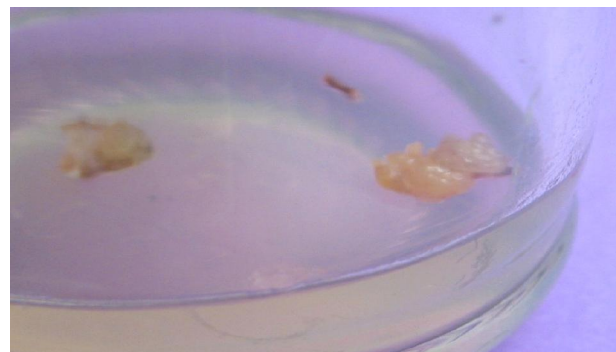
**FIG.3.** Embryogenic callus developed 40 days after transferring the callus derived from hypocotyls explant to medium lacking plant growth regulators.



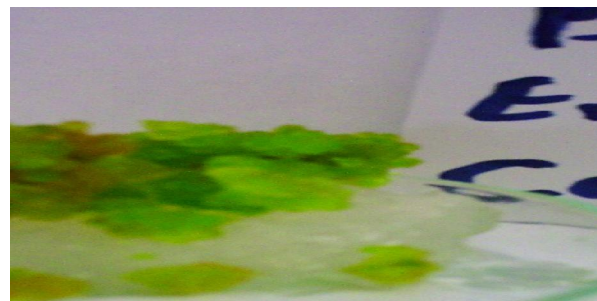
**FIG.5.** Plants regenerated when the embryogenic callus derived from immature embryo explant was subcultured on medium lacking plant growth regulators.



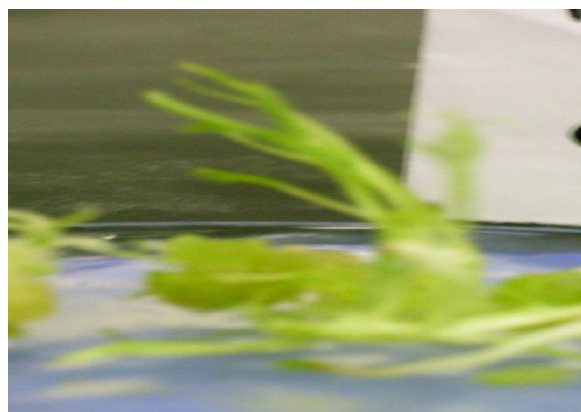
**FIG.2.** Callus induction from hypocotyls explant of *Cuminum setifolium* (Boiss.) Kos.- Pol, Apiaceae



**FIG.4.** Callus initiation from immature embryo explant of *Cuminum setifolium* (Boiss.) Kos.- Pol, Apiaceae.



**FIG.6.** Embryogenic callus developed 30 days after subculture of callus derived from embryo to B5 medium supplemented with 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP.



**Fig.7.** Regeneration and shoot induction from embryo explant of *Cuminum setifolium* (Boiss.) Kos.- Pol, *Apiaceae*.

**TABLE1.** Callus induction and regeneration percentage in media containing different concentrations of plant growth regulators (PGRs).

PGRs Treatments (mg l <sup>-1</sup> )	Treatment Code	Medium	Explant	Callus Induction (%)	Regeneration (%)	Shoot number mean
-	B	B5	Embryo	6.67 ab	56.67 a	45
0.2 NAA+0.2BAP	BBN	B5	Embryo	40.00 a	43.23 ab	64
1 2,4-D+1KIN	BMC	MS	Embryo	20.00 ab	0 c	0
1 2,4-D+2KIN	BMB	MS	Embryo	13.33 ab	0 c	0
1 2,4-D+0.5KIN	C	B5	Embryo	20.00 ab	3.33 c	2
-	B cold	B5	Embryo	1.33 b	14.29 bc	1
0.2 NAA+0.2BAP	BBN cold	B5	Embryo	1.25 b	28.57 abc	2
1 2,4-D+1KIN	BMC cold	MS	Embryo	0 b	0 c	0
1 2,4-D+2KIN	BMB cold	MS	Embryo	0 b	0 c	0
1 2,4-D+0.5KIN	C cold	B5	Embryo	14.29 ab	28.57 abc	2

## DISCUSSION

Tissue culture is useful for multiplying and conserving the species, which are difficult to regenerate by conventional methods and save them from extinction. Efficient development and germination of somatic embryos are prerequisite for commercial plantlet production. In this experiment growth regulators were selected according to previous studies on regeneration of cumin (Ebrahimie *et al.* 2003; Hussein and Batra, 1998). Results indicated that suitable combination of auxins and cytokinins are important for organogenesis of *Cuminum setifolium* from embryo explants. The best treatment for callus initiation and regeneration simultaneously was B5 medium containing 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP. The highest shoot number was also observed on B5 medium containing 0.2 mg l<sup>-1</sup> NAA and 0.2 mg l<sup>-1</sup> BAP. Lowering of growth regulator concentrations in culture media has improved embryo development and germination of many medicinal plants (Arumugam and Bhojwani, 1990; Kumar, 1992; Wakhlu *et al.*, 1990). Germination of the somatic embryos is achievable on MS medium without the growth regulator (Zhou *et al.*, 1994; Choi *et al.*, 1997). However, Arumugam and Bhojwani (1990) noted that inclusion of BA (2 µM) and gibberellic acid (GA3, 2.8 µM) in the medium stimulated embryo development of *Podophyllum hexanrum*, although 75% of the embryo germinated on basal MS medium devoid of growth regulator. Similar

results were reported on the germination of embryos of *Psoralea corylifolia* (Chand and Sahrawat, 2002). Wakhlu *et al.* (1990) have reported that the somatic embryos of *Bunium persicum* matured and germinated on the basal MS medium supplemented with 1.0 mg l<sup>-1</sup> kinetin. Further, Kunitake and Mii (1997) reported that 30-40% of somatic embryos of *A. officinalis* germinated after being treated with distilled water for a week; they were subsequently transferred to half-strength MS medium supplemented with 1.0 mg l<sup>-1</sup> IAA, 1.0 mg l<sup>-1</sup> GA3 and 1% sucrose. However, the somatic embryo of *Typhonium trilobatum* have been germinated on MS medium supplemented with 0.01 mg l<sup>-1</sup> NAA and 2% (w/v) sucrose after 2 weeks of culture (Das *et al.*, 1999).

Somatic embryogenesis during callus induction step is usual in some *Umbeliferae* species such as carrot and fennel (Hunault *et al.* 1989). 2,4-D and NAA only or together with Kin is essential for continuity of callus induction and its reduction leads to organogenesis and adventitious embryo formation in *Bunium persicum*. Regeneration occurred in some treatments without kinetin, showing that kinetin is not essential for *Bunium persicum* regeneration like other *Umbeliferae* species (carrot, fennel, celery and etc.) (Hunault *et al.* 1989, Wakhlu *et al.* 1990), whereas it is essential for cumin (*Cuminum cyminum* L.) regeneration (Agrawal, 1996, Tawfik and Noga 2002, Ebrahimie *et al.* 2003). However the composition of auxin



and cytokinin has been observed about other plants and it is essential to embryogenesis and organogenesis (Tripathi and Tripathi, 2003). In some other species, induced somatic embryos might need a little cytokinin or other plant growth regulators for germination and growth (Satheesh Kumar and Bhavanandan, 1988). This technique have some advantages including higher regenerated plantlet number, simultaneous regeneration and callus induction, shortening tissue culture period, less infection, subculture and chemical consumption reduction. With use of young embryo explant, the regeneration number significantly increased. Although somatic embryogenesis has been widely used in *Umbelifereae* family especially carrot (Vits *et al.* 1994, Chi *et al.* 1996, Hidetoshi *et al.* 2000, Tawfik and Noga 2001, 2002) but high capacity of organogenesis using younger explants in this family has not been considered except for cumin (*Cuminum cyminum* L.) (Ebrahimie *et al.* 2003).

Generally, there is not information about *in vitro* culture of *Cuminum setifolium*. For the first time, different explants were used and fragmented embryo was the best explant in *Cuminum setifolium* regeneration. It seems that embryo explants have better response to low concentrations of macro and micro elements because of faster response on B5 medium. In this technique, we can reach to complete rooted plants without any subculture 8 weeks after explant transferring and the total period is 50-60 days, counting cold period. By using hypocotyl explant, the total period is 150 days, counting 20 days cold period for germination as well as many subcultures.

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