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Study on shoot regeneration and somatic embryogenesis in cumin (*Cuminum cyminum* L.) landraces

Danial KAHRIZI[1,2,*] and Jahad SOORNI[2]

1. Biotechnology for Drought Resistance Research Department, Razi University, Kermanshah, Iran, E-mail: dkahrizi@razi.ac.ir
2. Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran,
E-mail: d.soorni@yahoo.com
*Corresponding author, D. Kahrizi, E-mail: dkahrizi@razi.ac.ir

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Abstract. To study on different *in vitro* morphogenesis pathways (shoot regeneration and somatic embryogenesis) in four cumin accessions, three experiments (direct and indirect regeneration and indirect somatic embryogenesis) were conducted. Factorial experiment based on completely randomized design (CRD) with three replications was carried out. Direct shoot regeneration of cumin accessions on shoot explant was tested in different concentrations of TDZ (1, 2, 5, 10 and 20 µM) on B5 medium. Indirect shoot regeneration was conducted in B5 medium supplemented with different combinations of BAP (0.1, 0.2, 0.4 and 1 mg/l) in combination with NAA (0.2 and 0.4 mg/l) from shoot explant derived calli. In indirect somatic embryogenesis experiment, the induced calli were transferred to MS medium free of any plant growth regulators. Statistical analysis showed that the highest (57.5%) and lowest (6.67%) direct shoot regeneration were found in 10 µM and 1 µM of TDZ respectively. Results showed that 0.1mg/l NAA plus 0.4mg/l BAP and 0.1mg/l NAA plus 1mg/l BAP combinations determined as the highest level for indirect shoot regeneration (with 25.83 % and 25 % respectively). Furthermore, Shahdad and Afghanistan accessions had the highest (14.38 and 13.75 % respectively) and Koohbanan and Badrood the lowest (10.42 and 10.21 % respectively) indirect shoot regeneration. There was a relationship between 2,4-D concentration (that was applied for callus induction stage) and somatic embryo numbers in the somatic embryogenesis experiment. Also, we observed the more number of somatic embryos with increasing of 2,4-D concentration that were used for callus induction.

Key words: callus, cumin, direct and indirect regeneration, explant, plant growth regulators.

Introduction

Cumin (*Cuminum cyminum* L.) belonging to Apiaceae family, is one of the most important medicinal plants in the world. This plant family is one of the well-known families among flowering plants because of its worth properties (Masoumi et al. 2012). Since differentiation of embryoids was observed in a member of Apiaceae family (*Daucus carota*) from callus for the first time, this family becomes a model for study on morphogenesis (specially somatic embryogenesis) in tissue culture (Jha et al. 1983).

Wilt and dry root rot are the most serious diseases of cumin that causes great losses in seed yield and quality (Tawfik 1998). Conventional plant breeding procedures for improve the resistance of cumin to these stresses is very limited. Thus, production of transgenic cumin plants through genetic engineering procedures may resistances this plant to mentioned biotic stresses (Tawfik & Noga 2001).

Several studies have been conducted on shoot regeneration and somatic embryogenesis of cumin so far (Tawfik & Noga 2001, 2002, Ebrahimie et al. 2003, Ebrahimie et al. 2007, Valizadeh et al. 2007), but study on different explant such as root, has not been reported up to now, or is very limited.

The aims of this study were to develop efficient shoot regeneration and somatic embryogenesis procedure that can be used in efficient gene transfer method and synthetic seed production.

Abbreviations

2,4-D = 2,4-Dichlorophenoxyacetic acid
B5 = Gamborg's medium
BAP = 6-benzylaminopurine
Kin = Kinetin
MS = Murashige and Skoog's medium
NAA = α-Naphthaleneacetic acid
TDZ = 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (Thidiazuron)

Materials and methods

This study was conducted in Medicinal Plants Tissue Culture Lab., Agriculture and Natural Resources Campus, Razi University during 2012.

Mature seeds of four heterogeneous cumin accessions, collected from Shahdad, Koohbanan, Badrood (three small province of Iran) and Afghanistan regions. Seeds were surface sterilized and cultured following the procedure previously described (Soorni et al. 2012).

Direct shoot regeneration

When the cumin seedlings grew about 5 cm in long, shoot explant was prepare for direct shoot regeneration experiment. The experiment was carried out as factorial using a completely randomized design with two factors and three replications (as Petri dishes). Five explant segments were placed in each petri dish containing 25 ml B5 basal medium, then sealed with parafilm. Investigated factors included:

A: Accession (Shahdad, Koohbanan, Badrood and Afghanistan)
B: TDZ concentrations (1, 2, 5, 10 and 20 µM)

After five weeks (35 days) in culture each frond was scored for shoot regeneration percentage. B5 media supplemented with 1, 2, 5, 10 or 20 µM TDZ were used then transferred to growth chamber. The frequency of shoot regeneration per each treatment was calculated by dividing the number of shoots to the original number of plated explants. Mean of replications was used for statistical analysis.

Indirect shoot regeneration

Callus was induced from shoot segments of cumin seedlings on MS medium supplemented with 1 mg/l 2,4-D plus 0.1 mg/l Kin.

For indirect shoot production, calli were transferred to B5 medium supplemented with different combinations of BAP and NAA. The experiment was carried out as factorial using a completely randomized design with three factors and three replications (as Petri

dishes) and five explant segments in each petri dish. Investigated factors included:

A: Accession (Shahdad, Koohbanan, Badrood and Afghanistan)

B: BAP concentrations (0.1, 0.2, 0.4 and 1 mg/l)

C: NAA concentrations (0.1 and 0.4 mg/l)

In both direct and indirect shoot regeneration, basal B5 media without any PGRs used as control.

Eight different combinations of PGRs (0.1 mg/l NAA+0.1 mg/l BAP; 0.1 mg/l NAA+0.2 mg/l BAP; 0.1 mg/l NAA+0.4 mg/l BAP; 0.1 mg/l NAA+1 mg/l BAP; 0.4 mg/l NAA+0.1 mg/l BAP; 0.4 mg/l NAA+0.2 mg/l BAP; 0.4 mg/l NAA+0.4 mg/l BAP; 0.4 mg/l NAA+1 mg/l BAP) were investigated.

Somatic embryogenesis

For induction of somatic embryogenesis, calli of different explants of cumin accessions that were grown in different concentrations of 2,4-D plus 0.1 mg/l Kin, transferred to MS media with no PGRs (PGRs free). For this purpose an experiment was conducted in a factorial based on completely randomized design with three factors and three replications (as Petri dishes) and five calli segments in each petri dish. Investigated factors included:

A: Accession (Shahdad, Koohbanan, Badrood and Afghanistan)

B: 2,4-D concentrations (0.5, 1 and 2 mg/l)

C: Explant type (root, shoot, leaf and mature embryo)

Both shoot regeneration and somatic embryogenesis experimental cultures were at 23°C under a 16 h light photoperiod of approximately 40 µmol m^{-2} s^{-1} illumination provided by Gro-Lux fluorescent lights.

Statistical analysis

Specific analyses and the results are noted in the appropriate figure and table footnotes and text. Mean separation was performed using Duncan's Multiple Range test at 0.05 probability level after ANOVA. Statistical analyses were done using Excel 2010, SPSS Ver. 19 and SAS Ver. 9.1 software.

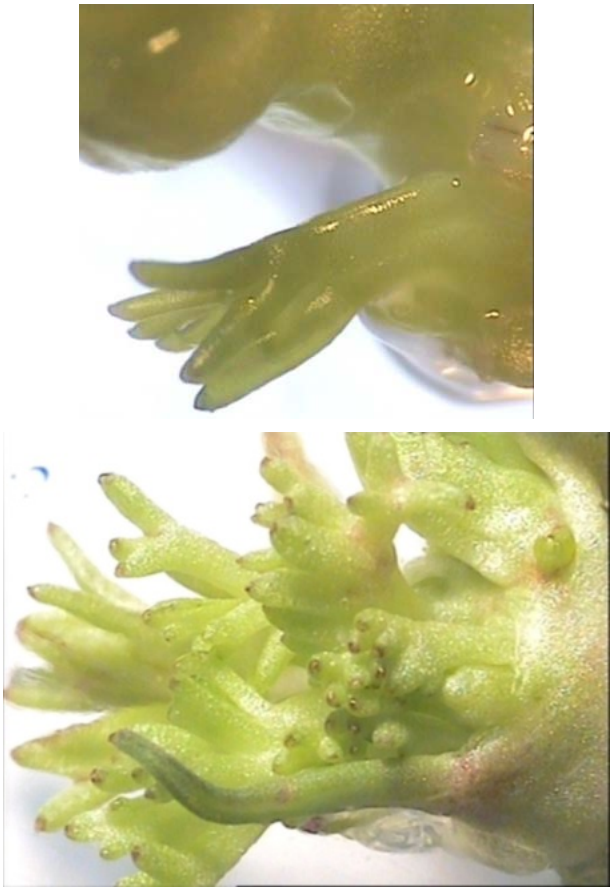

Figure 1. Direct shoot regeneration on 10 µM TDZ in cumin (*Cuminum cyminum* L.).

Results and Discussion

Direct shoot regeneration

Results showed that accessions have same response to direct shoot regeneration. This result reported by Ebrahimie et al. (2007) also. On the contrary, Beiki et al. (2011) reported that direct shoot regeneration is genotype-dependent.

On the contrary of accession, TDZ levels have high significant effect ($p<0.01$) on direct shoot regeneration (Table 1).

Table 1. Analysis of variance for direct shoot regeneration percentage in cumin (Cuminum cyminum L.).

Source of variation	degree of freedom	Mean squares
Accession	3	0.26 ^{ns}
TDZ	4	57.88 **
Accession × TDZ	12	0.18 ^{ns}
Error	40	0.72
CV %	14.39 %	

<ns> and <**> means non-significant and Significant at 0.01 level of probability, respectively

The highest response to direct shoot regeneration (Fig. 1), with the mean of 57.5 %, was obtained from 10 µM TDZ that it was not significant with 5 and 20 µM TDZ concentrations (Fig. 2). Regarding to this result, we offer the 5 µM TDZ concentration for direct shoot regeneration in cumin.

Gupta and Bhargava (2001) achieved shoot regeneration from hypocotyl explant in cumin genotype RZ-19 with a frequency up to 30% on MS medium supplemented with 0.5 and 0.1 mg/l concentration of TDZ.

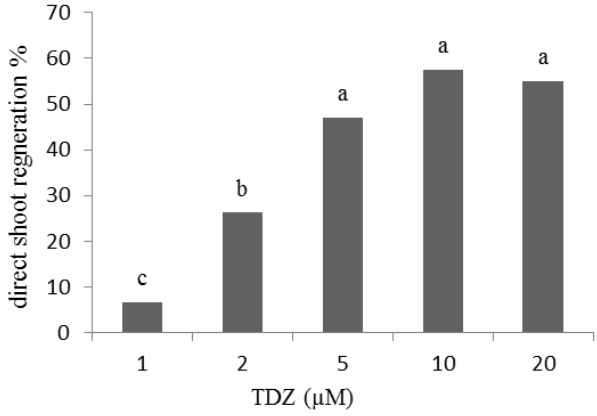

Figure 2. Mean comparisons of TDZ concentration on direct shoot regeneration in cumin (*Cuminum cyminum* L.).

Indirect shoot regeneration

Analysis of variance showed significant effect of accession, NAA, BAP and NAA× BAP interactions on B5 medium (Fig. 3) on indirect shoot regeneration ($p<0.01$) (Table 2).

Mean comparisons of indirect shoot regeneration percentage of cumin accessions showed that Shahdad and Afghanistan accessions with an average of 14.38% and 13.75% respectively were the highest percentage of indirect shoot

Table 2. Analysis of variance for indirect shoot regeneration percentage in cumin (*Cuminum cyminum* L.).

Source of variation	degree of freedom	Mean squares
Accession	3	2.03**
NAA	1	37.30**
BAP	3	88.21**
Accession × NAA	3	0.08 ns
Accession × BAP	9	0.42 ns
NAA× BAP	3	10.70**
Accession × NAA × BAP	9	0.56 ns
Error	64	0.34
CV%	19.43 %	

<ns> and <**> means non-significant and Significant at 0.01 level of probability, respectively

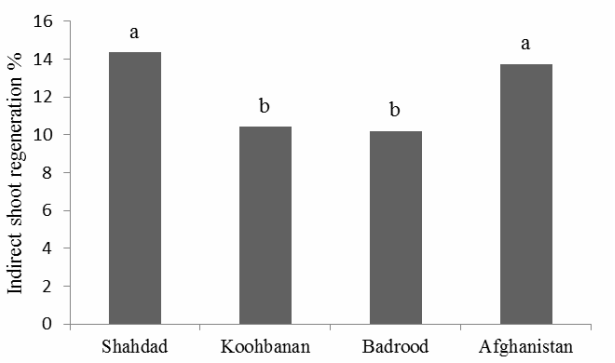

Figure 4. Mean comparisons of indirect shoot regeneration percentage of cumin (*Cuminum cyminum* L.) accessions under NAA and PAP treatments.

regeneration (Fig. 4).

Since, effects of NAA and BAP solely were not correct for consideration, the combination of these PGRs (i.e. NAA× BAP) had been considered.

Eight different combinations of NAA plus BAP were compared for their responses to shoot regeneration. It shows that B5 medium supplemented with 0.1 mg/l NAA+0.4 mg/l BAP, 0.1 mg/l NAA+1 mg/l BAP or 0.4 mg/l NAA+1 mg/l BAP with the mean of 25.8%, 25.0% and 22.0% respectively produced high frequency of shoot regeneration (Fig. 5).

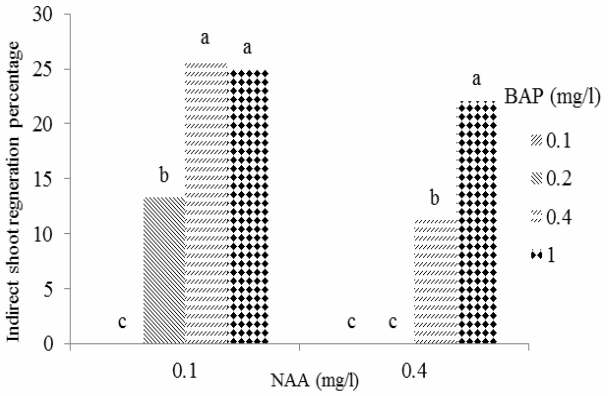

Figure 5. Mean comparisons of BAP levels in each level oe NAA on indirect shoot regeneration percentage in cumin (*Cuminum cyminum* L.).

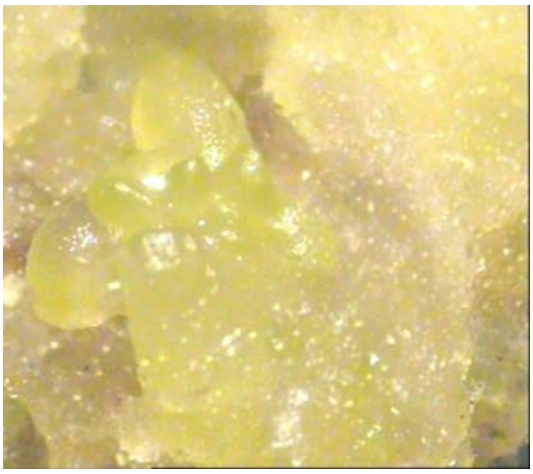

Figure 3. Indirect shoot regeneration on B5 medium suplemented with 0.1 mg/l NAA+0.4 mg/l BAP in cumin (*Cuminum cyminum* L.).

Somatic embryogenesis

Somatic embryos differentiated 14 days after transferring calli into free MS medium without any PGRs (Fig. 6). Tawfik and Noga (2002) obtained cumin somatic embryos in 7 days after transferring the cell suspension into liquid medium lacking plant growth regulators.

Dave and Batra (1995) inoculated different cumin explants (root, hypocotyl and cotyledon) on MS medium inoculated with 8 mg/l of BA. They were the first that studied the somatic embryogenesis of cumin and reported that hypocotyl was found to be the source to somatic embryos. This experiment supports the need of cytokinin for somatic embryogenesis.

Analysis of variance for number of somatic embryos in cumin showed high significant effect of 2,4-D on this trait (Table 3). This table also showed that the various organs (i.e. root, shoot, leaf and mature embryo) and accessions were produced somatic embryos.

Higher frequencies of somatic embryo number resulted in higher concentrations of 2,4-D that used for callus induction as from 0.5 up to 2 mg/l 2,4-D the number of somatic embryos were increase from 11.68 up to 16.29 (Fig. 7). This shows that the high effect of 2,4-D on somatic embryogenesis of *Cuminum cyminum* L. medicinal plant.

Furthermore, high number of somatic embryo production potential in each callus segment, makes somatic embryogenesis for high plant production in time.

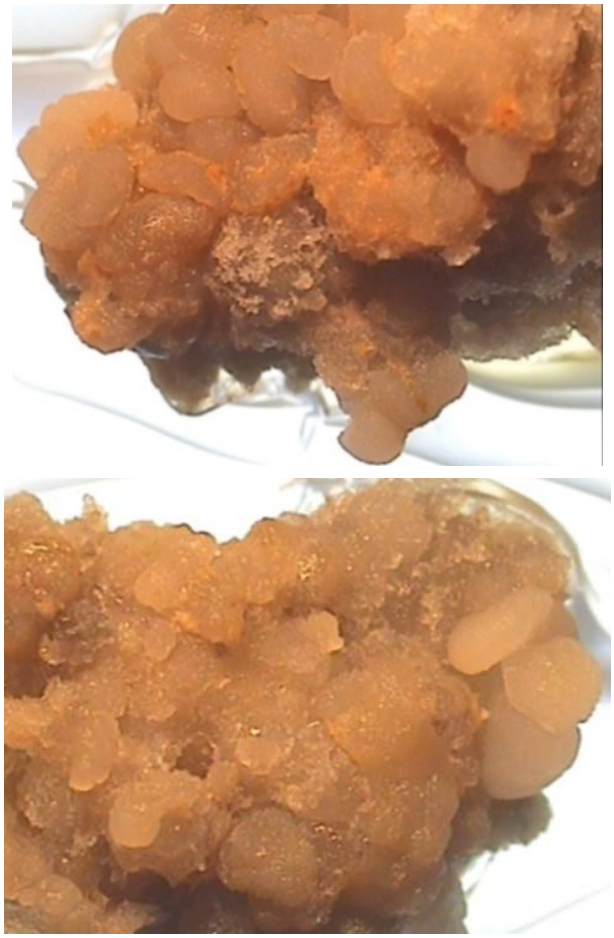

Figure 6. Some globular somatic embryos of cumin (*Cuminum cyminum* L.) that formed on calli segments after transferred to free MS basal medium.

Table 3. Analysis of variance for number of somatic embryos in cumin (*Cuminum cyminum* L.)

Source of variation	degree of freedom	Mean squares
Accession	3	4.76 ^{ns}
2,4-D	2	254.67**
Explant	3	50.83 ^{ns}
Accession× 2,4-D	6	18.82 ^{ns}
Accession× Explant	9	7.07 ^{ns}
2,4-D × Explant	6	32.98 ^{ns}
Accession×2,4-D×Explant	18	7.16 ^{ns}
Error	96	19.85
Coefficient of Variation	31.95 %	

<ns> and <**> means non-significant and Significant at 0.01 level of probability, respectively.

Although induction and regeneration of direct pathways (i.e. direct shoot organogenesis and direct somatic embryogenesis) were more frequent and rapid than indirect pathways such as organogenesis and somatic embryogenesis through callus (Ebrahimie et al. 2007), but sometimes indirect pathways are useful in biological researches (such as transferring of target genes to somatic embryos or calli segments). Thus we have to study these pathways carefully.

The cumin accessions of this study had been considered in some aspects such as SDS-PAGE and molecular markers

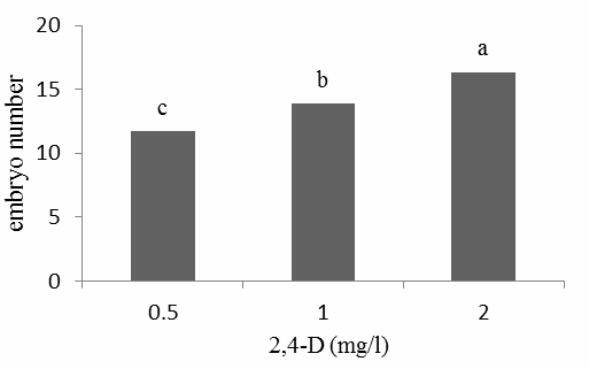

Figure 7. Mean comparisons of 2,4-D concentrations (that used for callus induction) on number of somatic embryos of cumin (*Cuminum cyminum* L.)

in prior studies (Masoumi et al. 2012, Rostami-Ahmadvandi et al. 2013), but observed no logical relationship between those results.

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