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The Effects of Photoperiod and 2,4-D Concentrations on Callus Induction of *Cuminum cyminum* Leaf Explant: An Important Medicinal Plant

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ABSTRACT

Plant tissue culture setting up is one of the most important stages for modern plant breeding. Availability of a tissue culture protocol for genetic transformation toward resistance to diseases would be an important step for the establishment of a genetic transformation system in most plants. In current research, callus induction was investigated in *Cuminum cyminum*. The experiment was arranged in a factorial experiment with two factors. The first factor was 2,4-Dichlorophenoxyacetic acid (2,4-D) plant growth regulator in four levels (0, 0.5, 1 and 2 mg LG^{1} plus 0.1 mg LG^{1} kinetin) in the MS basal medium. The second factor was photoperiodic status in two levels (dark and 16/8 h light/dark). The experiment was layed out based on completely randomized design with three replications. Statistical analysis results showed that the highest percentage of callus induction was resulted at 1.0 mg LG^{1} 2,4-D in both photoperiod conditions. Interaction effect of photoperiod×2,4-D concentrations showed significant (p#0.05) difference of callus induction of cumin. It is concluded that 1 mg LG^{1} 2,4-D treatment showed the most efficient influence on leaf explants callus induction.

Key words: Leaf explant, cumin, callus induction, kinetin

INTRODUCTION

Cumin (*Cuminum cyminum* L.) is a member of the Apiaceae family and one of the oldest and economically most important spices. Recent studies have indicated pharmaceutical, medicinal importance and antimicrobial effect of its oil extract (Tawfik and Noga, 2002). This plant prefer hot and tropical climate for growth, mainly in India, China, Iran and southern Egypt but can also be cultivated in cooler region under greenhouse (Tawfik and Noga, 2002). Cumin production has increases manifolds in recent decades in regions of Iran (Azizi and Kahrizi, 2008).

Cumin stands second after pepper due to diverse applications. Seed of this plant is good source for iron and manganese (Singh *et al*., 2010). Cumin seeds are precious for their typical popular aroma because to an aromatic alcohol (aminol) and spicy taste (Deshwal and Kumari, 2012). The plant can be development on every soil type also due to tap roots is a drought resistant plant (Koocheki *et al*., 2008; Singh *et al*., 2010).

Breeding via classical methods in cumin has some limitation due to laborious and time consuming of these methods (Ebrahimie *et al*., 2007a). Consequently plant genetic engineering

may offer an efficient new approach to improve the secondary metabolites of cumin for the medicinal industry and to overcome production problems, especially diseases (Heath-Pagliuso and Rappaport, 1990; Tawfik and Noga, 2001, 2002).

There is one study on genetic transformation of cumin through direct gene transfer using microprojectile bombardment (Singh *et al*., 2010), while *Agrobacterium*-mediated genetic transformation is not reported in cumin so far.

Availability of a tissue culture protocol for genetic transformation toward resistance to diseases would be an important step for the establishment of a genetic transformation system in this plant (Tawfik and Noga, 2001).

For genetic improvement of plants, setting up well a tissue culture protocol in utilize the advantage of cell and tissue culture is an essential prerequisite (Ashakiran *et al*., 2011).

Up to now, several researches have been conducted on callus induction of cumin (Tawfik and Noga, 2001, 2002; Ebrahimie *et al*., 2003, 2007a, b; Valizadeh *et al*., 2007) but study on effect of photoperiod on callus induction of leaf explant has not been reported or is very limited or is very limited so far.

Ultimate goals of this study was the development of an efficient callus culture system to use in gene transfer methodology especially to study a protocol for callus induction in cumin with 2,4-D plus kinetin in optimum photoperiodic conditions.

MATERIALS AND METHODS

For reach to the purpose of this research an experiment was conducted in Medicinal Plants Tissue Culture Lab., Agriculture and Natural Resources Campus, Razi University in 2012.

Plant material: The cumin seeds were collected from Shahdad region (57°42'N-30°25'E) in Kerman state of Iran. The seeds were surface disinfested by stirring in 2% (w/v) sodium hypochlorite for 15 min and were rinsed three times with sterile distilled water under aseptic conditions. The sterilized seeds were cultured in baby food jars (200 mL) containing 50 mL MS (Murashige and Skoog, 1962) hormone-free medium supplemented with 30 g L^{-1} sucrose and 8 g L^{-1} agar. The pH of the all media was adjusted to 5.7 prior to autoclaving at 120°C and 1.2 kg cm^{-2} for 20 min.

Callus induction: The experiment was carried out as factorial using a completely randomized design with two factors and three replications (as Petri dishes).

Investigated factors included:

- **H:** 2,4-D Hormone: 0, 0.5, 1 and 2 mg L^{-1}
- **P:** Photoperiodic status: 16 h light/8 h dark and 24 h dark

When the seedlings were about 4-6 cm height (1 month after seed culture), leaf segments (about 8 mm long) was excised and cultured horizontally on MS medium supplemented with 0.1 mg L^{-1} Kinetin plus different concentrations of 2,4-D. Five explant segments were cultured in each petri dish containing 25 mL MS basal medium then sealed with parafilm. After four weeks in culture each frond was scored for callus induction. MS media supplemented with 0, 0.5, 1 or 2 g L^{-1} 2,4-D plus 0.1 mg L^{-1} Kinetin, were placed at 24°C under the standard 16 h light photoperiod or darkness (by wrapping the plates in aluminum foil). The experiment was replicated three times

giving a total of 15 observations per treatment. The frequency of callus induction per each treatment was calculated by dividing the number of calli to the original number of plated explants.

Experimental cultures for photoperiod conditions were incubated at 23°C under a 16 h light photoperiod of approximately 40 µmol mG^{2} secG^{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. All tests for significance were conducted at " = 0.05 level. Mean separation was performed using Duncan's Multiple Range test at 0.05 probability level after ANOVA.

Because of the non-normally distributed data for callus induction, a data transformation was performed using the following Eq. 1:

$$Y = (C+0.5)^{0.5} \quad (1)$$

where, C is the callus induction percentage and Y is the result of the data transformation.

All statistical analyses were done using SPSS Ver. 19 and SAS Ver. 9.1 software.

RESULTS

Callus production was significantly influenced by concentration of 2,4-D and photoperiodic status. Callus induction was not observed on MS medium without any Plant Growth Regulator (PGR) in this study. This shows that 2,4-D is critical for callus induction of cumin (Table 2).

In this study the highest percentage of callus induction in both photoperiodic status were observed at 1 mg LG^{1} 2,4-D. This suggests that the rate of calli are induced depends on the concentration of 2,4-D. The results showed that there were significant effects for interaction effects of photoperiod and 2,4-D concentrations on callus induction of cumin (Table 1). Effects of 0.5 and 2 mg LG^{1} 2,4-D concentrations on callus induction percentages were significant in two photoperiod conditions (Table 2). Callus induction percentage in 0.5 mg LG^{1} 2,4-D concentration in darkness conditions (47%) was more than photoperiod one (0%). But in 2 mg LG^{1} 2,4-D concentration application in photoperiod conditions (80%) was superior to darkness status (40%) (Table 2). This result supports the interaction effect of 2,4-D concentrations and photoperiod conditions in leaf explant.

Table 1: Analysis of variance for the effects of photoperiod, 2,4-D and explant on percentage of the callus induction in cumin

Source of variation	df	Mean square
Photoperiod (P)	1	0.88^{ns}
2,4-D Hormone (H)	3	89.31**
P×H	3	19.17*
Error	16	4.98

ns: Non-significant, *,**Significant at 5 and 1% levels, respectively

Table 2: Mean comparison for the callus induction percentage of leaf explant in different concentrations of 2,4-D in cumin

	Callus induction	
2,4-D (mg LG^{1})	Photoperiod	Darkness
0	0^{c}	0^{c}
0.5	0^{c}	47^{b}
1	87^{a}	87^{a}
2	80^{a}	40^{b}

Means followed by different letters are significantly different at p<0.05 using Duncan's multiple range test

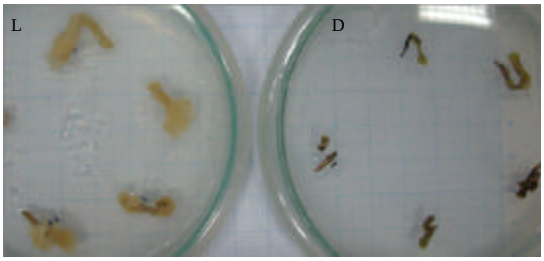

Fig. 1: Effect of photoperiod on callus induction in leaf explant on MS medium supplemented with 0.5 mg LG^{1} 2,4-D and 0.1 mg LG^{1} kinetin grown in 16/8 h L/D photoperiod (L) and darkness (D)

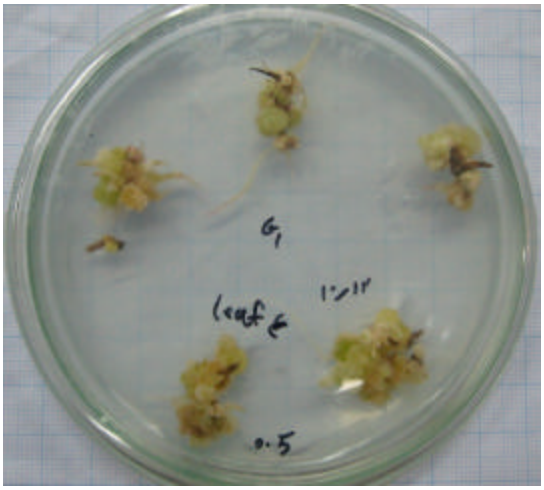

Fig. 2: Organogenic callus derived from cumin leaf explant on light conditions

Callus was induced at 4 weeks after incubation of leaf explant. Callus induction began from the cut ends of leaf explants then extended to the other parts (Fig. 1).

The highest percentage of callus induction was realized at 1 mg LG^{1} 2,4-D in either photoperiod conditions.

Although callus induction percentage in some treatments was not different in both photoperiod and darkness conditions but produced calli in darkness condition has more qualities (Fig. 1). Moreover darkness conditions produced more favorite and embryogenic calli.

The calli that are resulted from leaf explant in photoperiod conditions were green, compact and more rhizogenic (Fig. 2) that are not practical for cell suspension culture, callus sub-culturing or somatic embryogenesis researches.

DISCUSSION

The results of current experiment is in agreement with (Ebrahimie *et al*., 2007b) that reported callus formation started from the cut ends of embryo explants and extended to the other parts.

Effects of 0.5 and also 2 mg LG^1 2,4-D in two photoperiodic status on callus induction percentages in leaf explant were significant. This is disagreement with Ebrahimie *et al*. (2007a) that reported the majority of *in vitro* morphogenesis pathways derived from different cumin embryo explants were light-independent at the investigated light regimes. This disagreement maybe is due to the different types of explants (leaf and embryo) in these studies. In confirmation with this, result of this study showed that photoperiod conditions not affected on shoot and root explants callus induction (data not shown).

In agreement with present study, leaf explant of cumin produced friable callus on B_5 medium supplemented with 0.5 to 2 mg LG^1 2,4-D under 16/8 h of light/dark (Jha *et al*., 1983) but Tawfik (1998) reported that segments of leaf (3-5 mm long) dried and callus was not produced on MS medium supplemented with kinetin and/or 2,4-D concentrations. The reason for this contrast maybe is the status of culture such as explant long. Tawfik and Noga (2002) also induced callus production from primary leaf segment of cumin on a medium with 4 µM 2,4-D alone or plus 2 or 4 µM kinetin.

Among three explants that (Ebrahimie *et al*., 2007b) considered, the third explant type which passed the pre-culture period (older than other explants), produced much extent greater callus. This shows that when an explant becomes older, the probability of its callus production increases. This result maybe demonstrations older explant such as leaf segments produced better callus.

The results indicated that 2,4-D significantly affected callus induction of cumin. This callus production method may be useful in both particle bombardment and Agrobacterium-mediated genetic transformation approaches. The protocol was optimized by manipulations of different 2,4-D concentration and tow photoperiod conditions for enhanced callus production of cumin. Recommended protocol for callus induction of cumin might be applied to produce secondary metabolites, pharmaceutical industry and genetic transform to callus (Valizadeh *et al*., 2007).

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