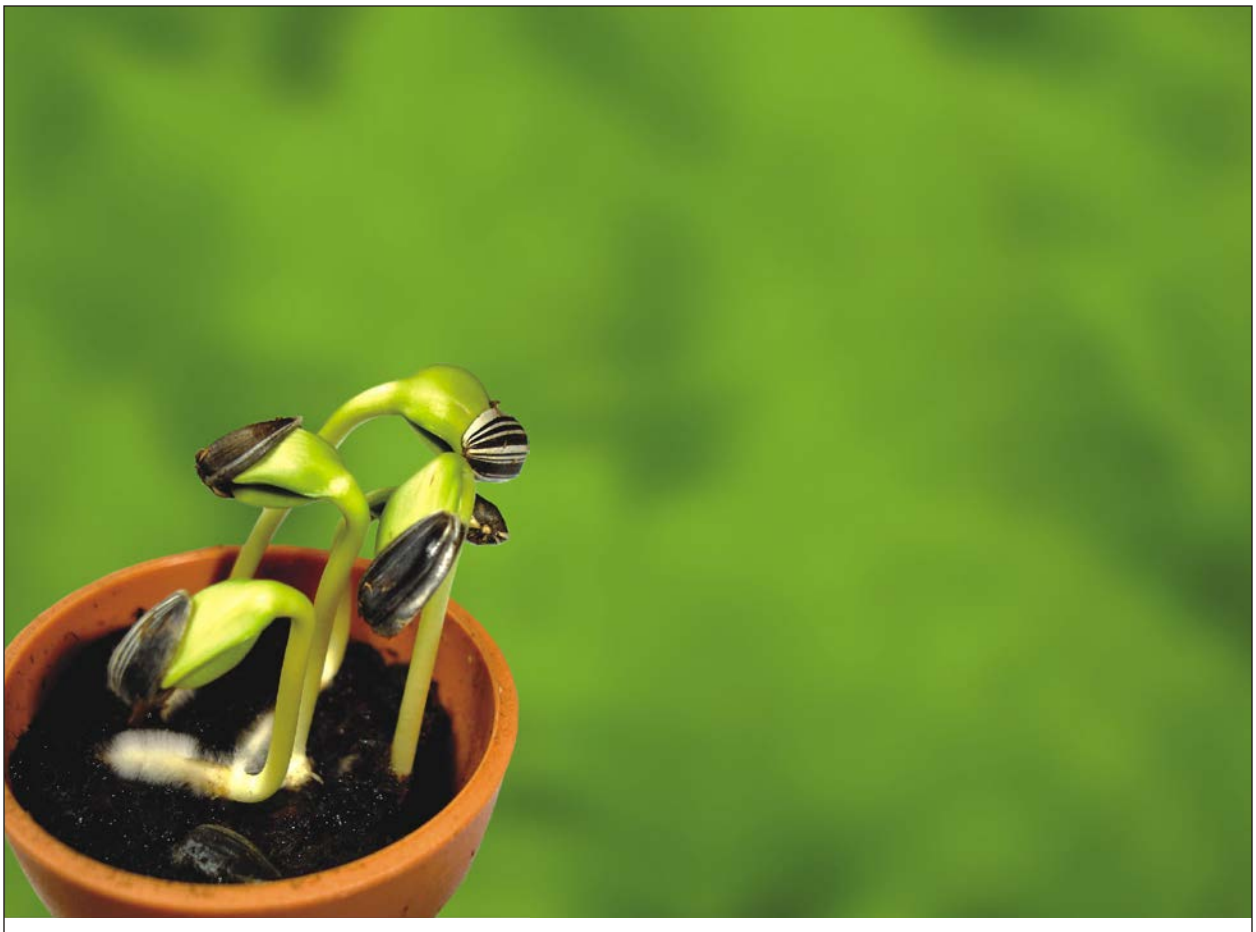

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A Novel Method for Regeneration of Plantlets from Embryo Explants of *Bunium persicum* (Boiss.) B. Fedtsch

[1]M. Valizadeh, [2]S.K. Kazemi Tabar and [3]G.A. Nematzadeh
[1]Department of Plant Breeding, [2,3]Department of Plant Breeding and Agronomy,
Faculty of Agriculture, Mazandaran University, Iran

Abstract: A new simple method was developed for regeneration of Parsi Zira. This method yielded a large number of shoots within short period of time (30-40 days) without any subculturing. The effect of various combinations of Plant Growth Regulators (PGRs) on callus formation and shoot regeneration were investigated on B5 medium. Simultaneous callus and root formation and shoot regeneration were obtained. The experiment was conducted in a completely randomized design with 30 treatments and 10 replications per treatment. The best treatments for callus induction were the media containing 0.1 mg L^{-1} 2,4-D and 2 mg L^{-1} Kin or 1 mg L^{-1} NAA and 2 mg L^{-1} Kin. The best treatment for regeneration was the medium supplemented with 0.1 mg L^{-1} NAA and 4 mg L^{-1} Kin. The treatment containing 0.1 mg L^{-1} NAA and 4 mg L^{-1} Kin was the best treatment for callus and root induction and regeneration simultaneously. The highest somatic embryogenesis was observed on the medium containing 2 mg L^{-1} 2,4-D.

Key words: *Bunium persicum* (Boiss.) B. Fedtsch., embryo explant, regeneration, tissue culture

INTRODUCTION

Parsi Zira, *Bunium persicum* (Boiss.) B. Fedtsch., is a native plant of limited zones of the west asia and grows in northern areas of Khorasan, Kerman, east of Zagros to Bandar Abbas and south of Alborz in Iran. Production of this plant is limited due to seed dormancy and several biotic stresses of which wilt diseases are the most serious. Only cold treatments are effective in seed germination. Other treatments such as giberellic acid, cytokinin, potassium nitrate, washing and light treatments are not useful (Bonianpoor, 1995).

Generally *Umbeliferae* species including *Bunium persicum* have antimicrobial properties (Shetty *et al*., 1994). Potential genetic variability for conventional breeding is limited in *Bunium persicum* (Hunault *et al*., 1989). Genetic transformation may enable the development of transgenic plants with enhanced resistance to wilt diseases. However, an efficient micropropagation system with high regeneration frequency is required.

Wakhlu *et al*. (1990) obtained callus from mericarps of *Bunium persicum* on MS (Murashige and Skoog, 1962) medium supplemented with 2 mg L^{-1} 2,4-D and 4 mg L^{-1} Kin. In this report, small white clumps of compactly packed cells developed on the callus on a medium containing 1.0mg L^{-1} 2,4-D and 0 mg L^{-1} Kin. These cell clumps differentiated into numerous globular embryos on the same medium. Embryo maturation was achieved on the basal as well as on 1 mg L^{-1} Kin supplemented medium.

Sharifi (1995) used hypocotyl and cotyledon explants in *Bunium persicum* tissue culture. The callus growth was faster on B5 (Gamborg *et al*., 1968) medium containing 2 mg L^{-1} NAA and

Corresponding Author: S.K. Kazemi Tabar, Department of Plant Breeding and Agronomy, Faculty of Agriculture, Mazandaran University, Iran

2 mg L^{-1} Kin. Plumule and shoot formation from hypocotyls and somatic embryogenesis were higher on medium supplemented with 0.1 mg L^{-1} NAA and 2 mg L^{-1} Kin and MS medium containing 0.5 mg L^{-1} 2,4-D, respectively.

Ebrahimi *et al*. (2003) used embryo explants for cumin tissue culture yielding a large number of shoots within short period of time without any subculturing. In this report, the best treatments were B5 medium containing 0.2 mg L^{-1} IAA and 1 mg L^{-1} BAP or 0.2 mg L^{-1} NAA and 0.2 mg L^{-1} BAP.

To obtain plant material in reported methods for Parsi Zira regeneration, seeds have to be germinated *in vitro* and then hypocotyls and cotyledon leaflets of seedlings used as explant. The reported methods were also laborious and time consuming due to the long callus phase which is associated with using hypocotyl and cotyledon leaflet as explants. Plant regeneration from explants in a short period of time bypassing a long intermediate callus phase could reduce somaclonal variation (Skirvin *et al*., 1994). Availability of a tissue culture protocol with such criteria would be an important step towards the establishment of a genetic transformation system in this species. The objective of this study was to determine the possibility of using younger explants like embryo for simultaneous callus and root formation and shoot regeneration without any subculturing and to optimize growth regulators for increasing regeneration efficiency and reducing the time.

MATERIALS AND METHODS

Parsi Zira seeds were collected from Kalat at Khorasan province in Iran. Seeds were surface sterilized in 1.5% (w/v) sodium hypochlorite solution for 15 min and rinsed three times with sterile distilled water. Seeds were then soaked for 20 days in sterile distilled water at 4°C in the dark. Embryos were extracted from the turgid seeds by cutting the end of the seeds and pressing the middle of them. The embryos were cut and only the hypocotyl were used. B5 (Gamborg *et al*., 1968) medium containing full-strength of macro-and micro-elements, vitamins and sucrose (30 g L^{-1}) were used. The different plant growth regulators, NAA (0.1, 1 and 2 mg L^{-1}), 2,4-D (0.1, 1 and 2 mg L^{-1}) only or together with Kin (0, 0.5, 1, 2 and 4 mg L^{-1}) were added to the medium.

Prior to autoclaving at 121°C for 15 min pH was adjusted to 5.7. The medium were solidified with 8% (w/v) agar (Sigma). The explants were cultured (one explant per each dish or replication) in sterile dishes (7×12 mm) each containing 15 mL of culture medium, sealed with Parafilm and maintained at 25±2°C under 16 h photoperiod (30 μmoles m^{-2} s^{-1}). After eight weeks, the number of explants producing callus and regeneration and the number of shoots regenerated from each replication were counted. Then the frequency of callus induction and shoot regeneration were calculated by dividing the number of calli and shoots to the original number of plated explants. The experiment was conducted in a completely randomized design with 30 treatments and 10 replications per treatment. Mean of replications was used for statistical analysis.

ANOVA was used to analyze the frequency of callus formation and shoot regeneration. Mean separation was conducted using Duncan's Multiple Range test at 0.05 probability.

RESULTS

Callus initiation and regeneration (Fig. 1) were observed on the medium 7 and 20 days after explant transfer to medium, respectively. A great yellow callus was observed after 3 weeks (Fig. 2). The results showed significant effects of treatments on regeneration and callus induction (Table 2). Due to PGR treatments the size of callus was different. Generally, the amount of callus in treatments without cytokinin was lower. The best treatments for callus induction were the media containing

Table 1a: The effect of PGRs on the frequency of shoot regeneration, root induction and shoot, root mean number on B5 and MS media

PGRs Treatments (mg L^{-1})	Shoot mean number	Root mean number	Root induction frequency	Callus induction frequency	Somatic embryogenesis frequency	Shoot regeneration frequency
0.1 NAA	0b	0.17d	0.08d	0.58a-e	0.17bc	0b
0.1NAA+0.5KIN	0.17b	1.92a-d	0.42abc	0.75a-d	0.5ab	0.17ab
0.1 NAA+1KIN	0.40b	0.90bcd	0.20dc	0.5b-e	0.3abc	0.20ab
0.1 NAA+2KIN	0b	0.73dc	0.18dc	0.45b-e	0.1c	0b
0.1 NAA+4KIN	4.10a	1.50a-d	0.50abc	0.7a-e	0c	0.30ab
1NAA	0.75b	4.33a	0.25bdc	0.75a-d	0.25abc	0.25ab
1NAA+0.5KIN	0.36b	3.55abc	0.45abc	0.73a-e	0.1c	0.09ab
1NAA+1KIN	0.67b	1.67a-d	0.44abc	0.78a-d	0c	0.22ab
1NAA+2KIN	0b	3.40a	0.60a	1a	0.2bc	0b
1NAA+4KIN	0.36b	2.82a-d	0.55ab	1a	0c	0.18ab
2NAA	0.64b	3.73ab	0.45abc	0.91abc	0c	0.27ab
2NAA+0.5KIN	0b	0d	0d	0.44cde	0.33abc	0b
2NAA+1KIN	0.70b	2.40a-d	0.50abc	0.8abc	0c	0.30ab
2NAA+2KIN	0.92b	1.92a-d	0.25bdc	0.92ab	0c	0.42a
2NAA+4KIN	1.33b	0d	0d	0.56a-e	0.22bc	0.11ab

Different letter(s) within each column indicate significant differences

Table 1b: The effect of PGRs on the frequency of shoot regeneration, root induction and shoot, root mean number on B5 and MS media

PGRs Treatments (mg L^{-1})	Shoot mean number	Root mean number	Root induction frequency	Callus induction frequency	Somatic embryogenesis frequency	Shoot regeneration frequency
0.1 2,4-D	0.82b	0.36d	0.09d	0.73a-e	0.36abc	0.27ab
0.1 2,4-D+0.5KIN	0b	0d	0d	0.33de	0.11c	0b
0.1 2,4-D+1KIN	0.91b	0d	0d	0.73a-e	0.1c	0.27ab
0.1 2,4-D+2KIN	1.18b	0d	0d	1a	0.1c	0.36a
0.1 2,4-D+4KIN	0.09b	0d	0d	0.27e	0c	0.09ab
1 2,4-D	0b	0d	0d	0.67a-e	0.25abc	0b
1 2,4-D+0.5KIN	0b	0d	0d	0.9abc	0.2bc	0b
1 2,4-D+1KIN	0b	0d	0d	0.73a-d	0.07c	0b
1 2,4-D+2KIN	0.55b	0d	0d	0.91abc	0.1c	0.18ab
1 2,4-D+4KIN	0b	0d	0d	0.75a-d	0.17bc	0b
2 2,4-D	0b	0d	0d	0.9abc	0.6a	0b
2 2,4-D+0.5KIN	0b	0d	0d	0.44cde	0c	0b
2 2,4-D +1KIN	0b	0d	0d	0.44cde	0.33abc	0b
2 2,4-D +2KIN	0b	0d	0d	0.8abc	0.1c	0b
2 2,4-D +4KIN	0.14b	0d	0d	0.57a-e	0.14bc	0.14ab

Different letter(s) within each column indicate significant differences

Table 2: Variance analyse of somatic embryogenesis, shoot and root induction frequency, shoot and root mean number

Source of variation	Degrees of freedom	Shoot regeneration frequency	Root induction frequency	Somatic embryo frequency	Root mean number	Shoot mean number	Callus induction frequency
Treatment	29	0.19*	0.48**	0.25**	22.63**	6.42**	0.42**
Error	287	0.10	0.11	0.12	8.54	2.80	0.19

*Significant difference at 0.05 probability level; ** Significant difference at 0.01 probability level

0.1 mg L^{-1} 2,4-D and 2 mg L^{-1} Kin or 1 mg L^{-1} NAA and 2 mg L^{-1} Kin (Table 1). The best treatment for regeneration was the medium supplemented with 0.1 mg L^{-1} NAA and 4 mg L^{-1} Kin (Table 1). In this treatment, it was counted to 20 plantlets in one of replications. The treatment containing 0.1 mg L^{-1} NAA and 4 mg L^{-1} Kin was the best treatment for callus and root induction and regeneration simultaneously (Table 1). The highest root induction was observed on treatments supplemented with 1 mg L^{-1} NAA or 1 mg L^{-1} NAA and 2 mg L^{-1} Kin. The highest somatic embryogenesis was observed on the medium containing 2 mg L^{-1} 2,4-D (Table 1a, b).

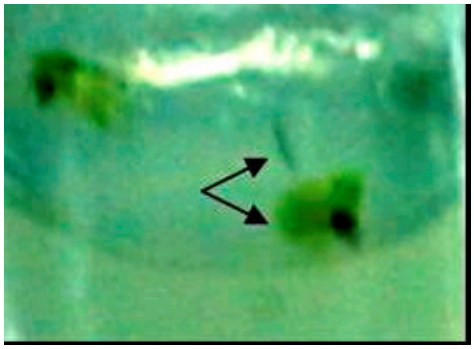

Fig.1: Early regeneration from embryo explant after 20 days

Fig. 2: Great yellow callus formation after 3 weeks

Fig. 3: Somatic embryo growth after transfer to medium without PGRs

Somatic embryo growth and stem elongation occurred after transfer to medium without PGRs (Fig. 3). In some treatments, simultaneous regeneration, root and callus induction occurred in the same

medium. The above results were obtained eight weeks after explant transfer to media. The number of regenerated plantlets per explant can be significantly increased by subculturing the regenerated calli.

DISCUSSION

Somatic embryogenesis during callus induction is not unusual in *Umbeliferae* family specially carrot and fennel (Hunault *et al*., 1989). 2,4-D and NAA only or in combination with Kin are essential for continuity of callus induction. Auxin reduction leads to organogenesis and adventitious embryo formation in Parsi Zira. Regeneration occurred in some treatments without kinetin, showing that kinetin is not essential for Parsi Zira regeneration like other *Umbeliferae* species such as carrot and fennel (Hunault *et al*., 1989), but it is essential for cumin regeneration (Ebrahimie *et al*., 2003). The previous studies suggest that callus transfer to media supplemented with 1 mg L^{-1} 2,4-D (Wakhlu 1990) or 0.5 mg L^{-1} 2,4-D (Bonianpoor, 1995) leads to embryogenesis. However suitable combination of auxins and cytokinins are important for embryogenesis and organogenesis (Guohua, 1998). In some other species, induced somatic embryos might need a little cytokinin or other plant growth regulators to grow (Kumar *et al*., 1988). The method which has been practiced here revealed some advantages in comparison with others including higher regeneration frequency and regenerated plantlet number, simultaneous regeneration, root and callus induction in the same medium, shortening tissue culture time, less infection, no subculturing and less chemical consumption. The higher frequency of shoot regeneration from embryo explants compared with previously reports (hypocotyl segments) may be explained by the fact that the former explants are too old to respond to growth regulators rapidly and efficiently, but the later is younger and more sensitive, responding more quickly to PGRs. Embryo culture has been used for olive and iris (Canas *et al*., 1992). In other plant species, embryo explants have been used for micropropagation and genetic transformation (Yong *et al*., 1999).

This method yielded a large number of shoots within short period of time (30-40 days) without any subculturing. The total period is 50-60 days, considering cold treatment. Previously reported methods are consisted of *in vitro* seed germination, obtaining seedling, cutting hypocotyls and culturing hypocotyls in callus formation medium and transferring callus to regeneration medium. These processes are time consuming (210 days considering 90 day-cold treatment) and laborious to be used as an efficient and economical micropropagation system. The above results show the importance of explant type. The presented method may also be useful for future studies on other spices like *Cuminum setifulium*. As mentioned above, Parsi Zira production are highly limited because of seed dormancy and fungal diseases. By application of this method, a large number of desirable and pathogene free genotypes can be propagated. A high frequency of plantlet regeneration increases the chance of obtaining transformed plant on genetic transformation procedures and short tissue culture time is helpful in reducing the time and cost. This method could also be used to produce essence and raw materials for pharmaceutical industry.

Abbreviations

B5-Gamborg *et al*. (1968) medium; BAP-6-benzylaminopurine; 2,4-D-2,4-dichlorophenoxyacetic acid; IAA-indol-3-acetic acid; Kin-kinetin; MS-Murashige and Skoog (1962); NAA-α-Naphthaleneacetic acid; PGRs-plant growth regulators

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