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Organogenesis in anise (*Pimpinella anisum* L.)

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Abstract

High frequency organogenesis was achieved in anise (*Pimpinella anisum* L.) using hypocotyl explants from *in vitro* germinated seedlings. Both cytokinins, benzylaminopurine and kinetin induced shoot regeneration but the effect of benzylaminopurine was more pronounced. High frequency shoot regeneration (45 shoots explant^{-1}) was obtained on benzylaminopurine 1 mg l^{-1}. Both cytokinins were also tested in combination with auxins (naphthaleneacetic acid/ indoleacetic acid). Interaction of benzylaminopurine/kinetin with naphthaleneacetic acid/ indoleacetic acid increased the length of regenerated shoots. Different kinds of callus morphology was observed but it had no relationship with regeneration potential. The regenerated plants were normal and healthy.

Keywords: callus, organogenesis, shoot regeneration

Abbreviations: BAP=Benzylaminopurine; IAA=Indoleacetic acid; KN=Kinetin; NAA=Naphthaleneacetic acid

Application of tissue culture techniques in improvement of anise (*Pimpinella anisum* L.) (Apiaceae) is limited. A reproducible and highly regenerating *in vitro* protocol is a pre- requisite before a tissue culture system is used in crop improvement programmes for gene insertion and expression. Limited information is available on response of somatic tissues to differentiate and develop plantlets *in vitro* from callus culture of anise. There are some protocols of regeneration in anise using various explants (root, shoot apics, seeds, leaf) and plant growth regulators (Chand *et al*. 1997; Bela & Shetty 1999; Eguchi *et al*. 1998; Ernst & Oesterhelt 1984). The present investigation was undertaken with the objective of realizing the regeneration ability of hypocotyl explants from *in vitro* germinated seedlings of anise.

Clean and healthy seeds of anise were surface sterilized with 0.1% mercuric chloride for 4 min and thoroughly washed with sterilized distilled water 4–5 times. These surface sterilized seeds were placed in a sterilized test tube on a filter paper for germination in a BOD at 25^{o}C; the lower half of filter paper was immersed in distilled water. Hypocotyl segments from germinated seedlings (10–15 days old) were used as explants and inoculated on MS medium (Murashige & Skoog 1962) supplemented with various concentrations of BAP and KN (0.5–6.0 mg l^{-1}), NAA and IAA (0.1–1.0 mg l^{-1}) alone and in combination. Cultures were incubated

at 27±1°C under fluorescent light in a 14/10 h photo period. Light intensity was maintained at 35 μmol $M^{-2}S^{-1}$ at bench level. Observations were recorded periodically on number of explants inoculated and responded, morphology of callus, number of shoots $explant^{-1}$ and length of shoots.

At low concentration of BAP (0.5–2.0 mg l^{-1}), 100% of explants responded which was reduced to 83.3% at BAP 3.0 and 4.0 mg l^{-1} and up to 50% at BAP 6 mg l^{-1}. Callus induction took seven days in all the treatments with good amount of callus. Except minimum and maximum concentrations of BAP, where the callus was yellow and friable, all the other treatments produced green friable callus which was organogenic. A good number of shoots per explant was regenerated in all the treatments ranging from 15 to 45 shoots $explant^{-1}$ on BAP 6 and 1 mg l^{-1} respectively. The length of regenerated shoots ranged from 1.5 to 3.0 cm (Table 1).

Presence of KN alone in the medium in place of BAP elicited more or less similar response. All explants (100%) responded on MS medium supplemented with KN 1 and 2 mg l^{-1}. Number of shoots regenerated per explant ranged from 4 to 25 which was slightly less than treatments containing only BAP (Table 1).

NAA when supplemented in the medium at a higher concentration i.e., 1.0 mg l^{-1}, produced maximum response of cultures. White friable callus produced in most treatments was organogenic. All the treatments produced fairly good amount of shoots ranging from a minimum of eight at highest concentration of BAP and NAA (4 and 1 mg l^{-1}) to a maximum of 45 shoots $explant^{-1}$ at 0.5 mg l^{-1} each of BAP and NAA. Average length of regenerated shoot ranged from 1 to 4 cm. When NAA was replaced with IAA keeping the BAP concentration same, it exhibited slightly less pronounced response. Number of shoots $explant^{-1}$ ranged from 7 to 33 with no shoots in higher concentration of BAP and IAA 0.1–0.1 mg l^{-1}. Average shoot length was also less as compared to NAA (Table 2).

On replacement of BAP with kinetin, keeping the NAA or IAA as source of auxin, IAA proved to be more effective than NAA. Number of shoots $explant^{-1}$ was higher on KN plus low concentration of NAA (0.1 mg l^{-1}). As the concentration of NAA increased from 0.1 to 0.5 and 1.0 mg l^{-1}, number of shoots $explant^{-1}$ were drastically reduced. Almost 100% explants responded on combination of low concentration of both KN and IAA with very good quantity of callus. Each treatment

Table 1. Effect of cytokinins (BAP and KN) on regeneration in anise

MS medium + BAP/KN (mg l^{-1})	Response (%)		Days to callus induction		No. of shoots $explant^{-1}$		Length of shoot (cm)	
	BAP	KN	BAP	KN	BAP	KN	BAP	KN
0.50	100.00	33.33	7.00	7.00	17.00	4.00	1.50	0.50
1.00	100.00	100.00	7.00	7.00	45.00	18.00	2.50	1.50
2.00	100.00	100.00	7.00	7.00	22.00	22.00	2.00	5.00
3.00	83.33	66.66	7.00	7.00	30.00	25.00	2.00	2.00
4.00	83.33	50.00	7.00	7.00	20.00	20.00	1.50	1.00
5.00	66.66	33.33	7.00	7.00	16.00	15.00	2.00	1.50
6.00	50.00	83.33	7.00	7.00	15.00	16.00	3.00	3.00
Mean	83.33	66.66	7.00	7.00	23.57	17.14	2.07	2.07
SD (±)	17.82	26.73	0.00	0.00	9.93	6.24	0.49	1.40

BAP=Benzylaminopurine, KN=Kinetin

Table 2. Effect of cytokinins and auxins on regeneration in anise

MS medium + BAP/KN and IAA/NAA (mg l^{-1})	Response (%)				No. of shoots explant^{-1}				Length of shoot (cm)			
	BAP+ IAA (mg l^{-1})	BAP+N AA (mg l^{-1})	Kn + IAA (mg l^{-1})	Kn + NAA (mg l^{-1})	BAP + IAA (mg l^{-1})	BAP + NAA (mg l^{-1})	KN + IAA (mg l^{-1})	KN + NAA (mg l^{-1})	BAP + IAA (mg l^{-1})	BAP + NAA (mg l^{-1})	KN + IAA (mg l^{-1})	KN + NAA (mg l^{-1})
0.5 + 0.1	50.00	50.00	100.00	83.30	22.00	22.00	25.00	37.00	1.50	2.00	3.50	4.00
1.0 + 0.1	66.60	50.00	100.00	16.60	17.00	13.00	27.00	18.00	1.00	1.50	3.50	2.00
2.0 + 0.1	33.30	66.60	66.60	50.00	7.00	35.00	22.00	32.00	1.00	3.00	1.50	1.00
3.0 + 0.1	66.60	33.30	100.00	83.30	8.00	27.00	13.00	22.00	1.00	2.50	1.00	3.50
4.0 + 0.1	33.30	66.60	100.00	83.30	0.00	25.00	25.00	35.00	-	3.00	1.00	3.00
0.5 + 0.5	83.30	83.30	100.00	66.60	30.00	45.00	35.00	6.00	4.00	4.00	4.50	2.00
1.0 + 0.5	83.30	66.60	66.60	33.30	27.00	30.00	35.00	1.00	3.00	2.50	4.00	0.30
2.0 + 0.5	66.60	100.0	66.60	83.30	20.00	17.00	32.00	6.00	3.00	1.50	2.00	1.50
3.0 + 0.5	50.00	83.30	100.00	66.60	10.00	13.00	33.00	0.00	2.00	2.00	2.00	-
4.0 + 0.5	66.60	83.30	66.60	100.00	0.00	12.00	30.00	20.00	-	2.00	2.00	3.70
0.5 + 1.0	50.00	100.0	66.60	66.60	17.00	22.00	15.00	0.00	4.00	2.50	3.00	-
1.0 + 1.0	100.0	100.0	100.00	50.00	27.00	18.00	22.00	3.00	3.00	3.00	4.00	3.00
2.0 + 1.0	83.30	83.30	66.60	50.00	33.00	12.00	25.00	5.00	1.00	1.00	1.50	2.00
3.0 + 1.0	50.00	83.30	100.00	16.60	22.00	22.00	23.00	5.00	1.00	3.00	2.50	1.50
4.0 + 1.0	50.00	100.0	33.30	50.00	12.00	8.00	10.00	4.00	0.50	4.00	1.00	2.00
Mean	62.19	76.64	82.19	59.97	16.80	21.40	24.80	12.93	2.00	2.50	2.47	2.27
SD (±)	19.38	20.71	21.36	25.05	10.41	9.96	7.71	13.27	1.24	0.87	1.20	1.10

BAP=Benzylaminopurine; IAA=Indoleacetic acid; KN=Kinetin; NAA=Naphthaleneacetic acid

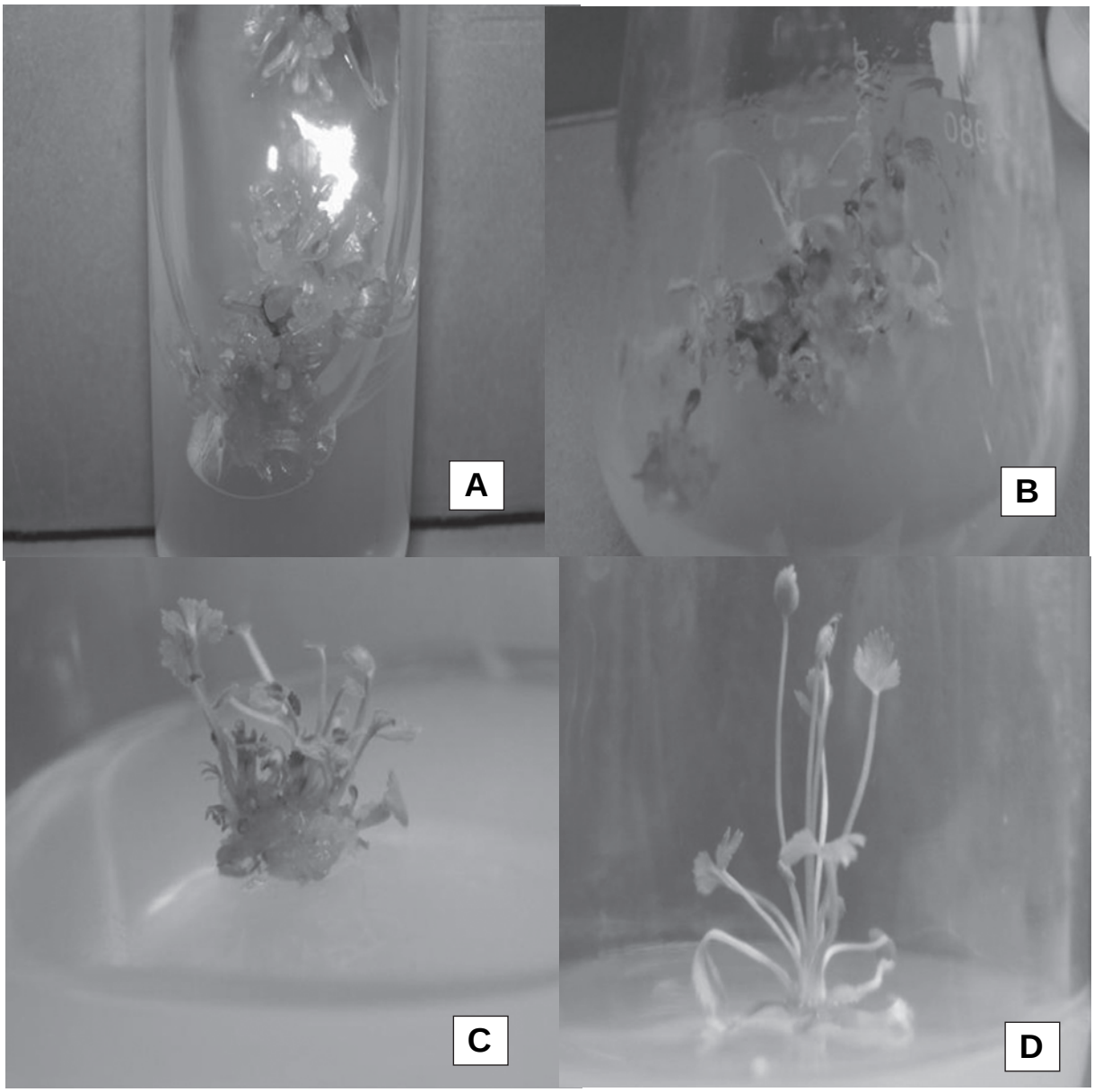

Fig 1. Organogenesis in anise. A. Organogenic callus developing from hypocotyl explant showing shoot buds B & C. Developing shoots from callus D. Healthy and normal regenerated shoot

produced good number of shoots ranging from 10 to 35 shoots explant^{-1}. IAA at 0.5 mg l^{-1} with KN (1–4 mg l^{-1}) produced maximum number of shoots explant^{-1}.

Plant regeneration from cultured cells and tissues is achieved largely by application of exogenous plant growth regulators. High frequency of multiple shoot formation in anise was also achieved by Chand *et al*. (1997) from callus cultures derived from shoot apices, root and stem explants, and also from seed-derived callus. Bela & Shetty (1999) also reported somatic embryogenesis in anise but the process involved two stages and different plant growth regulators including abscisic acid.

In the present study, both cytokinins induced shoot regeneration but the effect of BAP was more pronounced than KN. Maximum number of shoots explant^{-1} (45) was obtained with BAP 1 mg l^{-1}. BAP is the most commonly used

synthetic cytokinin in tissue culture. Being a synthetic hormone it remains stable in the system for longer time. The cytokinins were also tested in combination with IAA/NAA to see their synergistic effect on regeneration frequency. Interaction of BAP with NAA/IAA was, however, at par with treatments containing only cytokinin as far as number of shoot explant^{-1} was concerned, but length of shoots was higher in combination treatments. Similarly, KN and IAA combination was also equally good for number of shoot explant^{-1} and length of shoots. But combination of KN with NAA was only effective when NAA was supplemented with low concentration of KN. It was also observed that callus morphology is an important trait for assessment of morphogenic potential of callus. Friable callus generally proved non organogenic while green compact callus is indicative of organogenic callus. In the present investigation we observed almost all types of callus morphology and it had no relation with regeneration potential in anise. The regenerated plants were normal and such high frequency regeneration protocol can be effectively used for gene transformation.

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