In-vitro propagation of the multipurpose Egyptian medicinal plant *Pimpinella anisum*

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Background and objective
Plant tissue culture technology offers a solution for meeting the increasing commercial demand for medicinally important plants, especially cross-pollinating species, whose genetic heterogeneity presents difficulties when using traditional propagation methods. Herein, we describe an effective, rapid, and simple protocol for the micropropagation of anise (*Pimpinella anisum*).
Materials and methods
We investigated the effect of the type of explant and the type and concentration of plant growth regulator, either individually or in combination, on plant micropropagation.
Results and conclusion
Although multiple shoot formation rate was higher for nodal than for shoot tip explants, there was no significant difference in rooting response between shoots arising from either of them. Maximum shoot response, number of shoots per explant, and shoot length were observed in nodal explants grown on Murashige and Skoog medium supplemented with 5 μmol/l 6-benzylaminopurine, 1 μmol/l kinetin, and 0.5 μmol/l naphthalene acetic acid. The most effective medium for root regeneration contained 3 μmol/l of either indole-3-butyric acid or naphthalene acetic acid. Interestingly, there was no evidence for hyperhydricity, which is commonly found in cultured anise, using our method. Plantlets were successfully hardened and transferred to the greenhouse, with an 85% survival rate. This protocol provides an efficient means for large-scale production of anise, as well a basis for further research aimed at genetic improvement of this plant.

Keywords:
anise, growth regulators, micropropagation, multiple shoot formation, vitrification

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Introduction

For centuries, medicinal plants and their extracts have been used to treat various human ailments. In developing countries, they continue to be used as primary remedies for historical, cultural, and economical reasons: between 56 and 80% of the population in these countries depends on medicinal plants to meet their primary health-care needs [1]. Additionally, owing to the emergence of drug-resistant pathogens, many people in developed countries have begun to use alternative therapies comprising medicinal herbs [2]. Accordingly, there has been a substantial increase in research focused on medicinal plants in the last few decades. Various medicinal plants with bioactive compounds have been investigated with regard to their therapeutic potential. Currently, more than 50% of all clinically used drugs are derived from natural sources [3].

The anise plant (*Pimpinella anisum* L.) is one of the oldest known medicinal plants that has been used since antiquity [4]. This annual aromatic herb of the Apiaceae family is native to Egypt, and is used as flavoring and as a carminative as well as to promote digestion and relieve gastrointestinal spasms [5]. *P. anisum* has numerous biological properties including antibacterial, antiviral, antifungal, insecticidal, anti-inflammatory, and antioxidant activities [5,6]. The fruit (aniseed) is the main material used for medicinal purposes and contains 2–6% (w/w) volatile oil consisting primarily of trans-anethole [7]. Egypt and Spain are the world's major producers of this essential oil [8]. In addition to pharmaceuticals, aniseed and its oils have many applications in the food, nutrition, and cosmetics industries [9]. As such, there is a need for large quantities of homogeneous and true-to-type plant populations to meet commercial demand. However, the traditional production of anise by sexual propagation (seeds) does not guarantee uniformity of the progeny as the plant is a cross-pollinating species and is thus genetically heterogeneous.

Plant tissue culture technology offers an alternative method for the propagation of medicinal plants such as anise. Unlike conventional methods of propagation, plant tissue culture strategies enable a high multiplication rate and the production of pathogen-free plants. In addition, such strategies provide raw materials, in the form of plants and their bioactive compounds, throughout the year in a short duration and only require limited space; moreover, mass cloning of a particular plant is possible, thereby ensuring consistency in the quality and quantity of the chemical constituents. Finally, *in-vitro* plant tissue culture systems allow the production of medicinal plants with specific, desirable characteristics via transgenic technologies [10].

The geographic origin of a plant is one of the many factors that can influence the successful propagation of plants in culture. Despite the importance of anise, information on anise micropropagation – especially of the Egyptian accession – is lacking. A few earlier studies have focused on the development of a system for anise micropropagation through indirect regeneration via an intermediate callus phase; however, this approach increases the probability of somaclonal variation, which is undesirable for large-scale clonal multiplication [11]. In contrast, direct regeneration has the advantages of rapid growth rate, ease of operation, and production of true-to-type clones.

The aim of the present study was to develop an efficient protocol for the micropropagation of Egyptian anise. We investigated the shoot induction ability of different explants as well as the effects of various plant growth regulators (PGRs) on shoot and root induction. Our findings provide an efficient method for the mass production of anise and a basis for future genetic transformation studies.

Materials and methods

Plant material and explant preparation

Seeds of Egyptian anise were obtained from the Agricultural Research Center of the Ministry of Agriculture and Land Reclamation, Egypt. The seeds were surface sterilized with 20% Clorox (5% NaOCl) containing 0.1% Tween-20, for 20 min, and thoroughly washed three times with sterilized distilled water. Aseptic seeds were cultured on Murashige and Skoog (MS) medium [12] without growth regulators and were allowed to germinate under laboratory conditions. The medium was supplemented with 3% sucrose and solidified with 0.8% agar. The pH of the culture medium was adjusted to 5.7 before adding the agar, followed by autoclave sterilization at 121°C for 20 min. The medium (50 ml) was distributed in 350-ml glass jars (6 cm in diameter) that were covered with plastic lids. Each jar contained five seeds. The cultures were incubated at 25±2°C under a 16 : 8-h light/dark photoperiod at 40 μmol/m^2/s provided by white fluorescent lamps. Shoot tips (0.5–1 cm in length) and nodal segments (0.5–1 cm in length, with two axillary buds) from 3-week-old seedlings were used as explants.

Multiple shoot formation

Both types of explants were separately cultured on MS medium supplemented with various concentrations of either 6-benzylaminopurine (BAP), kinetin (Kin), or thidiazuron (TDZ) (0, 1, 2, 3, 4, and 5 μmol/l) (Table 1). The combined effect of the most potent cytokinin (5 μmol/l BAP) determined from the previous experiment with an auxin [0.1, 0.5, and 1 μmol/l naphthalene acetic acid (NAA)], cytokinin (1, 3, and 5 μmol/l Kin), or both was examined using new explants (from the 3-week-old seedlings) (Table 2). The culture vessels and the growth conditions were the same as those described above, except that each jar contained two explants. The shoot response (%), number of shoots per explant, and length of shoots were recorded after 5 weeks.

Rooting and acclimatization of plantlets

Shoots that developed from each kind of explant (obtained only from the optimal treatments – i.e. 5 μmol/l BAP+1 μmol/l Kin+0.5 μmol/l NAA for nodal explants and 5 μmol/l BAP+3 μmol/l Kin +0.5 μmol/l NAA for shoot tip explants) – were separately cultured on either full-or half-strength MS medium supplemented with various levels of either indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or NAA (0, 1, 2, 3, 4, and 5 μmol/l). The culture vessels and growth conditions were the same as described above for multiple shoot formation experiments. Rooting was recorded as a percentage after 4 weeks. For acclimatization, healthy plantlets with well-developed roots were gently removed from the jars to avoid damaging the tissue, and washed with tap water to remove traces of medium adhering to the roots. The plantlets were then transferred to pots containing a sterilized mixture of peat moss, garden soil, and sand (1 : 1 : 1) and cultured for a transition period (7–10 days) under the same growth conditions. Then, plantlets were transferred to the greenhouse where they were allowed to grow to maturity. Percent survival was determined 4 weeks after transplanting the plantlets.

Table 1 Effect of cytokinin type and concentration on shoot initiation and proliferation from various anise explants after 5 weeks of culture

Cytokinin concentration (μmol/l)	Shoot response (%)		Number of shoots/explants		Shoot length (cm)	
	Shoot tip explants	Nodal explants	Shoot tip explants	Nodal explants	Shoot tip explants	Nodal explants
Control	21.33±4.33^{f}	28.67±4.33^{f}	1.00±0.00^{f}	1.90±0.10^{f}	2.20±0.17^{cde}	2.13±0.18^{bcd}
1 BAP	59.33±2.33^{d}	68.67±2.33^{d}	2.13±0.12^{d}	3.03±0.12^{de}	2.03±0.14^{cde}	2.10±0.11^{bcd}
3 BAP	76.33±2.67^{bc}	90.67±2.33^{ab}	2.63±0.12^{c}	3.97±0.20^{bc}	2.37±0.18^{abcde}	2.43±0.09^{abc}
5 BAP	93.00±4.04^{a}	95.33±2.33^{a}	3.23±0.19^{a}	4.87±0.14^{a}	2.77±0.12^{ab}	2.70±0.15^{a}
7 BAP	81.33±2.33^{b}	86.00±4.04^{b}	3.33±0.14^{a}	4.73±0.20^{a}	2.23±0.18^{bcde}	2.03±0.14^{cd}
1 Kin	47.67±2.33^{e}	54.67±2.33^{e}	1.63±0.09^{e}	2.67±0.09^{e}	2.30±0.11^{bcde}	2.27±0.18^{abc}
3 Kin	68.67±2.33^{cd}	73.67±2.67^{d}	2.10±0.11^{d}	3.20±0.17^{cd}	2.53±0.14^{abc}	2.47±0.14^{abc}
5 Kin	81.33±2.33^{b}	83.67±2.33^{bc}	2.67±0.14^{c}	4.07±0.18^{b}	2.87±0.19^{a}	2.77±0.12^{a}
7 Kin	78.67±4.33^{b}	76.33±2.67^{cd}	2.73±0.12^{bc}	4.00±0.21^{b}	2.77±0.14^{ab}	2.33±0.18^{abc}
1 TDZ	61.67±2.33^{d}	71.33±4.33^{d}	2.20±0.10^{d}	3.40±0.10^{cd}	1.97±0.17^{de}	2.07±0.17^{cd}
3 TDZ	83.67±2.33^{b}	88.33±2.33^{ab}	3.10±0.10^{ab}	4.97±0.12^{a}	2.57±0.20^{abc}	2.60±0.17^{ab}
5 TDZ	76.33±2.67^{bc}	73.67±2.67^{d}	3.37±0.23^{a}	4.10±0.11^{b}	2.43±0.18^{abcd}	2.17±0.20^{bcd}
7 TDZ	64.00±4.04^{d}	52.33±2.33^{e}	2.40±0.21^{cd}	3.13±0.19^{de}	1.87±0.12^{e}	1.73±0.13^{d}
P value of explants	0.002**		0.000**		0.176^{ns}	
P value of cytokinins	0.000**		0.000**		0.000**	
P value of explants×cytokinins	0.014*		0.008**		0.933^{ns}	

Values represent mean±Standard error of three independent experiments with 14 replicates each. Means with the same superscript letter within columns are not significantly different at the 5% probability level ($P<0.05$) according to Duncan's test. BAP, 6-benzylaminopurine; Kin, kinetin; ns, not significant; TDZ, thidiazuron. *P value less than 0.05. **P value less than 0.01.

Table 2 Effect of optimal concentration of 6-benzylaminopurine (5 μmol/l) in combination with different plant growth regulators on shoot initiation and proliferation from various anise explants after 5 weeks of culture

PGR concentration (μmol/l)	Shoot response (%)		Number of shoots/explants		Shoot length (cm)	
	Shoot tip explants	Nodal explants	Shoot tip explants	Nodal explants	Shoot tip explants	Nodal explants
Control	93.00±4.04^{abc}	95.33±2.33^{ab}	3.23±0.19^{cd}	4.87±0.14^{b}	2.77±0.18^{cd}	2.70±0.14^{cde}
1 Kin	93.00±4.04^{abc}	95.33±2.33^{ab}	3.33±0.15^{bcd}	5.43±0.19^{a}	2.93±0.14^{bcd}	3.00±0.21^{bcde}
3 Kin	95.33±2.33^{ab}	88.33 ±2.33^{abcd}	3.93±0.14^{a}	4.77±0.13^{b}	2.97±0.12^{abcd}	2.63±0.18^{e}
5 Kin	88.33±2.33^{abcd}	81.33±2.33^{de}	3.17±0.14^{cd}	4.10±0.11^{cd}	2.13±0.20^{ef}	1.97±0.12^{f}
0.1 NAA	95.33±2.33^{ab}	95.33±2.33^{ab}	3.30±0.11^{cd}	4.97±0.18^{b}	3.20±0.17^{abc}	3.20±0.21^{abcd}
0.5 NAA	90.67±2.33^{abcd}	97.67±2.33^{a}	2.87±0.18^{de}	4.70±0.15^{b}	3.17±0.18^{abc}	3.53±0.09^{a}
1 NAA	78.67±4.33^{e}	90.67 ±2.33^{abcd}	2.13±0.14^{f}	4.03±0.12^{d}	2.53±0.22^{de}	2.83±0.12^{cde}
1 Kin+0.1 NAA	95.33±2.33^{ab}	95.33±2.33^{ab}	3.57±0.22^{abc}	5.67±0.14^{a}	3.10±0.11^{abc}	3.23±0.20^{abc}
1 Kin+0.5 NAA	90.67±2.33^{abcd}	97.67±2.33^{a}	3.13±0.14^{cd}	5.53±0.12^{a}	3.47±0.20^{a}	3.43±0.18^{ab}
1 Kin+1 NAA	83.67±2.33^{cde}	93.00±4.04^{abc}	2.60±0.17^{ef}	4.73±0.18^{b}	2.77±0.12^{cd}	3.17±0.14^{abcde}
3 Kin+0.1 NAA	95.33±2.33^{ab}	86.00±4.04^{bcd}	3.97±0.14^{a}	4.80±0.11^{b}	3.33±0.14^{ab}	2.90±0.17^{bcde}
3 Kin+0.5 NAA	97.67±2.33^{a}	88.33 ±2.33^{abcd}	3.80±0.13^{ab}	4.57±0.20^{bc}	3.43±0.12^{ab}	2.97±0.13^{bcde}
3 Kin+1 NAA	88.33±2.33^{abcd}	83.67±2.33^{cde}	3.10±0.15^{cd}	4.20±0.17^{cd}	3.13±0.14^{abc}	2.67±0.18^{de}
5 Kin+0.1 NAA	86.00±4.04^{bcde}	81.00±5.00^{de}	3.00±0.11^{de}	4.07±0.15^{b}	2.17±0.18^{ef}	2.03±0.20^{f}
5 Kin+0.5 NAA	90.67±2.33^{abcd}	76.33±2.67^{e}	3.17±0.14^{cd}	3.73±0.18^{d}	2.20±0.15^{ef}	1.90±0.15^{f}
5 Kin+1 NAA	81.33±2.33^{de}	66.33±4.67^{f}	2.37±0.18^{f}	3.20±0.17^{e}	1.97±0.14^{f}	1.63±0.13^{f}
P value of explants	0.063^{ns}		0.000**		0.123^{ns}	
P value of PGRs	0.000**		0.000**		0.000**	
P value of explants×PGRs	0.000**		0.000**		0.109^{ns}	

Values represent mean±standard error of three independent experiments of 14 replicates each. Means with the same superscript letter within columns are not significantly different at the 5% probability level ($P<0.05$) according to Duncan's test. Kin, kinetin; NAA, naphthalene acetic acid; ns, not significant; PGR, plant growth regulator. **P value less than 0.01.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Subscription (IBM, Armonk, New York, USA). Factorial two-way and three-way analysis of variance (ANOVA) were used to evaluate the effects of two and three independent factors, respectively, on the shoot response and rooting. The variables were PGR treatment and explant type for shoot response; and PGR treatment, explant type, and strength of medium for rooting. Each treatment included 14 replicates and was repeated three times. Data are presented as mean ±SE and were compared with Duncan's multiple range test at a 5% probability level.

Results

Effect of individual cytokinins on multiple shoot induction

The morphogenetic responses of shoot tip and nodal explants treated with different types of cytokinin (BAP, Kin, or TDZ) at various concentrations (1, 3, 5, and 7 µmol/l) were evaluated. Explants cultured on MS medium without PGRs served as control and did not form multiple shoots, although one or two axillary shoots developed from preexisting buds (Table 1). The presence of cytokinin was indispensable for the development of multiple shoots. After 5 weeks, multiple shoot formation was observed for all cytokinin treatment groups and both explant types, although the percentage exhibiting a shoot response, mean number of shoots per explant, and mean shoot length varied significantly. Factorial ANOVA showed a significant effect of explant type, cytokinin treatment, and the interaction between them on the shoot response and number of shoots per explant. However, only cytokinin treatment had significant effect on shoot length ($P<0.001$). Apical buds in shoot tip explants developed into a shoot in no more than 7 days, whereas each axillary bud in nodal explants formed a shoot within 10 days. Additional shoot buds were induced from their bases after 8–10 days. Although shoots arising from shoot tip explants were more rapidly initiated than those from nodal explants, completion of shoot development required a longer time. Overall, multiple shoot buds were initiated from both explant types, starting from the third week of culture. There was no callus formation in the cytokinin treatment groups; 7 µmol/l TDZ occasionally (5%) caused slight callusing in nodal explants.

The shoot response ranged from 21.33±4.33 to 93.00 ±4.04% for shoot tip explants and from 28.67±4.33 to 95.33±2.33% for nodal explants (Table 1). Maximum shoot response (93.00±4.04%) was recorded for shoot tip explants on MS medium supplemented with 5 µmol/l BAP. MS medium enriched with either 3 or 5 µmol/l BAP or 3 µmol/l TDZ yielded the three highest shoot responses for nodal explants, with no significant difference between these groups (95.33 ±2.33, 90.67±2.33, and 88.33±2.33%, respectively). The highest numbers of shoots per explant were observed on MS medium supplemented with either 5 or 7 µmol/l BAP or 3 µmol/l TDZ for both explants, or on MS medium containing 5 µmol/l TDZ for shoot tip explants. Nodal explants had the highest average shoot length on MS medium enriched with either 5 µmol/l BAP or Kin; the same media yielded the highest mean shoot length for shoot tip explants along with MS medium supplemented with 7 µmol/l Kin. As 5 µmol/l BAP induced the strongest shoot response, highest shoot number per explant, and highest shoot length for both explant types, it was determined to be the optimal concentration for anise propagation. In general, although nodal explants showed stronger shoot response and proliferation than shoot tip explants, there was no significant difference between them in terms of shoot length.

Effect of optimal 6-benzylaminopurine concentration (5 µmol/l) in combination with cytokinin and/or auxin on multiple shoot formation

The combined effect of either cytokinin (Kin), auxin (NAA), or both, and the optimal BAP concentration (5 µmol/l) was also evaluated to determine whether the multiplication rate could be improved. MS medium supplemented with 5 µmol/l BAP served as a control. In general, the addition of PGRs along with BAP at the optimal concentration did not further enhance the shoot response of either type of explant, and in fact had adverse effects in some cases (Table 2). Although low concentrations slightly increased the shoot response, high concentrations had the opposite effect. Increases in the number of shoots obtained from a single explant and shoot height were observed with some treatments. The mean shoot number per shoot tip explant increased from 3.23±0.19 to 3.93±0.14 with 3 µmol/l Kin, 3.97±0.14 with 3 µmol/l Kin+0.1 µmol/l NAA, and 3.80±0.13 with 3 µmol/l Kin+0.5 µmol/l NAA in MS medium containing 5 µmol/l BAP. Additionally, the largest number of shoots from nodal explants was obtained in the presence of either 1 µmol/l Kin, 1 µmol/l Kin+0.1 µmol/l NAA, or 1 µmol/l Kin +0.5 µmol/l NAA (almost 5.5 shoots/explant). Shoot length was also enhanced by adding 1 µmol/l Kin +0.5 µmol/l NAA, 3 µmol/l Kin+0.1 µmol/l NAA, or 3 µmol/l Kin+0.5 µmol/l NAA for shoot tip explants and 0.5 µmol/l NAA or 1 µmol/l Kin+0.5 µmol/l NAA for nodal explants. Based on the above results, 5 µmol/l BAP combined with either 3 µmol/l Kin+0.1 µmol/l NAA or 3 µmol/l Kin+0.5 µmol/l NAA were

considered the ideal combinations for anise propagation from shoot tip explants, with no significant difference between them. Similarly, MS medium supplemented with 5 µmol/l BAP+1 µmol/l Kin+0.5 µmol/l NAA – which induced 5.53 shoots per explant with a mean shoot length of 3.43 cm – was selected as the optimal medium for direct shoot induction from nodal explants. There was no need for an additional elongation step – which is the standard procedure in many plant species – as the shoots derived from both types of explant under optimal treatment conditions were healthy and of an appropriate length (~3.5 cm).

Root formation and acclimation

Shoots longer than 3 cm were aseptically excised and transferred to a rooting medium to investigate the effects of auxin type and concentration, medium strength, and origin of regenerated shoots on rooting (Table 3). Factorial ANOVA showed that neither medium strength nor shoot origin affected the rooting frequency of regenerated shoots; only auxin treatment had a statistically significant effect. Irrespective of medium strength or shoot origin, shoots failed to induce rooting on any medium lacking auxin (control treatment). Although there was an initial swelling of the cut shoot ends, no roots developed even after 4 weeks of culture. Roots were initiated directly from the base of shoots within 2–3 weeks of culture on a rooting medium (i.e. containing auxin). Callusing was completely absent in all treatment groups except for 7 µmol/l NAA (21%) and 7 µmol/l IBA (17%). Despite the formation of slight calluses under these conditions, roots emerged and proliferated in 95% of the cases. Among the three tested auxins, 3 µmol/l IBA and NAA induced the strongest rooting response with no significant difference between them. There was no callus formation upon application of IAA even at the highest concentrations. In contrast to NAA and IBA, the concentration of IAA that produced the highest rooting rate was 5 µmol/l. After 4 weeks, healthy plantlets were temporarily transferred to pots for 7–10 days in the same growth chamber, and then moved to the greenhouse where they were allowed to grow to maturity. About 85% of micropropagated plants survived without obvious phenotypic changes. Figure 1 illustrates the proposed micropropagation scheme for anise.

Discussion

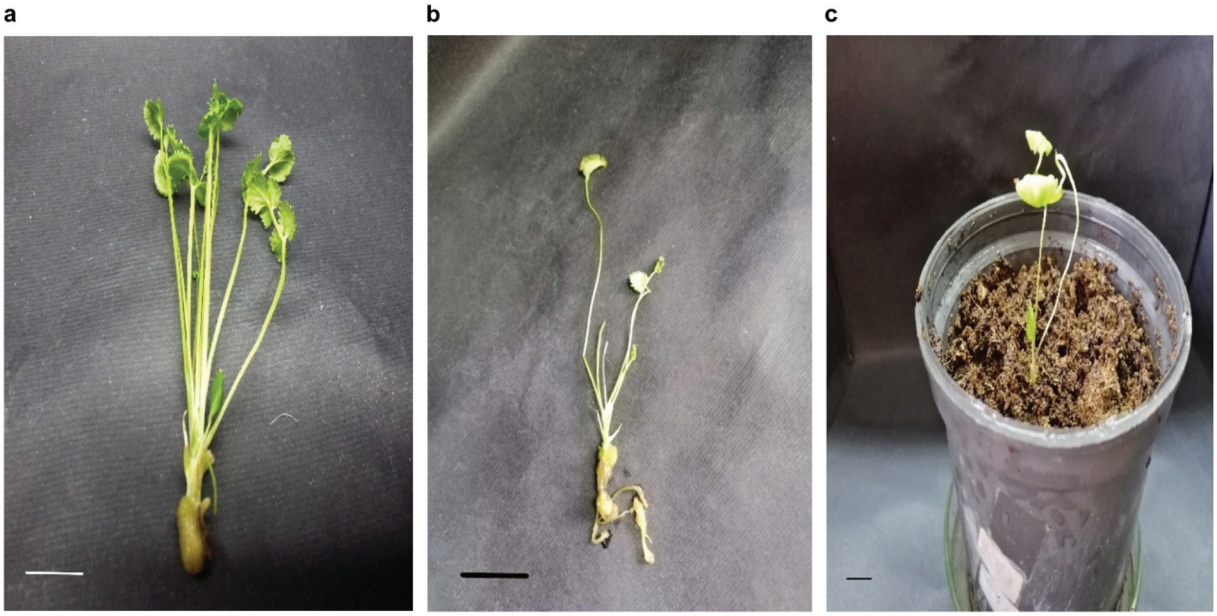
Micropropagation is among the most commercially efficient and practical plant propagation technologies. *In-vitro* propagation methods rely on the totipotency of plant cells. Direct organogenesis is

Table 3 Effect of different auxin types and concentrations and Murashige and Skoog medium strength on the frequency (%) of root induction from various explant-derived anise shoots

Auxin concentration (µmol/l)	Shoot tip-derived shoots		Node-derived shoots	
	½ MS medium	MS medium	½ MS medium	MS medium
Control	–	–	–	–
1 IAA	45.33±4.67^{e}	50.00±4.04^{f}	43.00±4.04^{f}	47.66±2.33^{f}
3 IAA	71.33±4.33^{cd}	71.33±4.33^{bcd}	69.00±5.00^{cde}	68.67±2.33^{cde}
5 IAA	83.67±2.33^{b}	81.33±2.33^{b}	83.67±2.33^{b}	81.00±5.00^{bc}
7 IAA	68.67±2.33^{cd}	64.00±4.04^{cde}	66.33±2.33^{de}	64.00±4.04^{de}
1 IBA	71.33±4.33^{cd}	73.67±2.67^{bc}	68.67±2.33^{cde}	71.33±4.33^{cd}
3 IBA	97.67±2.33^{a}	95.33±2.33^{a}	95.33±2.33^{a}	93.00±4.04^{a}
5 IBA	78.67±4.33^{bc}	76.33±2.67^{b}	76.33±2.67^{bc}	74.00±5.00^{cd}
7 IBA	66.33±2.33^{d}	61.67±4.67^{de}	64.00±4.04^{de}	61.67±2.33^{de}
1 NAA	74.00±5.00^{bcd}	71.33±4.33^{bcd}	73.67±2.67^{cd}	71.33±4.33^{cd}
3 NAA	95.33±2.33^{a}	93.00±4.04^{a}	95.33±2.33^{a}	90.67±2.33^{ab}
5 NAA	78.67±4.33^{bc}	76.33±2.67^{b}	76.33±2.67^{bc}	71.33±4.33^{cd}
7 NAA	64.00±4.04^{d}	59.33±2.33^{ef}	61.67±2.33^{e}	57.00±4.04^{ef}
P value of explants	0.078^{ns}			
P value of auxins	0.000**			
P value of media	0.082^{ns}			
P value of explants × auxins	1.000^{ns}			
P value of explants × media	0.989^{ns}			
P value of auxins × media	0.810^{ns}			
P value of explants × auxins × media	1.000^{ns}			

Values represent mean±SE of three independent experiments of 14 replicates each. Means with the same superscript letter within columns are not significantly different at the 5% probability level ($P<0.05$) according to Duncan's test. IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog; NAA, naphthalene acetic acid; ns, not significant. **P value less than 0.01.

Figure 1



In-vitro micropropagation of anise: (a) multiple shoot induction from nodal explants cultured on MS medium supplemented with BAP (5 μmol/l), Kin (1 μmol/l), and NAA (0.5 μmol/l) for 5 weeks; (b) root induction from nodal-derived shoots cultured on MS medium enhanced with IBA (3 μmol/l) for 4 weeks; (c) *in-vitro* propagated plantlet grown in a pot containing a sterilized mixture of peat moss, garden soil, and sand (1 : 1: 1) for 10 days. BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid; Kin, kinetin; MS, Murashige and Skoog; NAA, naphthalene acetic acid.

generally considered the safer route for micropropagation of clonal, true-to-type plants [13]; this complex process involves synergistic interactions between physical and chemical factors [14] and is initiated within the shoot (or root) meristem of an explant [15]. This is mainly controlled by the endogenous and exogenous balance of PGRs. Genotype, explant type, and physiological status modulate endogenous levels of these regulators. In particular, the endogenous levels of these regulators are influenced by the composition of the culture medium (especially the concentration and type of PGRs), which in turn greatly influences the direction and efficiency of organogenesis. In this study, we examined the influence of PGR and explant type on multiple shoot formation in anise explants in order to determine the ideal conditions for anise micropropagation.

Irrespective of the explant type, we observed that multiple shoots formed to varying degrees on all media (Table 1), implying that different types of explant with preexisting meristems are suitable for multiple shoot induction in anise. In fact, the use of preexisting meristems is one of the simplest, most reliable, and most successful means of micropropagation of many plant species, including other members of the Apiaceae family [16–19]. Moreover, recalcitrant species such as grain legumes were successfully regenerated from explants harboring preexisting meristems including embryo axes, cotyledonary nodes, shoot tips, and nodal explants [20,21]. The differential responses of the two explant types are likely due to differences in endogenous hormone levels. Nodal explants were superior to shoot tip explants in terms of multiple shoot induction and proliferation (Tables 1 and 2); this may be attributed to the absence of apical dominance, in which the shoot apex inhibits the growth of axillary buds. It is well known that auxin synthesized in the apical meristem moves basipetally along the length of the growing shoot and prevents axillary bud activation, although the precise underlying mechanism remains unclear. Cytokinins antagonize auxin in apical dominance; application of exogenous cytokinin promotes shoot formation even in the presence of a growing shoot apex or apically supplied auxin [22]. This is another possible explanation for the present observation that nodal explants required smaller amounts of cytokinin than shoot tip explants for maximum growth (Table 2). The superiority of nodal over shoot tip explants for multiple shoot proliferation has been demonstrated in other medicinal plants including *Teucrium scorodonia* [23], *Agastache foeniculum* [18], *Withania somnifera* [24], and *Mentha piperita* [25].

Cytokinins play a major role in many aspects of plant growth and development, including shoot formation and multiplication [26,27]. Consistent with our findings (Table 1), many studies have reported the positive effect of cytokinins on multiple shoot induction [22,28,29]. However, depending on the

species or cultivar, cytokinin type and concentration are critical factors that must be controlled for successful shoot initiation and proliferation. In the present study, BAP at a concentration of 5 μmol/l yielded maximal shoot induction and sprouting in both types of explant. The advantages of using BAP over other cytokinins for multiple shoot formation has been demonstrated in a number of plant species [24,30]. The naturally occurring ribosides and nucleotides in BAP makes it more stable than other cytokinins [31], accounting for the enhanced response induced by this treatment. There was a positive relationship between the increase in BAP concentration up to the optimal level (5 μmol/l) and multiple shoot formation in both nodal and shoot tip explants; however, increases in BAP concentration beyond the optimal level did not further improve any parameter associated with shoot multiplication, and instead suppressed the shoot response in both explant types as well as shoot length in nodal explants. This reduction in shoot formation may be related to the toxicity of BAP at higher concentrations. Narayanaswamy [32] reported that the toxicity caused by an excess of or prolonged exposure to growth regulators in the culture medium leads to genetic, physiological, and morphological changes and consequent inhibition of proliferation. The inhibitory effects of BAP at supraoptimal concentrations have been observed in other medicinal plants such as *W. somnifera* [24], *Veronica anagallis-aquatica* [33], *Cicer arietinum* [21], and *Canscora decussata* [30].

Plant regeneration and development are regulated by plant hormones; indeed, most physiological processes involve the interplay of several phytohormones [34] that act synergistically or antagonistically [35]. As a result, the use of multiple hormones versus a single hormone is more effective, in most cases, for plant regeneration. In this study, Kin and NAA in combination with BAP increased both the number of shoots per explant as well as the shoot length (Table 2). A similar synergistic effect between BAP and Kin or BAP and NAA in the induction of multiple shoot formation has been demonstrated in several plant species [30,36–38].

The exogenous application of auxin hormones (IAA, IBA, and NAA) had a significant effect on root formation (Table 3); shoots failed to sprout roots on any medium lacking auxin irrespective of medium strength or shoot origin. Auxins control nearly every aspect of plant growth and development [39,40] and are the principal regulators of lateral root development [41,42]. Recent reports indicate that this process comprises at least four distinct phases – that is, priming, initiation, patterning, and emergence [43,44] – that are modulated by auxins [45]. Several studies have shown that the exogenous application of auxins results in increased lateral root initiation, and that root development is highly dependent on auxin type and concentration [22,46]. In the present study, 3 μmol/l IBA or NAA elicited the strongest rooting response, with no significant differences between them. However, other studies have reported contradictory findings regarding their effects on root formation. For example, IBA was shown to enhance root formation in *C. decussata* [30], but the opposite was also demonstrated [47]. Furthermore, among the three auxins, IAA was found to be more effective than IBA and NAA in inducing root formation in *T. scorodonia* [22]. The variability in these results suggest that the effect of a given auxin is largely dependent on plant species.

Hyperhydricity (also known as vitrification) –a physiological disorder that causes shoots and leaves to become brittle, with a glassy appearance – presents a challenge for the culture-based multiplication of many plant species, including anise [48]. Several factors have been suggested to induce hyperhydricity such as a high level of cytokinins (especially BAP), excess of minerals, low concentration of gelling agents, and inadequate microenvironment (e.g. poor aeration/gaseous exchange and high humidity) within the culture vessels [49,50]. Notably, we did not find any sign of hyperhydricity in shoots – which is common in cultured anise – using our micropropagation system. This may be attributable to the genetic background of the Egyptian anise used in this study, which exhibits resistance to hyperhydricity. Factors that cause disorders in one plant type often do not affect other cultivars or species [51]. Our results suggest that the predisposition toward hyperhydricity is mainly genotype dependent, and that variations might occur not only among species but also between different varieties or cultivars within the same plant species, thus necessitating studies testing different plant species, varieties, and cultivars. The present findings are consistent with previous observations that different cultivars of pear and tree peony cultivars exhibit variable tendency for hyperhydricity [51,52], although none were found to show complete tolerance. In this context, future studies should aim to screen Egyptian anise for factors associated with resistance to hyperhydricity.

Conclusion

The results of this study demonstrate an efficient, rapid, and simple protocol for the *in-vitro* propagation of

P. anisum. We found that shoot tip and nodal explants can be used for multiple shoot formation, without an intervening callus phase or shoot elongation step. Another important finding was the absence of hyperhydricity, which is often observed in cultured anise. The present micropropagation system is suitable for application in the large-scale production of this important medicinal plant to meet commercial needs.

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Conflicts of interest

There are no conflicts of interest.

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