In vitro propagation and *Agrobacterium*-mediated genetic transformation of caraway (*Carum carvi L.*)

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Background and objective
Carum carvi is one of the oldest-known cultivated herbs around the world. The caraway seeds are regarded as antispasmodic, astringent, and carminative, and are used in treating somatic stimulants, dyspepsia, colic, flatulent indigestion, diarrhea, and improved liver function. Tissue culture is a suitable strategy for producing large-scale plantlets with a high potential to produce superior-quality plants. Plant transformation methods help to improve food quality and help plants to resist biotic and abiotic stresses. The current study aimed to optimize *in vitro* propagation system and genetic transformation protocol by using the *Agrobacterium*-mediated method for caraway.
Materials and methods
The shoot tip was used as an explant. We investigated the effect of growth hormones, carbon sources, gelling agents, bacteria optical density, inoculation period, acetosyringone concentration, and cocultivation period on caraway regeneration and transformation system.
Results and conclusion
Maximum shoot response, numbers of shoots per explant, and shoot length were observed when placing shoot tips on Murashige and Skoog media supplemented with 5 μmol/l BA (6-benzyladenin), 1 μmol/l NAA (1-naphthaleneacetic acid), and 30 g/l of sucrose. Gellan gum products (gelrite and phyta gel) were superior to agar products (agar and bactoagar), especially when used with a concentration of 2.5 or 3 g/l. For transformation protocol, *Agrobacterium* infection was maximum at an optical density of 0.8 when inoculated with explant for 5 min in the presence of 100 μmol/l acetosyringone and cocultivated for 3 days. In this study, we presented a productive technique for propagation and an *Agrobacterium*-mediated transformation system that can be beneficial in genetic transformation and other plant biotechnology techniques.

Keywords:
caraway, gelling agent, genetic transformation, growth regulators, micropropagation, shoot response, sugar type

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Introduction

Throughout history, medicinal plants have been essential in the treatment of various diseases around the world [1,2]. Over the past few decades, there has been a marked increase in studying medicinal plants. The therapeutic potential of various medicinal plants with bioactive substances has been studied. Currently, more than half of the drugs come from natural sources [3,4]. One of the most important medicinal plants is caraway (*Carum carvi L.*), it is considered one of the most vastly used medicinal, spice, and essential oil herbs in the world [5]. Egyptian caraway oil contains main components such as limonene (53.4%), β-selinene (11.1%), β-elemene (10.1%), and caryophyllene oxide (9.8%).

The chemical components of caraway showed up antibacterial activity against several types of bacteria such as *Salmonella typhi*, *Staphylococcus aureus*, *Aspergillus niger*, *Candida albicans*, and *Listeria innocua* [6]. The most important application of caraway is its use for treating digestive problems [7]. Constipation, nausea, and stomach aches are treated with caraway fruit. It stimulates digestion by increasing the secretion of digestive fluids and promoting the evacuation of bile [8]. Caraway seeds have diuretic effects that increased the urine output and total volume of extracted urine [9]. Caraway seeds' aqueous extract also has an antiglycemic effect [7] and increased the level of Thyroid-Stimulating Hormone (TSH) in patients with papillary thyroid carcinoma [10]. Caraway essential oil has antidiabetic effects that reduce glucose levels in

serum by a mechanism independent of insulin secretion [11].

Plant tissue culture technology is considered a method for the propagation of plants and this method enables a high multiplication rate and production of pathogen-free plants [12]. In addition, it required only limited space and takes short time compared with alternative methods of propagation [13]. Plant tissue culture technology allows the production of plants with desirable characteristics via transgenic technologies [14] and it considered the prerequisite step for transformation protocols. The most frequent way of obtaining transgenic plants is genetic transformation using *Agrobacterium tumefaciens*, it is low cost and relatively simple when compared with other methods. Furthermore, compared with direct approaches, this genetic transformation process produces fewer copies of the transgene and is regarded as more efficient [15]. Bacterial strains, bacteria concentrations, phenolic compounds added to the plant culture medium, plant genotypes, explant type, temperature during cocultivation, light, wounding of the target tissue, antibiotics, and the method used to select transgenic cells are all factors that can influence transformation efficiency [16].

Inspite of the multiple uses of caraway, only limited research had focused on transformation of caraway. To the best of our knowledge, only [17] in 1997 established a regeneration and transformation system of caraway. However, their protocol gave low efficiency of transformation rate and they used indirect organogenesis way that takes long time. The present study focused on developing a reliable and efficient method for the regeneration of caraway by optimizing various parameters for the maximum gene-transfer rates of caraway using *Agrobacterium* method.

Materials and methods

In vitro seed germination and explant preparation

Mature seeds were washed with tap water for 5 min, then inside the laminar airflow cabinet, they were washed with sterilized distilled water for 10 min, then the water was discarded, 70% ethanol was added for 2 min, and then seeds were washed with sterilized distilled water for 5 min. Seeds were soaked in 70% Clorox (5.25% NaOCl) containing 0.1% Tween-20 for 10 min, and then washed three times with sterilized distilled water. Surface-sterilized seeds were cultured on Murashige and Skoog (MS) medium supplemented with 3% sucrose and 8 g/l agar as solidifying agent. The medium was adjusted to 5.8 before adding the solidifying agent, followed by autoclave for sterilization at 121°C for 20 min The cultures were incubated at 25±2°C and exposed to a 16: &9618;8-h light/dark cycle. In all, 14-day-old seedlings were used to get the explant. Shoot tips with 4–6-mm height were used in the upcoming experiments.

Factors affecting shoot micropropagation

Shoot tips were cultured on a full MS medium supplemented with different concentrations of 6-benzyladenin (BA) (1, 3, 5, and 7 μmol/l), 1-naphthaleneacetic acid (NAA) (0.5, 1, and 2 μmol/l), and a combined effect for both was measured to know the effect of these growth regulators on shoot micropropagation. The pH of all prepared media was adjusted to 5.8 before autoclaving and fortified with 3% sucrose and 2 g/l gelrite.

For estimating the best type and concentration of carbohydrate source, full MS medium supplemented with 5 μmol/l BA and 1 μmol/l NAA was used and we examined four different types of carbon sources with different concentrations of sucrose (10, 20, 30, 40, and 50 g/l), maltose (10, 20, 30, 40, and 50 g/l), glucose (10, 20, 30, 40, and 50 g/l), or sorbitol (10, 20, 30, 40, and 50 g/l). The pH of all media was adjusted to 5.8 and 2 g/l gelrite has been used as solidifying agent.

To study the effect of gelling agents, shoot tips were cultured on 4.4 g/l MS medium supplemented with 5 μmol/l BA, 1 μmol/l NAA, and 3% sucrose, and then the pH of all media was adjusted to 5.8. Different types of gelling agents were utilized with various concentrations of agar (6, 7, 8, and 9 g/l), bactoagar (6, 7, 8, and 9 g/l), gelrite (1.5, 2, 2.5, and 3 g/l), or phyta gel (1.5, 2, 2.5, and 3 g/l). All cultures were then incubated at 26±1°C under 16/8-h (light/dark) conditions for 4 weeks. We estimated different parameters such as shoot response (SR), number of shoots per explant, and shoot length for each experiment to estimate the factors that give us the highest shoot micropropagation frequency.

Root induction and acclimatization of plantlet

The well-regenerated shoots were cultured on MS supplemented with 5 μmol/l indole-3-acetic acid (IAA) to enhance root formation for 4 weeks. Healthy and well-rooted plantlets were gently removed from jars and washed with tap water to clear away excess media from roots. The plantlets were then transferred to pots containing sterilized peat moss. Pots were covered with plastic bags and cultured for 7 days under the same growth conditions

in the growth room. After that, small openings were made in plastic bags gradually. The plastic bags were removed totally within a month and moved to greenhouse.

Agrobacterium strain and binary vector

The *A. tumefaciens* strain LBA4404 harboring standard binary vector P CAMBIA 1301 was used in this study. This plasmid vector is containing the hygromycin-resistance gene *(hpt)*, β-glucuronidase *(gus)* gene, and *DREB2A* gene under the control of cauliflower mosaic virus 35S promoter and sequence of poly-A signal at 3' end as terminator (Fig. 1).

Hygromycin-resistance sensitivity

Full MS media supplemented with 3% sucrose, 5 µmol/l BA, 1 µmol/l NAA, and 2.5 g/l gelrite was sterilized using autoclave and then different concentrations of hygromycin from 0, 10, 20, and 30 mg/l was added. The shoot tips were cultured on the media and incubated at 26±1°C under 16/8-h (light/dark) conditions for 3 weeks and the number of survived explants was finally recorded for each hygromycin concentration to determine its lethal dose to the nontransgenic plants.

Establishing of factors affecting *Agrobacterium*â mediated transformation

A. tumefaciens LBA4404 harboring the *DREB2A* gene that was stored at −80°C was revitalized by streaking on Luria–Bertani (LB) agar medium (25 g/l LB medium +14 g/l agar and then the pH was adjusted to 7) containing 50 mg/l streptomycin, 50 mg/l kanamycin, and 50 mg/l rifampicin, then incubated at 28°C in dark conditions for 2 days. A single bacterial colony obtained from streaked LB agar plate was grown for 40 h in 50 ml of LB broth medium containing 50 mg/l kanamycin, 50 mg/l streptomycin, and 50 mg/l rifampicin as antibiotics at 28°C with continuous shaking of 170 rpm to obtain an optical density (OD_{600}) of 0.6–1.2.

The factors 'Bacteria OD_{600}, Inoculation duration, cocultivation period and acetosyringone concentration' which affect the transformation efficiency, were evaluated. Different bacterial optical densities (0.6, 0.8, 1, and 1.2) were measured by using a spectrophotometer at the wavelength of 600 nm and used to infect Caraway shoot tips. Four different infection durations (2.5, 5, 10, and 20 min) were evaluated. The effect of acetosyringone at different concentrations (50, 100, 200, and 300 µmol/l) was also examined. The inoculated seedlings were cocultivated for different periods of 1, 2, 3, and 4 days.

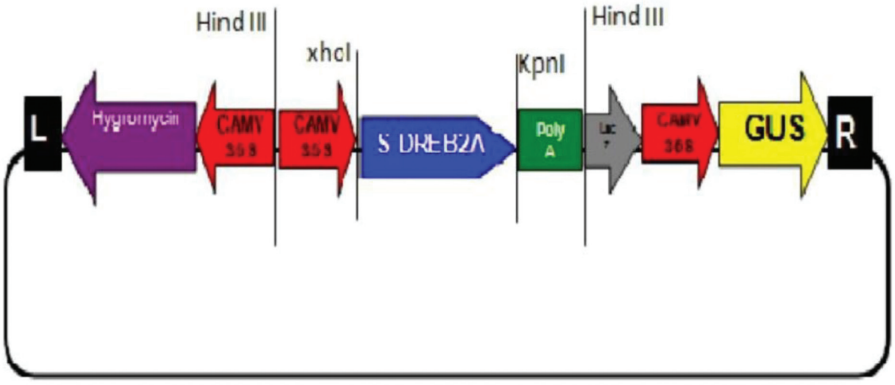
Detection of putative transgenic by β-glucuronidase assay

β-glucuronidase (GUS) staining has been regarded as the method of choice for assessing gene expression in plants because it is a sensitive, accurate, and simple to use. Histochemical GUS assay was carried out to determine the expression of the *gus* gene in the transformed plant tissues. According to Jefferson *et al.* [18] method, GUS enzyme activity was used to determine the efficiency of transformation process in plant tissues.

Caraway genomic DNA extraction and PCR

Caraway genomic DNA extraction was carried out by using Quick-DNA Plant/Seed Miniprep Kit. Leaves of putative transgenic plants were dissected using PCR with *DREB2A* primers that were designed to amplify the relevant sequence of genes. The PCR reaction was carried out as the following: 2 µl from DNA template, 1.5 µl of forward primer (5'-GAGTACCTCG AGATGGAGCGGGGGGAGGG-3'), 1.5 µl from

Figure 1



Construct map of vector PCAMBIA 1301 DNA *DREB2A*.

reverse primer (5'-GCAGCGGTACCGACTACT ACTCTAATAGGAG-3'), 12.5 µl from master mix, and 7.5 µl ddH_2O. The total volume of the reaction was 25 µl. All reaction steps were done in the laminar airflow hood by using ice rack. All eppendorfs were centrifuged for 10 s to get rid of any bubbles in them. The PCR reaction was carried out by using T100 thermal cycler (Bio-Rad). According to Amer *et al.* [19], we set the PCR program at 94°C for 5 min as initial temperature, then 94°C for 30 s as denaturation temperature, followed by 56.9°C for 30 s as annealing temperature, thereafter 72°C for 40 s, and 72°C for 10 min as extension temperature for 30 cycles. The result is visualized as distinct bands on agarose gels with concentration of 1%.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics Subscription (IBM,Armonk, New York, USA). One-way analysis of variance was used to evaluate the factors that affect the regeneration and transformation. Each experiment included ten replicates and was repeated three times. Data are presented as mean±SE and were compared with Duncan's multiple-range tests at a 5% probability level.

Results

Influence of growth regulators on shoot regeneration

Shoot tips cultivated on MS medium without plant growth regulators served as the control, and they only produced one axillary shoot from preexisting buds, but they did not produce numerous shoots. The shoot response ranged from 70.00±5.77 to 100.00±0.0. The presence of BA was indispensable for the development of multiple shoots and gave the maximum SR rate. It is worth mentioning that using combinations between BA and NAA showed a high SR rate, except in the case of 3 µmol/l of BA and 2 µmol/l of NAA. However, the SR rate was the lowest when using any concentration of NAA without BA (Table 1).

The number of shoots/explant ranged from 1.00±0.00 in the case of control to 3.23±0.20. MS medium supplemented with either 5 µmol/l BA+1 µmol/l NAA or 3 µmol/l BA+1 µmol/l NAA or 7 µmol/l BA+1 µmol/l NAA yielded the three highest numbers of shoots/explant with no significant differences between these groups (3.23±0.20, 3.17 ±0.19, and 2.80±0.17, respectively) (Table 1). The highest average of shoot length was observed when using MS media supplemented with 5 µmol/l BA +1 µmol/l NAA (3.67±0.12), 5 µmol/l BA+0.5 µmol/l NAA (3.60±0.11), 3 µmol/l BA+0.5 µmol/l NAA (3.57±0.09), 3 µmol/l BA (3.27±0.15), 1 µmol/l BA (3.23±0.15), or 5 µmol/l BA (3.20±0.15), with no significant differences between these groups (Table 1).

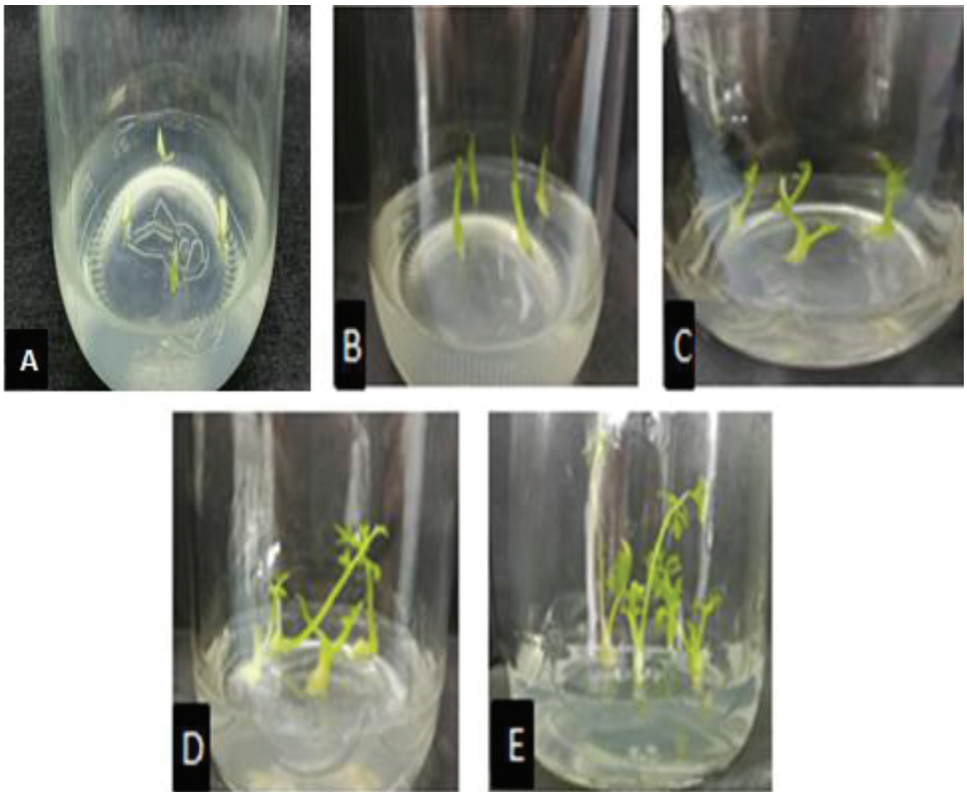
As a result, MS media enriched with 5 µmol/l BA and 1 µmol/l NAA induced the strongest shoot response, shoot numbers per explant, and the highest average shoot length, it was determined to be the optimal

Table 1 Effect of different concentrations of benzyladenin, naphthaleneacetic acid, and combination between them on direct shoot regeneration

BA (µmol/l)	NAA (µmol/l)	Shoot response	Number of shoots/explant	Shoot length
0	0	73.33±6.67c	1.00±0.00d	2.80±0.11b
1	0	96.67±3.33a	1.70±0.12c	3.23±0.15ab
3	0	93.33±3.33a	2.37±0.12b	3.27±0.15ab
5	0	100.00±0.0a	2.43±0.12b	3.20±0.15ab
7	0	96.67±3.33a	2.40±0.11b	2.83±0.09b
0	0.5	73.33±6.67c	1.10±0.06d	2.13±0.09c
1	0.5	93.33±0.67a	1.73±0.13c	2.83±0.15b
3	0.5	96.67±3.33a	2.40±0.17b	3.57±0.09a
5	0.5	93.33±3.33a	2.43±0.15b	3.60±0.11a
7	0.5	100.00±0.0a	1.73±0.09c	2.83±0.07b
0	1	70.00±5.77c	1.10±0.10d	2.07±0.09c
1	1	93.33±3.33a	2.43±0.12b	2.13±0.09c
3	1	100.00±0.0a	3.17±0.19a	2.87±015b
5	1	100.00±0.0a	3.23±0.20a	3.67±0.12a
7	1	96.67±3.33a	2.80±0.17ab	2.83±0.12b
0	2	56.67±3.33d	1.13±0.09d	1.40±0.11d
1	2	70.00±5.77c	1.70±0.11c	1.80±0.10 cd
3	2	83.33±3.33b	1.70±0.17c	2.10±0.06c
5	2	96.67±3.33a	2.40±0.06b	2.87±0.12b
7	2	93.33±3.33a	2.47±0.12b	2.83±0.09b

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level. BA, benzyladenin; NAA, naphthaleneacetic acid.

Figure 2



Effect of MS media supplemented with 5 μmol/l BA and 1 μmol/l NAA in shoot regeneration. (a) A 4-mm shoot tip in zero age of explant, (b) shoot regeneration after 1 week, (c) shoot regeneration after 2 weeks, (d)shoot regeneration after 3 weeks, (e)shoot regeneration after 4 weeks. BA, benzyladenin; MS, Murashige and Skoog; NAA, naphthaleneacetic acid.

concentrations for direct shoot regeneration of caraway by using shoot tips as explant (Figure 2).

Effect of different carbon sources on shoot regeneration

The maximum shoot response was observed when using 30 g/l sucrose, 30 g/l maltose, 40 g/l maltose, 40 g/l sucrose, 40 g/l sorbitol, 20 g/l sucrose, 20 g/l maltose, or 30 g/l sorbitol (Table 2) and there were no significant differences in shoot response between these treatments. Although several treatments have high SR rates, only half of these treatments gave the maximum number of shoots per explant. The numbers of shoots/explant ranged from 3.23±0.20 when using 30 g/l sucrose to 1.07±0.03 when using 10 g/l sorbitol (Table 2). As observed in statistical analysis, there were no significant differences in the number of shoots/explant when using 30, 40 g/l sucrose, 40, or 30 g/l maltose (3.23±0.20 g/l sucrose, 3.13±0.09 g/l sucrose, 3.07±0.15 g/l maltose, and 3.03±0.09 g/l maltose, respectively).

Among all the treatments, only 30 g/l sucrose (3.67 ±0.12 g/l sucrose) and 20 g/l sucrose (3.63±0.09 g/

Table 2 Influences of different types and concentrations of carbon source on direct shoot regeneration

Carbon source and concentration (g/l)	Shoot response	Number of shoots/explant	Shoot l ength
10 Sucrose	83.33±3.33b	1.73±0.09c	2.30±0.06c
20 Sucrose	93.33±3.33a	2.50±0.12b	3.63±0.09a
30 Sucrose	100.00±0.00a	3.23±0.20a	3.67±0.12a
40 Sucrose	96.67±3.33a	3.13±0.09a	3.03±0.09b
10 Glucose	66.67±3.33c	1.77±0.15c	1.53±0.07d
20 Glucose	66.67±6.67c	2.53±0.09b	2.27±0.09c
30 Glucose	80.00±5.77b	2.53±0.07b	3.00±0.15b
40 Glucose	83.33±3.33b	2.10±0.26bc	2.97±0.12b
10 Maltose	83.33±6.67b	1.73±0.09c	1.57±0.09d
20 Maltose	93.33±3.33a	2.47±0.15b	2.33±0.12c
30 Maltose	100.00±0.00a	3.03±0.09a	3.00±0.12b
40 Maltose	100.00±0.00a	3.07±0.15a	3.03±0.09b
10 Sorbitol	66.67±8.82c	1.07±0.03d	1.53±0.15d
20 Sorbitol	83.33±3.33b	1.77±0.12c	1.93±0.18 cd
30 Sorbitol	93.33±3.33a	1.77±0.17c	2.33±0.17c
40 Sorbitol	96.67±3.33a	1.73±0.09c	2.37±0.09c

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

l sucrose) showed the highest shoot length. When comparing all data from Table 2, we found that only 30 g/l sucrose gave high shoot response, number of shoots/explant, and shoot length. As a result, we recommend using 30 g/l sucrose as an ideal type and concentration for carbon source when using shoot tips as an explant for the caraway plant.

Effect of gelling agent on shoot regeneration

The results obtained from statistical analysis showed that there was no effect of using different types and different concentrations of gelling agent were remarked on shoot response, except when using 6 g/l bactoagar, which showed minimum SR rate (80.00±5.77). Although gelling agent type and concentrations had no effect on shoot response, but it had strong effect on multiplication rate and shoot length (Table 3). The findings showed that for shoot multiplication and shoot length, gellan gum products (gelrite and phyta gel) were superior to agar products (agar and bactoagar).

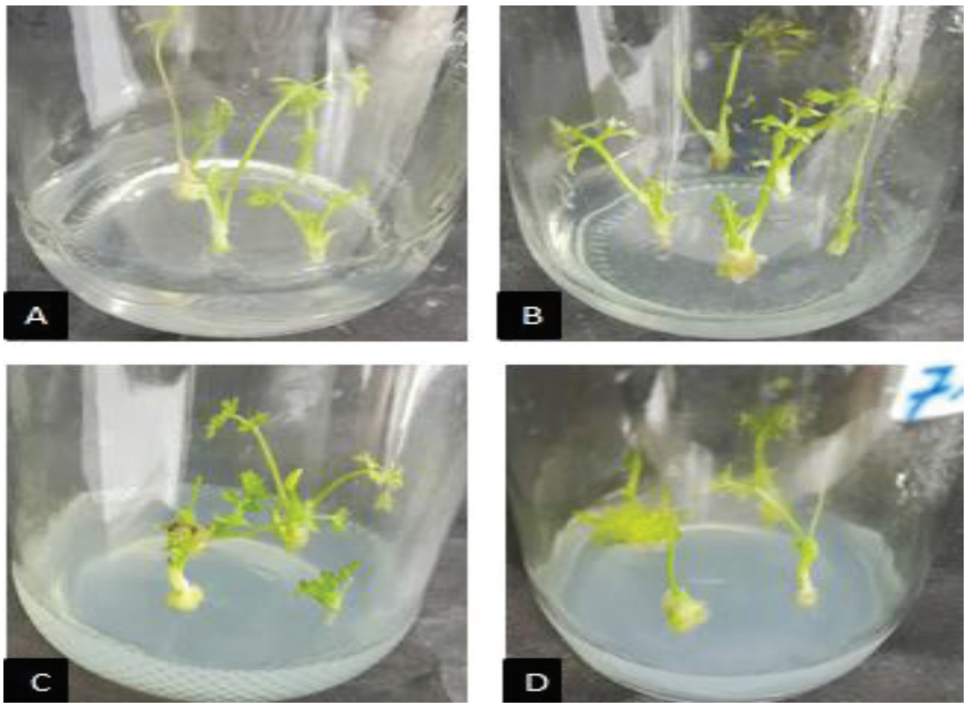
The maximum number of shoots/explant was observed when using 2.5, 3 g/l of gelrite, or 2.5, 3 g/l of phyta gel and there were no significant differences between

Table 3 The effect of gelling agents on direct shoot regeneration

Gelling agent	Concentration (g/l)	Shoot response	Number of shoots/explant	Shoot length
Agar	6	93.33±3.33a	2.50±0.17c	3.63±0.09c
	7	96.67±3.33a	3.23±0.20b	3.67±0.12c
	8	93.33±3.33a	3.27±0.09b	4.27±0.09b
	9	100.00±0.00a	3.20±0.12b	3.67±0.09c
Bactoagar	6	80.00±5.77b	3.23±0.15b	3.57±0.18c
	7	96.67±3.33a	3.30±0.12b	3.63±0.20c
	8	96.67±3.33a	3.23±0.15b	4.30±0.17b
	9	100.00±0.00a	3.17±0.15b	4.27±0.12b
Gelrite	1.5	96.67±3.33a	3.20±0.06b	4.30±0.12b
	2	100.00±0.00a	3.57±0.24ab	4.63±0.26ab
	2.5	100.00±0.00a	3.97±0.19a	4.93±0.12a
	3	100.00±0.00a	3.93±0.12a	4.90±0.10a
Phyta gel	1.5	100.00±0.00a	3.20±0.12b	4.30±0.12b
	2	93.33±3.33a	3,23±0.15b	4.30±0.10b
	2.5	100.00±0.00a	3.93±0.13a	4.97±0.15a
	3	100.00±3.33a	3.97±0.17a	4.90±0.12a

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

Figure 3



Shoot induction of caraway in media solidified with (a) 2.5 g/l gelrite, (b) 2.5 g/l phyta gel, (c) 7 g/l bactoagar, (d) 7 g/l agar.

them. Shoot length ranged from 3.63±0.09 to 4.97 ±0.15, also gelrite (with concentrations of 2.5 and 3 g/l) and phyta gel (with concentrations of 2.5 and 3 g/l) showed the highest shoot length (Table 3 and Figure 3).

Optimizing of transformation protocol using *Agrobacterium*-mediated transformation method

Sensitivity test of caraway to hygromycin

The toxicity level of hygromycin was optimized for caraway shoot tips. Shoot tips were cultivated on MS media supplemented with 5 µmol/l BA, 1 µmol/l NAA, 3% sucrose, and 2.5 g/l gelrite. Different concentrations of hygromycin (0, 10, 20, 30, and 40 mg/l) were evaluated. Evidently, from 30 mg/L and above was effective for completely deadening of explant. As a result, the concentration of 30 mg/l of hygromycin seemed suitable for the identification of putative transformants (Table 4). Healthy shoots that regenerated from the explant under hygromycin stress were then evaluated by PCR analysis to confirm the presence of the transgene.

Effect of different Agrobacterium optical density on transformation efficiency

The results showed that there was no significant difference when using *Agrobacterium* with an OD_{600} equal to 0.8 and 1, but transformation-efficiency percentage was slightly higher when OD_{600} equal to 0.8. The minimum transformation efficiency% was observed when bacteria OD_{600} equal to 0.6 or above 1 (Table 5).

Table 4 Hygromycin-resistance sensitivity on caraway shoot tips

Hygromycin concentration (mg/l)	Survival (%)
0	100.00±0.0a
10	70.00±5.77b
20	13.33±3.33c
30	0.0±0.00
40	0.0±0.00

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

Table 5 Effect of Agrobacterium OD on transformation-efficiency percentage

Agrobactrium OD_{600}	Transformation efficiency (%)
0.6	16.67±6.67b
0.8	30.00±5.77a
1	23.33±6.67ab
1.2	16.67±3.33b

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

Effect of inoculation period on transformation efficiency

Data regarding transformation-efficiency percentage showed significant differences among the inoculation times used. The maximum transformation efficiency% was observed when the inoculation period was 5 min (26.67±6.67 min) and 10 min (23.33±3.33 min) and statistical analysis indicated that no significant differences were detected between them. When the inoculation period was 2.5 or 20 min, the transformation-efficiency percentage was decreased to 13.33±3.33 (Table 6).

Effect of cocultivation days on transformation efficiency

The best treatments were 3 days with no significant difference with 2 days, while one and 4 days decreased the transformation efficiency (Table 7).

Effect of acetosyringone concentration on transformation efficiency

The minimum transformation efficiency% was observed when using 50 µmol/l acetosyringone, while there were no significant differences when using 100, 200, or 300 µmol/l from acetosyringone according to statistical analysis (Table 8).

PCR analysis for detection of putative-transformed caraway

Shoot tips infected by Agrobacterium were cultured on selection medium for 2 weeks. The hygromycin-resistance shoots that passed from selection stage were transferred to shooting medium for 4 weeks.

Table 6 Effect of inoculation period on transformation-efficiency percentage

Inoculation period (min)	Transformation efficiency (%)
2.5	13.33±3.33b
5	26.67±6.67a
10	23.33±3.33a
20	13.33±3.33b

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

Table 7 Effect of cocultivation days on transformation-efficiency percentage

Cocultivation days	Transformation efficiency (%)
1	20.00±5.77b
2	33.33±3.33a
3	36.67±6.67a
4	23.33±3.33b

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

Leaves of putative transgenic plants were evaluated using PCR with *DREB2A* primers. Lane 3–9 contained the PCR products from shoots transformed with *A. tumefaciens* strain LBA4404 carrying *DREB2A* gene. A single band of 825 base pair was observed on lanes 3–9 containing PCR product from putative-transformed caraway (Fig. 4). The detection of the *DREB2A* gene in putative-transformed caraway supported the observation that the transformed caraway survived on hygromycin-containing selection media and supported the success of the transformation event.

Discussion

The Carum genus is a very important genus that belongs to the Apiaceae family. Carum genus has ~25 species, of which caraway is the only annual and biennial economical one such as spice, aperitif, and carminative in food and pharmaceutical industries [7]. Caraway is used to treatment of digestive problems [20]. It also increases the urine output and total volume of extracted urine [7] and it has antifungal [21], antibacterial [6], antioxidant [11], anticonvulsant properties [22], anti-inflammatory [23], and antidiabetic activities [24]. Although the great importance of caraway, there were limited researches about its regeneration and transformation system. In this study, we established a regeneration and genetic transformation protocol by using the *Agrobacterium*-mediated method for caraway.

Table 8 Effect of different concentrations of acetosyringone on transformation-efficiency percentage

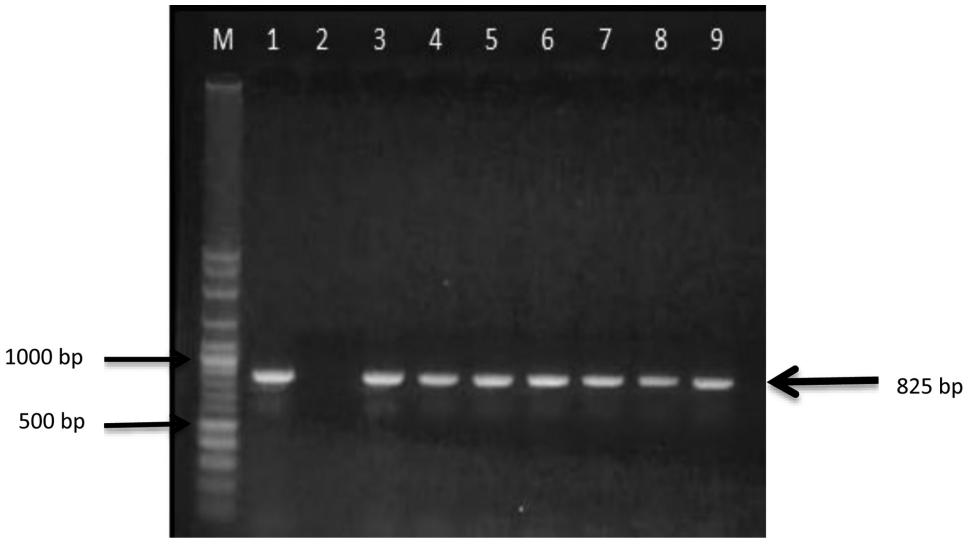
Acetosyringone concentration (mg/l)	Transformation efficiency (%)
50	26.67±3.33b
100	40.00±5.77a
200	36.67±3.33a
300	40.00±5.77a

Values represent mean±SE of three separate experiments with 10 replicates each. Values marked with the same letter within a column were not found to significantly differ using Duncan's multiple-range tests at a 5% probability level.

It is well known that the type of explant has a strong effect on the success of regeneration and transformation experiments. In fact, one of the easiest, most reliable, and effective methods for micropropagating of plant species, including other members of the Apiaceae family, is the use of preexisting meristems [25]. When using shoot tips as explants, they show high SR rate as they contain higher totipotency rate [26]. As a result, we used shoot tips as an explant for direct shoot micropropagation and we observed a high shoot response for most treatments, regardless to treatment type. According to Heidari *et al.* [27], shoot tips had a higher potential for shoot regeneration than other forms of explants and produced a high rate of shooting, which was complying with our results.

In our investigation, we found that the combined effect between BA and NAA not only improves the shoot multiplication rate but also promotes high shoot length. In agreement with our result, the combination between BA and NAA showed a great

Figure 4



PCR analysis using DREB2A primers. (M) DNA ladder (3000 base pair), lane 1 (positive control, plasmid), lane 2 (negative control, nontransformed plant), lane 3–9 (putative-transformed caraway plants).

effect on shoot regeneration with *Verbena officinalis L.* [28], *Thapsia garganica* [29], and *Pimpinella anisum L.* [30]. Also, the addition of BA alone or in combination with NAA improved the shoot-regeneration frequency in *Phragmites communis Trin* [31]. In general, auxins and cytokinins work effectively together to promote shoot and root development; higher cytokinin concentrations and lower auxin levels are frequently utilized as efficient stimulators for shoot regeneration.

The carbon source experiment supports our intention that it is very important to study the effect of different types of sugars. As observed in Table 2, the type and concentration of carbon source showed a high level of variations in shoot multiplication rate and shoot length. It was established that the plant kingdom's capacity to digest various kinds of carbohydrates is varied and this variation seems to be genotype-dependent [32]. According to studies evaluating the effects of various sugars on the growth of *in vitro* explants, sucrose has the most positive effect on plant development, which matches with our results. This discovery led to its widespread use as a carbon source in tissue culture [33,34]. Due to sucrose's high solubility in water, electrical neutrality, and absence of inhibitory effects on the majority of metabolic processes, sucrose has positive impacts on explant development under *in vitro* conditions [35].

The processes of plant tissue culture often take place in solid or semisolid nutrient media. A solid nutritional medium acts as soil and provides physical support for the culture to maintain contact with air for respiration. They affect important characteristics of the culture medium such as gel strength and each gelling agent has a different level of gel strength. For this reason, the type of gelling agent can influence the growth of the plant tissue culture. Plant growth will be directly influenced by the composition of gelling agents since it will favor the binding to some nutrients over others [36]. In our observation, gellan gum products were superior to agar products in shoot multiplication and this could happen for several reasons. The impurities present in agar may be the reason. The two polysaccharides that make up agar are linear agarose and agropectin, in which agarose forms about 70% of the mixture [37]. Agar that contains agropectins and other organic contaminants can limit the growth of explants. On the other hand, gellan gum is a highly purified natural gelling agent with high quality and it does not contain any of the impurities present in agar. In addition, smaller quantities of gellan gum are able to produce gels of comparable hardness to agar [38].

One of the most important parameters that affect transformation efficiency is Agrobacterium OD_{600}. When *Agrobacterium* concentration used to infect explant was extremely high, it was difficult to wash out the *Agrobacterium* from the explants after the infection process and would lead to frequent *Agrobacterium* contamination after cocultivation time, also, it will lead to the death of explant and decreasing the survival rate. Thus, it was preferable to select a suitable low concentration of bacteria with a high capacity for infection [39]. In this study, we found that 0.8 OD_{600} showed high transformation-efficiency percentage and appropriate *Agrobacterium* contamination level that could be easily removed by using cefotaxime. The same OD_{600} of *Agrobacterium* was successfully used with different plant types like sugarcane [40], winter jujube [41], and *Acacia crassicarpa* [42]. Although Krens *et al.* [17] reported that the preferred OD_{600} for the caraway-transformation system was 0.6, we found that this concentration was not effective in our protocol. This disagreement may arise for a number of reasons such as different types of explants and different bacterial strains used.

Cocultivation period was the most crucial period that enhanced the expression of transgenes. The time of cocultivation has a significant impact on the success of *Agrobacterium*-mediated transformation, according to numerous studies in diverse plant species [43]. Through our implementation, we found that cocultivation of 3 days gave the highest transformation-efficiency percentage and showed normal bacterial growth. For 4 cocultivation days, the explant started to convert to brown color and transformation efficiency was decreased. The browning of the explants might occur over a longer cocultivation period (>3 days), which agreed with our result [44]. Another study claimed that decreasing the temperature to 20°C and expanding the cocultivation period (5–7 days) greatly increased the transformation frequency [45]. The differences in cocultivation period may be greatly varied due to plant species, genotype, or type of explant used in the experiments.

Acetosyringone has been utilized frequently to increase the effectiveness of *Agrobacterium*-mediated transformation of plant species during infection and cocultivation [46]. The concentration of acetosyringone used for transformation varied from 20 to 400 μmol/l. It is noted that each explant type and genotype requires a different acetosyringone concentration in the inoculation and cocultivation media to achieve the best results for *Agrobacterium*-

mediated transformation [47]. In the present study, the addition of acetosyringone increased significantly the transformation efficiency when added during the infection process and in cocultivation media, but there is no significant difference when using 100, 200, or 300 µmol/l from it.

Throughout this study, we recommend that 5–10 min was the optimum period that allowed the infection process to happen. Other plant species, including sorghum, maize, and wheat, have reported a range of infection times, from 5 min (for maize and sorghum) to 50 min (for wheat), suggesting that the infection time varies according to the plant species and *Agrobacterium* strain [48]. The various *Agrobacterium* strains utilized would be the cause of this variation in infection time. Because of this, it is critical to detect an *Agrobacterium* strain with a high capacity for infection and to optimize the infection times for each plant species and cultivar.

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Conflicts of interest
There are no conflicts of interest.

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