Integrative effects of stress- and stress tolerance-inducing elicitors on in vitro bioactive compounds of ajowan [Trachyspermum ammi (L.) Sprague] medicinal plant

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Abstract
In the present study, the integrative effects of two sets of stress tolerance-inducing and stress-inducing elicitors, including polyethylene glycol–salicylic acid and NaCl–methyl jasmonate, were evaluated on regeneration efficiency, antioxidants activity and phytochemical profile of in vitro shoot cultures of ajowan. Combinations of salicylic acid (SA) (0, 10, 20, 40, 80 µM)–polyethylene glycol (PEG 6000) (0, 1, 2, 5%) and/or methyl jasmonate (MeJA) (0, 100, 200, 300 mg/L)–NaCl (0, 10, 15, 20 mM) were added to the shoot regeneration Murashige and Skoog medium containing KIN (1.50 mg/L) and NAA (0.25 mg/L) plant growth regulators. The number of regenerated shoots and the in vitro rooting decreased significantly with increasing PEG and NaCl levels. SA (40 µM) reduced the adverse effect of PEG on the number of regenerated shoots and in vitro rooting. The activities of catalase, superoxide dismutase and peroxidase enzymatic antioxidants were significantly increased in SA (80 µM)–PEG (5%) and MeJA (300 mg/L)–NaCl (20 mM) treated plants. The gas chromatography–mass spectrometry (GC–MS)-profiling revealed quantitative and qualitative phytochemical differences between control and SA–PEG and MeJA–NaCl treated plants. The greatest means of p-cymene and thymol bioactive compounds were obtained from in vitro shoots treated with 5% PEG + 40 µM SA. The interaction of PEG–SA was better than NaCl–MeJA elicitors in terms of the content of valuable γ-terpinene, p-cymene and thymol compounds. The inter-simple sequence repeats (ISSR) markers proved the genetic stability of in vitro regenerated plants. The presented protocol is useful for large-scale sustainable production of secondary metabolites (SMs) of medicinal plants.

Key message
Secondary metabolites are important plants defense molecules. Stress induction and/or defense machine stimulation are two practical strategies to increase the accumulation of secondary metabolites. The combined effects of stress tolerance-inducing...
salicylic acid and stress-inducing polyethylene glycol were evaluated in indirect shoot culture of ajowan. Levels of p-cymene and thymol increased in PEG–SA treated ajowan plants.

**Keywords** Antioxidants · Bioactive compounds · Elicitor · Indirect shoot · Secondary metabolites · Stress

**Abbreviations**

BBBMs  Biotechnology-based breeding methods  
CAT  Catalase  
DMRT  Duncan's multiple ranges test  
GC–MS  Gas chromatography–mass spectrometry  
ISSR  Inter-simple sequence repeats  
KIN  Kinetic  
MeJA  Methyl jasmonate  
MS  Murashige and Skoog medium  
NAA  Naphthalene acetic acid  
PEG  Polyethylene glycol  
PGR  Plant growth regulator  
POX  Peroxidase  
ROS  Reactive oxygen species  
SA  Salicylic acid  
SMs  Secondary metabolites  
SOD  Superoxide dismutase

**Introduction**

Secondary metabolites (SMs) are chemical defense molecules that secreted when plants are under biotic and abiotic stresses (Alvarado et al. 2019). Improving the growth and survival of plants under stressful conditions is the primary function of SMs (Khare et al. 2020). Accumulation of higher amounts of SMs is one of the plant responses to stressful conditions (Mahajan et al. 2020). Stress induction and/or defense machine stimulation are two practical strategies to increase the accumulation of plants defense molecules, mainly antioxidant SMs. Due to environmental, geographical and seasonal independency, in vitro culture systems have great potential to increase the accumulation of plant’s bioactive compounds in a sustainable manner (Chandran et al. 2020). In vitro culture systems provide controlled conditions for quantitative and qualitative alteration of SMs of medicinal plants. Different stress-inducing materials and defense-trigger biotic and abiotic elicitors can be easily added to the culture medium to stimulate the accumulation of SMs of medicinal plants. Polyethylene glycol (PEG), NaCl and heavy metal ions are examples of such stress-inducing additives. Adding cadmium chloride (CdCl₂) to the culture medium increased alliin content in in vitro cultures of garlic (*Allium sativum*) (Malik et al. 2020) and plumbagin in the callus culture of chitrak (*Plumbago zeylanica* L.) (Singh et al. 2020). The accumulation of secondary metabolites of shikimic acid, caffeic acid, p-coumaric acid and rosmarinic acid was increased significantly in the cell suspension culture of *Salvia miltiorrhiza* by adding 50 mmol/L of NaCl to the culture medium (Yu et al. 2019). The positive effect of PEG 6000 (4%) on the total phenolic content, total flavonoid content and total antioxidant capacity of shoot cultures of *Stevia rebaudiana* has been reported (Ahmad et al. 2020). Applying elicitors, which stimulate the plant stress response (defense-trigger elicitors), is another important strategy to enhance the accumulation of SMs of medicinal plants in in vitro culture systems. Stress hormones (signaling compounds), including abscisic acid, jasmonic acid, methyl jasmonate (MeJA), and salicylic acid (SA), are the most important group of elicitors that have been applied to improve SMs accumulation in medicinal plants in vitro cultures (Thakur et al. 2019). Application of SA (1.0 mg/L) resulted in a significant increase in bacoside content in the cell suspension culture of *Bacopa monnieri* (Koul and Mal-lubhotla 2020).

Simultaneous application of stress- and stress tolerance-inducing elicitors in the culture medium is the third strategy to increase the accumulation of SMs of medicinal plants in in vitro culture systems. The integrative application of these two types of elicitors may be more effective than their individual application. The stress tolerance-inducing elicitors alleviate the harmful effects of stress-inducing elicitors on plant growth and development and subsequently increase their efficiency on SMs accumulation (Razavizadeh et al. 2020). The positive effects of concomitant use of stress-inducing and stress tolerance-inducing elicitors on in vitro production of SMs of *Verbascum sinuatum* (Karamian et al. 2020) and basil (*Ocimum basilicum* L.) (Nazir et al. 2020) have been reported.

Ajowan (*Trachyspermum ammi* L.) is one of the valuable medicinal plants of the *Apiaceae* family. Ajowan seeds have essential oil that contains about 50% of thymol. Thymol is a valuable bioactive compound with strong germicide, anti-spasmodic and fungicidal effects (Niaizian et al. 2018). P-cymene and γ-terpinene are other majorly present bioactive compounds in the essential oil of ajowan, which also the major precursors for thymol and carvacrol biosynthesis (Soltani Howzyeh et al. 2018a). Ajowan has a little area under cultivation in some of its major habitats such as Iran. In vitro production systems are a promising alternative to meet the growing demands for valuable bioactive compounds of ajowan and prevent the risk of extinction in the future (Niaizian et al. 2017). Different in vitro-based methods, including artificial tetraploidy induction (Noori et al. 2017), genetic engineering (Niaizian et al. 2019; Nomani et al. 2019), and biotic–abiotic elicitation (Razavizadeh...
et al. 2020), have been applied to improve the valuable bioactive compounds of ajowan. Despite their adverse effects on yield and yield components, abiotic stresses can increase the SMs of ajowan. Therefore, increasing the stress tolerance of ajowan is an applicable strategy to increase its SMs content under stressful conditions (Niazian et al. 2019). The present study was conducted to evaluate the effect of SA and MeJA elicitors on regeneration efficiency, enzymatic antioxidants activity and secondary metabolite production in shoot cultures of ajowan under the artificially induced drought (PEG 6000) and salinity (NaCl) stresses, respectively.

Materials and methods

Plant material and indirect regeneration procedure

Fifteen days old in vitro-obtained hypocotyl segments of Shiraz ecotype of ajowan were used as initial explants for callus induction. Five hypocotyls segments (0.5–1 cm) were placed in Petri dishes containing 30 mL of full-strength Murashige and Skoog (MS) medium supplemented with 0.20 mg/L naphthalene acetic acid (NAA) and 1.50 mg/L 6-benzylaminopurine (BAP). Petri dishes were incubated in a phytotron with 16/8 h (light/dark) photoperiod, photosynthetic photon flux density (PPFD) of 40 µmol/m²/s, and 24 °C. Callus induction of established hypocotyls was initiated after 15 days and three rounds of subcultures (each for 4 weeks) were done in the same medium. For shoot regeneration, induced calli were transferred to MS medium supplemented with 1.50 mg/L of Kinetin (KIN) plus 0.25 mg/L NAA. The complete details of the indirect shoot regeneration procedure of ajowan have been elaborated in Niazian et al. (2017).

Elicitation of in vitro shoot cultures

Two separate experiments were conducted to evaluate the effect of SA and MeJA elicitation on enzymatic antioxidants and bioactive compounds of in vitro regenerated shoots of ajowan under artificially induced drought and salinity stresses, respectively. Combination of different concentrations of SA (0, 10, 20, 40, 80 µM)–PEG 6000 (0, 1, 2 and 5%) and/or MeJA (0, 100, 200, 300 mg/L)–NaCl (0, 10, 15, 20 mM) were applied in shoot regeneration medium. The MS medium containing KIN (1.50 mg/L), NAA (0.25 mg/L), 3% (w/v) sucrose and 0.7% (w/v) agar (pH 5.8) was used as control. The culture medium was autoclaved at 121 °C for 20 min and then filter-sterilized (0.20 µm) elicitors were added to the medium under the laminar airflow chamber. Five callus segments (~ 200 mg) were cultured in Petri dishes containing 30 mL of MS medium supplemented with the aforementioned concentrations of KIN and NAA plant growth regulators (PGR) along with different combinations of PEG–SA and/or NaCl–MeJA elicitors. Both experiments were arranged as factorial, based on a completely randomized design (CRD), with three replications (as Petri dishes) and five samples per replication. All cultures were incubated in phytotron with the aforementioned condition for the callus induction procedure. Subcultures were done with 2 weeks intervals and the main and interaction effects of applied elicitors on the number of regenerated shoots were investigated in the seventh week of the experiment.

For root induction, the regenerated shoots (~ 4–5 cm height) were transferred to a half-strength PGR-free MS medium. Culture vessels were maintained in a phytotron with the aforementioned condition for the callus induction and shoot regeneration. The combined effect of PEG–SA and NaCl–MeJA on the rooting percentage of in vitro regenerated shoots was evaluated after 2 weeks.

Acclimatization

The well-developed roots (~ 3 cm) of regenerated plants were first washed with distilled sterile water to remove the traces of agar. Then, plantlets were transferred to plastic pots (200 mL) containing 1:1 perlite:peat moss. The pots were covered with transparent plastic and irrigated with half-strength MS medium for 1 week and then covers were removed for complete acclimatization. The successfully acclimatized plantlets were then transferred to larger plastic pots (8 × 10 cm) filled with autoclaved farm soil. The greenhouse established plants were grown and after 2 months starts to flowering. In the next 2 months, reached seeds were gathered and used for essential oil extraction and phytochemical assessments.

Measurement of enzymatic antioxidants

Fresh leaf samples protein was extracted by Sudhakar et al. (2001) method. For this purpose, a leaf sample (0.1 g) of acclimatized plantlets was homogenized in 2 mL tubes containing 20 mg of polyvinylpyrrolidone (PVP). Then, 1 mL of extraction buffer [Potassium phosphate (50 mM) + sodium sulfite (1 mM)] was added to the samples. After vortexing, samples were incubated at 4 °C for 30 min and then centrifuged at 12,000 rpm (Sudhakar et al. 2001). After centrifuging, the supernatant was isolated to measure antioxidant enzyme activities. The activity of the catalase (CAT) enzyme was measured using the Aebi (1974) method based on the rate of hydrogen peroxide (H₂O₂) decomposition. The reduction of absorbance at 240 nm was measured and activity was expressed as unit/mg protein. The activity of peroxidase (POX) was estimated using Hemededa and Klein (1990) method. The reaction mixture was prepared by mixing 10 mmol/L H₂O₂, 0.05% guaiacol enzyme and 25 mmol/L phosphate buffer (pH 7) and the activity of the...
enzyme was determined by determining the absorbance at 470 nm. Superoxide dismutase (SOD) activity was measured using Beauchamp and Fridovich (1971) method. The assay mixture, including 300 µL of the extracts in addition to 50 mM sodium phosphate buffer (pH 7), 12 mM l-methionine, 50 mM Na2CO3, 1 µM riboflavin, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 75 µM p-nitro blue tetrazolium chloride was prepared. The SOD activity unit was identified as the amount of enzyme needed to prevent a 50% (w/v) p-nitro blue tetrazolium chloride photo-reduction increase in absorbance at 560 nm.

**Essential oil extraction and gas chromatography–mass spectrometry (GC–MS) analysis**

For essential oil extraction, a seed sample (20 g), consist of a mixture of three plants from each group of in vitro regenerated plants, was ground using an electric grinder, then achieved fine powder was added to 500 mL distilled water on top of a heater at 100 °C and oil was extracted using a Clevenger-type 5 apparatus (Noori et al. 2017) for 2.5 h. Essential oil extraction was conducted in three replications. For GC–MS, 1 µL of the essential oil samples was injected into the GC split-less with the injection port. The GC–MS analysis was conducted in three replications. A GC–MS apparatus using the HP (Agilent Technology): 6890 Network GC System gas chromatograph connected to a mass detector (5973 Network Mass Selective Detector) was used for GC–MS analysis. The gas chromatograph was equipped with an HP-5MS capillary column (fused silica column, 30 m × 0.25 mm i.d., Agilent Technologies) and an EI mode with ionization energy of 70 eV with a scan time of 0.4 s and mass range of 40–460 amu was used. Helium gas was a carrier and its flow rate was 1.0 mL/min. The oven temperature was linearly programmed from 40 to 250 °C at a rate of 3 °C/min. The composition of the essential oil constituents was identified based on the comparison of their retention time relative to n-alkanes and computer matching with the NIST (National Institute of Standards and Technology) Adams library spectra, Wiley 7 n.1 mass computer library, and with those reported in the literature (Adams 1997). In the final, area under the curve of GC/MS spectra was used to calculate the relative percentage of each component (Soltani Howyzeh et al. 2018b; Noori et al. 2017).

**Genetic stability assessment using inter simple sequence repeats (ISSR) markers**

DNA samples of 3 weeks old regenerated plants (mixed DNAs of leaves of 10 plants), were used to assess the genetic stability of in vitro regenerants under the effect of applied elicitors. DNA samples of seed obtained plants were used as control. The DNA extraction was done using a CTAB-activated charcoal protocol described by Križman et al. (2006). Ten UBC primers of Set #9 (University of British Columbia, Vancouver, Canada) were used as ISSR markers (Table 1). The PCR amplifications were carried out using a C-1000 thermal cycler (Bio-Rad, Hercules, USA) following the protocol described by Rawat et al. (2013). PCR amplified products were separated through an agarose gel (1.5%) in 0.5 × TBE buffer stained with ethidium bromide under 100 V constant power supply for 1.5 h and visualized under UV light in a gel documentation system (Cleaver Scientific Ltd., Rugby, UK). The polymorphism percentage was calculated as the fraction of total polymorphic bands on the total number of amplified bands (Table 1).

**Table 1 Information of UBC ISSR primers applied to evaluate the genetic stability of in vitro regenerated ajowan medicinal plants under the effect of applied elicitors**

| No. | Primer code | Primer sequence (5’–3’) | Tm (°C) | Number of allele/primer | Number of monomorphic bands | Number of polymorphic bands | Total number of amplified bands/primer | Size range (bp) |
|-----|-------------|-------------------------|---------|------------------------|-----------------------------|-------------------------------|--------------------------------------|----------------|
| 1   | UBC 815     | (CT)₈G                  | 52      | 4                      | 3                           | 1                            | 27                                   | 600–1400        |
| 2   | UBC 817     | (CA)₉A                  | 52      | 2                      | 2                           | 0                            | 14                                   | 600–1200        |
| 3   | UBC 820     | (GT)₉T                  | 52      | 2                      | 2                           | 0                            | 14                                   | 1000–1400       |
| 4   | UBC 826     | (AC)₉C                  | 52      | 4                      | 3                           | 1                            | 27                                   | 300–1400        |
| 5   | UBC 829     | (TG)₉C                  | 53      | 4                      | 4                           | 0                            | 28                                   | 500–1300        |
| 6   | UBC 836     | (AG)₈YA                 | 52      | 3                      | 2                           | 1                            | 20                                   | 400–1200        |
| 7   | UBC 845     | (CT)₉RG                 | 54      | 4                      | 4                           | 0                            | 28                                   | 300–1300        |
| 8   | UBC 857     | (AC)₉YG                 | 54      | 3                      | 3                           | 0                            | 21                                   | 350–1200        |
| 9   | UBC 859     | (TG)₉RC                 | 55      | 3                      | 2                           | 1                            | 20                                   | 400–1400        |
| 10  | UBC 860     | (TG)₉RA                 | 52      | 4                      | 3                           | 1                            | 27                                   | 500–1300        |
| Total| 33          | 28                      | 5       | 226                    |                             |                               |                        |
Statistical analysis

All statistical analyses of the present study, including analysis of variance (ANOVA) and means comparison analysis, were conducted using SAS® software (SAS Institute, Inc., Cary, NC). All experiments were performed with three biological replications. The normality test was conducted with SAS software before the analysis of variance. The Duncan’s multiple ranges test (DMRT) at a 5% ($P \leq 0.05$) probability level was used for the means comparisons analysis. A dendrogram was constructed by using NTSYSpc (V. 2.02e) software based on ISSR results.

Results

Indirect shoot regeneration and rooting of regenerated shoots under the effect of PEG–SA and NaCl–MeJA elicitors

Proliferated calli were obtained in MS medium supplemented with NAA (0.20 mg/L) and BAP (1.50 mg/L) at the end of the second week of the experiment (Fig. 1a). Induced calli were then transferred to the shoot induction medium. The first regenerated shoots were observed on MS medium containing NAA (0.25 mg/L) and KIN (1.50 mg/L) PGRs along with combinations of PEG and SA (Fig. 1b). Regenerated shoots showed visual symptoms of wilting in a culture medium containing PEG treatment (Fig. 1c).

The results of means comparison analysis, using DMRT at 5% probability level, showed a significant decrease in the number of regenerated shoots with increasing concentrations of PEG and NaCl in the culture medium (Fig. 2a, c). In both experiments, the greatest means of the number of regenerated shoots were observed in the control treatment (MS medium containing NAA and KIN PGRs), whereas, the lowest means of regenerated shoots were obtained in MS medium containing the highest concentration of PEG (5%)–SA (80 µM) (Fig. 2a) and NaCl (20 mM)–MeJA (300 mg/L) (Fig. 2c). At a certain concentration of PEG, SA treatment caused a significant increase in the number of regenerated shoots (Fig. 2a). The regenerated shoots were transferred to the root induction medium (Fig. 3a). Developed roots were observed after 2 weeks of establishment in PGR-free MS medium (Fig. 3b). The results of means comparison analysis revealed the significant adverse effect of both PEG and NaCl treatments on root induction percentage of in vitro regenerated shoots of ajowan. The lowest rooting percentage was obtained by the highest degree of drought (5% PEG 6000) (Fig. 2b) and salinity (20 mM NaCl) (Fig. 2d) stresses. The greatest rooting percentage was obtained in the control treatment. Salicylic acid showed a positive effect on the rooting percentage of PEG-treated shoots as the greatest mean of rooting percentage under severe drought stress (5% PEG 6000) was obtained by using 40 µM of SA (Fig. 2b). MeJA elicitor was not able to compensate for the harmful effects of NaCl on root induction of regenerated shoots and no rooting was observed at the highest level (20 mM) of NaCl (Fig. 2d).

The successfully acclimatized plantlets were transferred to a greenhouse (24 ± 1 °C and 90% relative humidity), along with intact (seed obtained) plants, for flowering and seed maturation. There was no obvious morphological difference between in vitro micropropagated and seed obtained plants of ajowan (Fig. 4). The acclimatization rate of in vitro regenerated ajowan plants was 85%.
Antioxidative enzymes activity under the effects of PEG and SA elicitors

A substantial increase in CAT activity was observed with increasing levels of PEG and SA in the shoot induction medium (Table 2). The greatest mean of CAT activity was observed in shoots regenerated in MS medium containing the highest concentrations of applied PEG 6000 (5%) and SA (80 µM). Under the severe artificial drought stress (5% PEG), there was no significant difference between 40 and 80 µM of SA, based on the DMRT test (Table 2). The highest POX activity was observed at severe artificial drought stress, whereas the lowest activity was observed in the control treatment. Salicylic acid under PEG treatment induced a significant increase in POX activity (Table 2). In non-stress condition (0% PEG), SA treatment increased the activity of POX, however, there was no significant difference between applied concentrations of SA at the 5% probability level (Table 2).

Antioxidative enzymes activity under the effects of NaCl and MeJA elicitors

The activity of CAT, SOD and POX antioxidative enzymes was significantly increased with increasing levels of NaCl and MeJA elicitors in the shoot induction medium (Table 3). The greatest means of mentioned antioxidative enzymes were obtained by the interaction of the highest concentrations of NaCl (20 mM) and MeJA (300 mg/L) (Table 3). The lowest means of CAT, SOD and POX were observed in control medium (0 mM NaCl + 0 mg/L MeJA) (Table 3).
The phytochemical profile of ajowan under the effects of PEG and SA elicitors

The results of GC–MS analysis showed significant quantitative and qualitative differences in the composition of the essential oils of in vitro PEG–SA treated plants with plants regenerated in the control medium (Fig. 5a, b). Thymol, γ-terpinene, and p-cymene were the main components in the essential oil of in vitro regenerated ajowan plants (Table 4). The major qualitative difference of in vitro regenerated plants was related to Sabinene that missed in the phytochemical profile of in vitro regenerated shoots treated with high levels of PEG 6000 (2 and 5%) (Table 4). The α-thujene percentage of non-treated plants was less than the PEG and SA treated shoots (Table 4).

Based on the results of GC–MS analysis, the p-cymene of in vitro micropropagated plants was increased with increasing levels of PEG and SA (Table 4). The greatest mean of p-cymene was observed when 5% PEG along with 40 µM of SA were added to the shoot induction medium (Table 4). Another major component of essential oil, γ-terpinene, in PEG and SA treated shoots was more than the control plants, however, there were no significant differences among applied PEG and SA concentrations in terms of this bioactive compound (Table 4). The combination of PEG and SA had a significant positive effect on the amount of thymol. The greatest mean of thymol content (53.15%) was obtained with the application of SA (40 µM) under severe drought stress treatment (5% PEG). The lowest mean of the thymol content was obtained in plants regenerated under control treatment (Table 4).

The phytochemical profile of ajowan under the effects of NaCl and MeJA elicitors

The results of GC–MS analysis revealed that NaCl and MeJA elicitors enhanced the contents of and p-cymene, γ-terpinene and thymol in in vitro regenerated shoots of ajowan (Table 5). The greatest mean of p-cymene was obtained from the interaction of NaCl (20 mM) and MeJA (300 mg/L), whereas the greatest means of γ-terpinene and Thymol were obtained from the interaction of NaCl (20 mM) and MeJA (200 mg/L) (Table 5). Sabinene was lost in the phytochemical profile of in vitro regenerated ajowan shoots treated with NaCl (Table 5).

Genetic stability assessment

Ten amplified ISSR primers produced 226 scorable bands from in vitro regenerated and control plants of ajowan. An average of 3 bands per primer, ranging from 2 to 4 bands, with only 2.21% of polymorphism was observed (Fig. 6a, S1, S2). The dendrogram obtained from ISSR primers analysis...
### Table 2
Effect of in vitro drought stress and salicylic acid elicitor on the catalase, superoxide dismutase and peroxidase activity in indirect regenerated shoots of ajowan

| PEG 6000 concentration (%) | Salicylic acid concentration (µM) | Catalase (unit/mg protein) | Superoxide dismutase (unit/mg protein) | Peroxidase (unit mg/protein/min) |
|---------------------------|----------------------------------|---------------------------|----------------------------------------|---------------------------------|
| 0                         | 0                                | 0.56±0.02<sup>m</sup>     | 127.33±0.27<sup>f</sup>               | 0.40±0.00<sup>g</sup>          |
| 10                        | 0                                | 0.76±0.11<sup>l</sup>     | 125.67±1.66<sup>g</sup>               | 0.52±0.02<sup>k</sup>          |
| 20                        | 0                                | 0.76±0.01<sup>i</sup>     | 130.67±0.66<sup>g</sup>               | 0.57±0.00<sup>b</sup>          |
| 40                        | 0                                | 0.81±0.01<sup>h</sup>     | 133.00±0.57<sup>g</sup>               | 0.65±0.01<sup>i</sup>          |
| 80                        | 1                                | 1.03±0.03<sup>j</sup>     | 131.67±0.88<sup>g</sup>               | 0.71±0.00<sup>f</sup>          |
| 0                         | 10                               | 0.93±0.03<sup>ik</sup>    | 135.33±1.66<sup>g</sup>               | 0.74±0.00<sup>b</sup>          |
| 10                        | 0.88±0.01<sup>il</sup>           | 97.33±3.66<sup>b</sup>    | 0.75±0.00<sup>h</sup>                 |                                 |
| 20                        | 0.93±0.03<sup>ik</sup>           | 138.67±0.88<sup>g</sup>   | 0.78±0.00<sup>b</sup>                 |                                 |
| 40                        | 1.13±0.03<sup>i</sup>            | 143.00±1.00<sup>g</sup>   | 0.76±0.01<sup>h</sup>                 |                                 |
| 80                        | 1.33±0.12<sup>h</sup>            | 145.67±0.66<sup>g</sup>   | 0.76±0.02<sup>i</sup>                 |                                 |
| 1                         | 0                                | 1.46±0.03<sup>eh</sup>    | 148.33±0.33<sup>d</sup>               | 0.82±0.00<sup>f</sup>          |
| 10                        | 1.53±0.03<sup>ef</sup>           | 150.67±0.66<sup>g</sup>   | 0.84±0.02<sup>g</sup>                 |                                 |
| 20                        | 1.60±0.00<sup>fg</sup>           | 153.00±0.00<sup>f</sup>   | 0.87±0.00<sup>f</sup>                 |                                 |
| 40                        | 1.71±0.01<sup>e</sup> f<sup>if</sup> | 167.00±0.01<sup>bo</sup>  | 0.88±0.00<sup>f</sup>                 |                                 |
| 80                        | 1.78±0.01<sup>ef</sup>           | 171.00±1.00<sup>ce</sup>  | 0.90±0.00<sup>f</sup>                 |                                 |
| 5                         | 0                                | 1.86±0.03<sup>cd</sup>    | 175.67±0.33<sup>d</sup>               | 1.06±0.03<sup>ef</sup>         |
| 10                        | 1.93±0.03<sup>bc</sup>           | 179.33±0.33<sup>bc</sup>  | 1.13±0.03<sup>cd</sup>                |                                 |
| 20                        | 2.03±0.03<sup>b</sup>            | 186.67±1.66<sup>b</sup>   | 1.26±0.03<sup>cd</sup>                |                                 |
| 40                        | 2.33±0.08<sup>a</sup>            | 240.00±0.00<sup>e</sup>   | 1.43±0.06<sup>cd</sup>                |                                 |
| 80                        | 2.46±0.03<sup>a</sup>            | 245.00±2.88<sup>d</sup>   | 1.73±0.03<sup>cd</sup>                |                                 |

Induced calli were cultured on MS medium containing 0.25 mg/L NAA and 1.5 mg/L KIN along with combinations of PEG 6000 (0, 1, 2, 5%) and salicylic acid (0, 10, 20, 40, 80 µM) for indirect shoot regeneration. Values represent the mean± standard error of three biological replicates. Values followed by the same letters are not significantly different at the $P < 0.05$.

### Table 3
Effect of in vitro salinity stress and methyl jasmonate elicitor on the catalase, superoxide dismutase and peroxidase activity in indirect regenerated shoots of ajowan

| NaCl concentration (mM) | Methyl jasmonate concentration (mg/L) | Catalase (unit/mg protein) | Superoxide dismutase (unit/mg protein) | Peroxidase (unit mg/protein/min) |
|-------------------------|---------------------------------------|---------------------------|----------------------------------------|---------------------------------|
| 0                       | 0                                     | 0.50±0.00<sup>k</sup>     | 126.33±0.33<sup>k</sup>               | 0.36±0.03<sup>l</sup>          |
| 10                      | 0.60±0.05<sup>d</sup>                 | 127.33±0.33<sup>k</sup>   | 0.43±0.03<sup>l</sup>                 |                                 |
| 200                     | 0.68±0.01<sup>h</sup>                 | 129.33±0.33<sup>jk</sup>  | 0.53±0.03<sup>h</sup>                 |                                 |
| 300                     | 0.74±0.00<sup>g</sup>                 | 132.33±0.33<sup>j</sup>   | 0.62±0.03<sup>g</sup>                 |                                 |
| 10                      | 0.86±0.01<sup>h</sup>                 | 140.33±0.33<sup>g</sup>   | 0.67±0.00<sup>h</sup>                 |                                 |
| 100                     | 0.94±0.01<sup>g</sup>                 | 142.33±0.33<sup>jh</sup>  | 0.70±0.00<sup>h</sup>                 |                                 |
| 200                     | 0.96±0.01<sup>g</sup>                 | 144.66±0.33<sup>h</sup>   | 0.72±0.00<sup>g</sup>                 |                                 |
| 300                     | 1.00±0.00<sup>g</sup>                 | 146.33±0.33<sup>jh</sup>  | 0.76±0.00<sup>g</sup>                 |                                 |
| 15                      | 0.32±0.00<sup>f</sup>                 | 149.33±0.66<sup>fg</sup>  | 0.86±0.00<sup>d</sup>                 |                                 |
| 100                     | 1.40±0.00<sup>f</sup>                 | 150.66±0.33<sup>gf</sup>  | 0.88±0.00<sup>d</sup>                 |                                 |
| 200                     | 1.54±0.01<sup>f</sup>                 | 154.00±1.00<sup>f</sup>   | 0.91±0.00<sup>d</sup>                 |                                 |
| 300                     | 1.53±0.00<sup>f</sup>                 | 160.00±5.00<sup>f</sup>   | 0.93±0.00<sup>d</sup>                 |                                 |
| 20                      | 1.76±0.03<sup>de</sup>                | 176.00±1.52<sup>c</sup>   | 1.06±0.03<sup>de</sup>                |                                 |
| 100                     | 1.93±0.03<sup>de</sup>                | 182.33±0.33<sup>e</sup>   | 1.23±0.03<sup>de</sup>                |                                 |
| 200                     | 2.06±0.06<sup>b</sup>                 | 240.66±0.66<sup>d</sup>   | 1.50±0.00<sup>e</sup>                 |                                 |
| 300                     | 2.26±0.03<sup>a</sup>                 | 243.00±1.00<sup>e</sup>   | 1.50±0.05<sup>e</sup>                 |                                 |

Induced calli were cultured on MS medium containing 0.25 mg/L NAA and 1.5 mg/L KIN along with combinations of NaCl (0, 10, 15, 20 mM) and methyl jasmonate (0, 10, 200, 300 mg/L) for indirect shoot regeneration. Values represent the mean± standard error of three biological replicates. Values followed by the same letters are not significantly different at the $P < 0.05$. 

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showed the similarity among in vitro regenerants and also their similarity with control plant of ajowan (Fig. 6b).

**Discussion**

The plant cell/tissue/organ culture is one of the promising biotechnology-based breeding methods (BBBMs) for the constant production of SMs of medicinal plants (Niazian 2019). The traditional in vitro techniques (adding different components such as PGRs, precursors, elicitors and other additives to the culture medium) are simpler and cost-effective than complicate BBBMs, such as genetic engineering and synthetic biology, to manipulate the phytochemical profile of medicinal plants.

Differentiated (organogenesis) and undifferentiated (callus) culture systems have been applied for in vitro production of the bioactive compounds of medicinal plants. Callus cultures have great potential in this regard (Koufan et al. 2020). However, undifferentiated calli are not as promising as organogenesis cultures in terms of SMs production (Santos et al. 2020). The superiority of differentiated cultures over the callus culture systems, in terms of the level of produced bioactive compounds, has been reported in *Schisandra henryi* (Jafernik et al. 2020), *Phellodendron chinense* (He et al. 2020) and *Argania spinosa* (L.) (Koufan et al. 2020). These observations could be due to the effect of developmental factors on SMs of medicinal plants, as the major of secondary metabolites often occur at a certain stage of plant growth. In addition, there is a relationship between morphogenesis and synthesis and accumulation of SMs in medicinal plants (Li et al. 2020).

The application of biotic and abiotic elicitors in culture medium, which induces defense-related metabolic pathways, is one of the creative strategies to change the biochemical profile of medicinal plants and enhance the accumulation of SMs. The key to successful elicitation is the identification of the best elicitor for a specific plant. The chemical composition and concentration of the elicitor, as well as the timing of its application, are critical factors in elicitation processes. The efficacy of elicitation can be assessed through the production of bioactive compounds, which can be quantified using various analytical techniques such as GC–MS. Figure 5 presents the GC–MS profiling of in vitro regenerated plants in control and under the effect of applied elicitors. The GC–MS chromatograms reveal the changes in the phytochemical profile of the plants, indicating the potential of elicitation in enhancing the production of bioactive compounds.
Table 4  The phytochemical profile of in vitro regenerated plants of ajowan under the effects of drought stress and salicylic acid elicitor

| PEG concentration (%) | Salicylic acid concentration (µM) | α-Thujene | α-Pinene | Sabinene | β-Pinene | β-Myrcene | α-Terpine | p-Cymene | β-Phellandrene | γ-Terpinene | Terpinene-4-ol | Thymol |
|-----------------------|-----------------------------------|-----------|----------|----------|----------|----------|----------|----------|----------------|-------------|---------------|--------|
| 0                     | 0                                 | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> | 0.24 ± 0.00<sup>b</sup> |
| 10                    | 0                                 | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> | 0.25 ± 0.00<sup>b</sup> |
| 20                    | 0                                 | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> | 0.26 ± 0.00<sup>b</sup> |
| 40                    | 0                                 | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> | 0.28 ± 0.00<sup>b</sup> |
| 80                    | 0                                 | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> | 0.31 ± 0.00<sup>b</sup> |
| 5                     | 0                                 | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> | 0.39 ± 0.00<sup>b</sup> |

Induced calli were cultured on MS medium containing 0.25 mg/L NAA and 1.5 mg/L KIN along with combinations of PEG 6000 (0, 1, 2, 5%) and salicylic acid (0, 10, 20, 40, 80 µM) for indirect shoot regeneration. Values represent the mean ± standard error of three biological replicates. Values followed by the same letters are not significantly different at the *P* < 0.05.
Table 5 The phytochemical profile of in vitro regenerated plants of ajowan under the effects of salinity stress and methyl jasmonate elicitor

| NaCl concentration (mM) | Methyl jasmonate concentration (mg/L) | α-Thujene | α-Pinene | Sabinene | β-Pinene | β-Myrcene | α-Terpinene | p-Cymene | β-Phellandrene | γ-Terpinene | Terpinene-4-ol | Thymol |
|------------------------|--------------------------------------|----------|---------|---------|---------|----------|------------|---------|----------------|------------|----------------|--------|
| 0                      | 0                                    | 0.21 ± 0.00  | 0.18 ± 0.00  | 0.21 ± 0.00  | 1.58 ± 0.00  | 0.43 ± 0.00  | 0.16 ± 0.00  | 0.22 ± 0.00  | 0.22 ± 0.00 | 27.72 ± 0.00  | 0.36 ± 0.00  | 41.36 ± 0.00 |
| 100                    | 0                                    | 0.23 ± 0.00  | 0.19 ± 0.00  | 0.21 ± 0.00  | 1.55 ± 0.00  | 0.43 ± 0.00  | 0.16 ± 0.00  | 0.23 ± 0.00  | 0.23 ± 0.00 | 27.12 ± 0.00  | 0.36 ± 0.00  | 41.36 ± 0.00 |
| 200                    | 0                                    | 0.23 ± 0.00  | 0.19 ± 0.00  | 0.18 ± 0.00  | 1.53 ± 0.01  | 0.44 ± 0.01  | 0.16 ± 0.00  | 0.24 ± 0.00  | 0.24 ± 0.00 | 27.35 ± 0.01  | 0.37 ± 0.00  | 41.66 ± 0.00 |
| 300                    | 0                                    | 0.23 ± 0.00  | 0.20 ± 0.00  | 0.16 ± 0.00  | 1.52 ± 0.00  | 0.47 ± 0.00  | 0.16 ± 0.00  | 0.25 ± 0.00  | 0.25 ± 0.00 | 27.35 ± 0.00  | 0.37 ± 0.00  | 41.64 ± 0.01 |
| 10                     | 0                                    | 0.25 ± 0.00  | 0.23 ± 0.00  | 0.23 ± 0.00  | 1.62 ± 0.00  | 0.48 ± 0.00  | 0.17 ± 0.00  | 0.26 ± 0.00  | 0.26 ± 0.00 | 27.44 ± 0.01  | 0.38 ± 0.00  | 43.43 ± 0.11 |
| 100                    | 0                                    | 0.25 ± 0.00  | 0.22 ± 0.00  | 0.23 ± 0.00  | 1.62 ± 0.01  | 0.49 ± 0.00  | 0.17 ± 0.00  | 0.27 ± 0.00  | 0.27 ± 0.00 | 27.10 ± 0.32  | 0.38 ± 0.00  | 43.87 ± 0.00 |
| 200                    | 0                                    | 0.26 ± 0.00  | 0.23 ± 0.00  | 0.23 ± 0.00  | 1.60 ± 0.00  | 0.50 ± 0.00  | 0.18 ± 0.00  | 0.28 ± 0.01  | 0.28 ± 0.01 | 27.37 ± 0.10  | 0.38 ± 0.00  | 43.99 ± 0.05 |
| 300                    | 0                                    | 0.27 ± 0.00  | 0.24 ± 0.00  | 0.24 ± 0.00  | 1.62 ± 0.02  | 0.52 ± 0.00  | 0.18 ± 0.00  | 0.30 ± 0.00  | 0.30 ± 0.00 | 27.55 ± 0.11  | 0.40 ± 0.00  | 44.09 ± 0.00 |
| 15                     | 0                                    | 0.32 ± 0.00  | 0.27 ± 0.00  | 0.27 ± 0.00  | 1.68 ± 0.00  | 0.56 ± 0.00  | 0.17 ± 0.00  | 0.33 ± 0.00  | 0.33 ± 0.00 | 27.62 ± 0.04  | 0.42 ± 0.00  | 44.84 ± 0.04 |
| 100                    | 0                                    | 0.32 ± 0.00  | 0.28 ± 0.00  | 0.28 ± 0.00  | 1.68 ± 0.00  | 0.57 ± 0.00  | 0.17 ± 0.00  | 0.35 ± 0.00  | 0.35 ± 0.00 | 27.66 ± 0.00  | 0.43 ± 0.00  | 44.93 ± 0.01 |
| 200                    | 0                                    | 0.34 ± 0.00  | 0.28 ± 0.00  | 0.28 ± 0.00  | 1.69 ± 0.00  | 0.58 ± 0.00  | 0.17 ± 0.00  | 0.38 ± 0.00  | 0.38 ± 0.00 | 27.45 ± 0.16  | 0.43 ± 0.00  | 45.38 ± 0.06 |
| 300                    | 0                                    | 0.35 ± 0.00  | 0.28 ± 0.00  | 0.28 ± 0.00  | 1.67 ± 0.00  | 0.58 ± 0.00  | 0.17 ± 0.00  | 0.38 ± 0.00  | 0.38 ± 0.00 | 27.66 ± 0.02  | 0.43 ± 0.00  | 46.38 ± 0.03 |
| 20                     | 0                                    | 0.36 ± 0.00  | 0.29 ± 0.00  | 0.29 ± 0.00  | 1.69 ± 0.00  | 0.60 ± 0.00  | 0.18 ± 0.00  | 0.36 ± 0.00  | 0.36 ± 0.00 | 27.71 ± 0.00  | 0.44 ± 0.00  | 48.27 ± 0.03 |
| 100                    | 0                                    | 0.31 ± 0.00  | 0.31 ± 0.00  | 0.29 ± 0.00  | 1.67 ± 0.05  | 0.57 ± 0.00  | 0.17 ± 0.00  | 0.36 ± 0.00  | 0.36 ± 0.00 | 27.76 ± 0.00  | 0.45 ± 0.00  | 48.59 ± 0.03 |
| 200                    | 0                                    | 0.33 ± 0.00  | 0.30 ± 0.00  | 0.30 ± 0.00  | 1.67 ± 0.00  | 0.58 ± 0.00  | 0.17 ± 0.00  | 0.37 ± 0.00  | 0.37 ± 0.00 | 27.86 ± 0.02  | 0.37 ± 0.00  | 49.91 ± 0.03 |
| 300                    | 0                                    | 0.33 ± 0.00  | 0.30 ± 0.00  | 0.30 ± 0.00  | 1.65 ± 0.06  | 0.62 ± 0.00  | 0.18 ± 0.00  | 0.42 ± 0.04  | 0.42 ± 0.04 | 27.40 ± 0.10  | 0.42 ± 0.00  | 46.34 ± 0.02 |

Induced calli were cultured on MS medium containing 0.25 mg/L NAA and 1.5 mg/L KIN along with combinations of NaCl (0, 10, 15, 20 mM) and methyl jasmonate (0, 10, 200, 300 mg/L) for indirect shoot regeneration. Values represent the mean ± standard error of three biological replicates. Values followed by the same letters are not significantly different at the P < 0.05.
valuable antioxidant bioactive compounds (Liu et al. 2018; Mahendran et al. 2018; Tonk et al. 2016). Elicitors are a group of plant biostimulants that can increase the accumulation of SMs of medicinal plants (Tonk et al. 2016). Stress hormones (SA, abscisic acid, jasmonic acid, MeJA), compatible solutes (proline), chitosan, and microbial extracts (bacterial, fungal and yeast) are the most applied elicitors in this regard. Some abiotic elicitors can be added to the culture medium for direct stress induction, especially osmotic stress. Polyethylene glycol, NaCl, heavy metal ions and nanoparticle-based metals are examples of abiotic stress-inducing elicitors (Ahmad et al. 2020; Yu et al. 2019; Malik et al. 2020; Mosavat et al. 2019).

In the present study, the combined effects of stress-inducing abiotic elicitors and stress tolerance-inducing elicitors of SA and MeJA were assessed on the SMs and enzymatic antioxidants activity of ajowan shoot cultures. PEG is a water-soluble polymer that widely has been applied to induce osmotic stress in plants by blocking the water-conducting channels of leaves, reducing the osmotic potential of cells and impairment of nutrient uptake (Castañeda and González 2021). Both applied PEG and NaCl significantly reduced the number of regenerated shoots and rooting percentage of the regenerated shoots. Salicylic acid treatment compensated for the negative effects of artificially induced drought stress on shoot regeneration and rooting of regenerated shoots and increased the regeneration efficiency of ajowan under stressful conditions. However, the greatest means of regenerated shoots and rooting percentage were related to the control medium. Miclea et al. (2020) have reported the adverse effect of elicitors on the number of in vitro shoots in Lavandula angustifolia. Salicylic acid is a stress tolerance-inducing compound that can improve plants growth under stressful conditions via modulation of the physiological parameters, reactive oxygen species (ROS) scavenging capacity, phytohormonal changes and antioxidant enzyme activity (Abdelaal et al. 2020; Rasheed et al. 2020; Torun et al. 2020). The positive effects of combinations of SA–PEG and MeJA–NaCl elicitors on SMs content and antioxidants activity of in vitro regenerated shoots were evident in the present study. Thymol, γ-terpinene and p-cymene considerably increased under the interaction of artificially induced drought and salinity stresses with SA and MeJA treatments. Salicylic acid is a signaling molecule that involved in the expression of stress-related genes in plant cells and the secretion of SMs in stress conditions induced by abiotic or biotic factors (Demirci et al. 2021). Therefore, to increase the accumulation of secondary metabolites, the use of salicylic acid under stressful conditions can be much more effective than its use in non-stressful conditions. Methyl jasmonate is another important signal molecule that its positive effect on bioactive compounds of different medicinal plants, including Macleaya cordata (Huang et al. 2021), S. miltiorrhiza (Wei et al. 2020), and S. rebaudiana (Rasouli et al. 2021), has been reported. Researchers have mainly applied the above mentioned biotic and abiotic elicitors individually in in vitro cultures of different medicinal plants. However, there are some examples of the combined use of these elicitors. Razavizadeh et al. (2020) applied different concentrations of chitosan (0, 10 and 20 mg/L) in callus and shoot cultures of ajowan, under artificially induced salinity stress (NaCl), and reported the positive effect of NaCl and chitosan in enhancing the contents of thymol and p-cymene. They also reported the increased activity of CAT, SOD and ascorbate peroxidase antioxidants under in vitro induced salt stress (100 mM NaCl). The integrative application of biotic (chitosan and SA) and abiotic (NaCl) elicitors led to enhanced total phenolics and total flavonoids in callus cultures of safflower (Carthamus tinctorius L.) (Golkar et al. 2019; Karamian et al. 2020) investigated the effect of

Fig. 6 a DNA fingerprinting pattern generated with UBC 815 ISSR primer. M molecular weight marker, C control plants, T1–T6 in vitro regenerated plants in MS medium supplemented with of KIN (1.5 mg/L) and NAA (0.25 mg/L) plant growth regulators and applied elicitors. b The dendrogram obtained from ISSR primers results for clustering in vitro regenerated and control plants of ajowan
in vitro methyl jasmonate elicitor on bioactive compounds of *V. sinusatum* under drought stress (PEG 6000) condition. They reported that the highest total phenol and flavonoid contents were obtained when 200 µM of methyl jasmonate was added to the culture medium under severe drought stress (− 0.5 MPa).

In addition to the stress tolerance-inducing elicitors, stress tolerance-enhancer compounds, such as melatonin, have been applied as elicitors to increase the accumulation of in vitro bioactive components of medicinal plants (Coskun et al. 2019; Duran et al. 2019). Other stress tolerance-enhancer additives, such as gibberellin inhibitors, ethylene inhibitors, osmoprotectants, antioxidant activators, ROS scavengers, and detoxification activators (Niazian and Shariatpanahi 2020), can potentially be used in combination with stress-inducing elicitors (PEG, NaCl, heavy metal ions) to increase the in vitro production of plants bioactive compounds. Silver nitrate (AgNO₃), as an ethylene inhibitor compound, has been applied to increase bioactive compounds of medicinal plants in different in vitro culture systems (Gonçalves et al. 2019; Yu et al. 2019; Açıkgöz 2020). It is obvious that the integrative application of these stress tolerance-enhancers and stress-inducing elicitors can be more effective than their individually application in terms of accumulation of antioxidant SMs.

Finding the best combination(s) of defense-trigger biotic and abiotic elicitors is very important to achieve the maximum levels of desired bioactive compounds of medicinal plants in different in vitro culture systems. However, in vitro culture is a multi-variable procedure with many influential factors (Niazian and Niedbała 2020). There are some useful advanced computational methods, such as machine learning algorithms, that can help researchers to overcome the complex nature of in vitro studies (Hesami et al. 2020; Hesami and Jones 2020; Niazian and Niedbała 2020). These advanced computational methods have also been applied for modeling and optimizing in vitro production of plant’s bioactive compounds, under the effect of various influencing factors (Kaur et al. 2020; Salehi et al. 2020, 2021).

Monomorphic patterns of ISSR primers showed high similarity between control and in vitro regenerated plants under the effect of applied elicitors. Production of genetic clones of the true-too-type plants is the main objective of an in vitro regeneration program. However, obtained plants from an indirect organogenesis pathway usually show slight divergence in genetic composition (Kshirsagar et al. 2021). Preservation of the original genetic background of medicinal plants during in vitro propagation is very important to protect valuable endemic genotypes/ecotypes (Niazian et al. 2017). Therefore, assessing the genetic fidelity of regenerated plants is an essential step in establishing an efficient protocol for the in vitro production of SMs of medicinal plants. Researchers often use molecular markers and flow cytometry to study the genetic stability of in vitro regenerated shoots. ISSR markers cover different genomic regions and offer an effective method for evaluating somaclonal variation in regenerated plants (Rawat et al. 2018; Raji and Farajpour 2020). While preserving the genetics of the in vitro regenerants, the optimized protocol in the present study increased the accumulation of valuable bioactive compounds of ajowan medicinal plant using integrated SA–PEG biotic and abiotic elicitors. This protocol can be widely used for the sustainable and safe production of SMs of other medicinal plants of the Apiaceae family.

**Conclusions**

In vitro culture systems provide an excellent opportunity for the sustainable production of valuable bioactive compounds of medicinal plants. Precursor feeding, elicitation and manipulation of culture medium parameters (basal culture medium, PGRs, carbon sources, additives and pH) are the simple and efficient traditional methods to increase contents of in vitro SMs. Elicitation of culture systems with defense-trigger compounds is a creative strategy to stimulate the accumulation of defense SMs.

In the present study, two combinations of stress tolerance-inducing and stress-inducing elicitors, including PEG–SA and NaCl–MeJA, were applied in indirect shoot regeneration of ajowan. Applied concentrations of SA reduced the adverse effects of PEG on shoot regeneration and rooting percentage of in vitro cultures. It did not happen for the interaction of NaCl–MeJA as shoot regeneration and rooting percentage was decreased with increasing levels of applied elicitors. The co-application of both PEG–SA and NaCl–MeJA led to the higher activity of enzymatic antioxidants (CAT, POX and SOD) and higher contents of valuable γ-terpinene, p-cymene and thymol bioactive compounds than non-stress (control) condition. Salicylic acid elicitation under artificially induced drought stress was more efficient than MeJA elicitation under salinity stress condition, in terms of in vitro production of SMs in ajowan medicinal plant. The genetic background of micropropagated ajowan plants showed high similarity with intact seed obtained plants. The results of the present study are useful for researchers who want to increase the amount of valuable bioactive compounds of their desired medicinal plants.

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Author contributions MN performed in vitro and greenhouse experiments and wrote the whole body of the manuscript. MSH contributed to study conception and project design, analysis and interpretation of data, GC/MS analysis, and revised the manuscript. SASN supervised the project and helped to improve the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest to disclose.

Ethical approval There is no any ethical standard related to the present article.

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