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

Bioactive compounds of medicinal plants have a wide range of applications in pharmaceutical, food and other industries. In vitro culture systems have great potential for sustainable production of bioactive compounds of medicinal plants. In the present study, the individual and combined effects of a stress tolerance-inducing (salicylic acid) and a stress-inducing elicitor (polyethylene glycol) were evaluated on regeneration efficiency, antioxidants activity and phytochemical profile of in vitro shoot cultures of ajowan. Different concentrations of salicylic acid (SA) (0, 10, 20, 40, 80 µM) and polyethylene glycol (PEG 6000) (0, 1, 2, 5%) were added to the shoot regeneration Murashige and Skoog medium containing Kin (1.5 mg/L) and NAA (0.25 mg/L) plant growth regulators. Salicylic acid reduced the adverse effect of PEG treatment on number of regenerated shoots and in vitro rooting. The activities of catalase, superoxide dismutase, and peroxidase enzymatic antioxidants were significantly increased in SA + PEG treated plants. The gas chromatography-mass spectrometry (GC-MS)-profiling revealed quantitative and qualitative phytochemical differences between control and SA + PEG 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 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. The same strategy (stress tolerance-inducing elicitor + stress-inducing elicitor) is applicable to increase valuable SMs in other production systems such as hydroponic, greenhouse and field conditions.

Introduction

There is an increasing demand for plant-derived natural products, especially the bioactive compounds of medicinal plants (Niazian et al. 2018; Soltani Howyzeh et al. 2019). These bioactive compounds are the basis for many commercial pharmaceutical drugs and have great values in pharmaceutical, food, perfume, agrochemical, cosmetic and other industries (Li et al. 2020). Therefore, finding efficient protocols for sustainable production of bioactive compounds of medicinal plants, including secondary metabolites (SMs), is very important to meet the growing demands in pharmaceutical and other industries.

Environmental factors such as drought, salinity, light intensity, high/low temperature, heavy metals and soil nutrients status have adverse effects on plant growth and photosynthesis. Osmolytes accumulation, antioxidants production and higher SMs accumulation are some of the plant responses to harsh environmental conditions (Mahajan et al. 2020). Secondary metabolites are chemical defense molecules that secreted when plants are under biotic and abiotic stresses (Alvarado et al. 2019). Improving growth and survival of plants under stressful conditions is the primary function of SMs (Khare 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 and stimulate the accumulation of SMs in different *in vitro* culture systems of medicinal plants. Polyethylene glycol (PEG), NaCl and heavy metal ions are examples of such stress-inducing compounds. Adding cadmium chloride ($CdCl_2$) 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 in *in vitro* culture system. Stress hormones (signaling compounds), including abscisic acid, jasmonic acid, methyl jasmonate, 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 salicylic acid (1.0 mg/L) resulted in a significant increase in bacoside content in cell suspension culture of *Bacopa monnieri* (Koul and Mallubhotla 2020).

Ajowan (*Trachyspemum 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). Different *in vitro*-based methods, including artificial tetraploidy induction (Noori et al. 2017), genetic engineering (Niazian 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. The present study was conducted to evaluate the effect of SA elicitation on regeneration efficiency, enzymatic antioxidants activity and secondary metabolite production in shoot cultures of ajowan under the artificially induced drought stress (PEG 6000).

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 in Murashige and Skoog (MS) medium supplemented with 0.2 mg/L naphthalene acetic acid (NAA) and 1.5 mg/L 6-benzylaminopurine (BAP). Callus induction of established hypocotyls was initiated after 15 days and three rounds of subcultures were done in the same medium. For shoot regeneration, induced calli were transferred to MS medium supplemented with 1.5 mg/L of Kin plus 0.25 mg/L NAA. The complete details of indirect shoot regeneration procedure of ajowan have been elaborated in Niazian et al (2017).

Elicitation of in vitro **cultures**

Combination of different concentrations of salicylic acid (SA) (0, 10, 20, 40, 80 µM) and PEG 6000 (0, 1, 2 and 5 %) were used to assess the effect of concomitant use of artificially induced drought stress and elicitation on enzymatic antioxidants and bioactive compounds of *in vitro* regenerated shoots of

ajowan. The MS medium containing Kin (1.5 mg/L), NAA (0.25 mg/L), 3% (w/v) sucrose and 0.7% (w/v) agar (pH 5.8) was used as control. Culture medium was autoclaved at 121°C for 20min and then filter-sterilized (0.2 µm) SA and PEG were added to medium under the laminar airflow chamber. Five callus segments were cultured in petri dishes containing 30 mL of MS medium supplemented with different combinations of PEG and SA. Experiment was arranged as factorial, based on a completely randomized design (CRD), with three replications (as petri dishes). All cultures were kept in a phytotron with 16/8 h (light/dark) photoperiod, photosynthetic photon flux density (PPFD) of 40 µmol $m^{-2}s^{-1}$, and 24°C. Subcultures were done with two weeks intervals and the combined effect of treatments on the number of regenerated shoots was investigated in the seventh week of the experiment.

For root induction, the regenerated shoots were transferred to half strength plant growth regulator (PGR)-free MS medium. Culture vessels were maintained in a phytotron with aforementioned condition for the shoot regeneration. The combined effect of PEG and SA on rooting of *in vitro* regenerated shoots was evaluated after two 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 containing 1: 1 perlite: cucurbit. The pots were covered with transparent plastic and irrigated with half strength MS medium for one week and then covers were removed for complete acclimatization. The successfully acclimatized plantlets were then transferred to plastic pots (8 × 10 cm) filled with autoclaved farm soil. The greenhouse established plants were grown and after two months starts to flowering. In the next two 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. The activity of catalase (CAT) enzyme was measured using Aebi (1974) method. The activity of peroxidase (POX) was estimated using Hemeda and Klein (1990) method. Superoxide dismutase (SOD) activity was measured using Beauchamp and Fridovich (1971) method.

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

For essential oil extraction, a seed sample (20g), 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 2.5h. 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 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. 2018; Noori et al. 2017).

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

To assess the genetic stability of *in vitro* regenerated plants different DNA samples from regenerated plants, each group contains mixed DNAs of leaves of ten plants (three weeks old), were used to assess the genetic stability. 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. 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 0.5X TBE buffer stained with ethidium bromide under 100 V constant power supply for 1.5 h and visualized under UV light in gel documentation system (Cleaver Scientific Ltd., Rugby, UK).

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). 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.

Results

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

Proliferated calli were obtained in MS medium supplemented with NAA (0.2 mg/L) and BAP (1.5 mg/L) at the end of the second week of the experiment (Fig. 1a). Induced calli were then transferred to shoot induction medium. The first regenerated shoots were observed on MS medium containing NAA (0.25 mg/L) and Kin (1.5 mg/L) PGRs along with combinations of PEG and SA (Fig. 1b). Regenerated shoots showed visual symptoms of wilting in 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 in culture medium (Fig. 2a). The greatest mean of number of regenerated shoots was observed in control treatment (MS medium containing NAA and Kin PGRs), whereas, the lowest regenerated shoots was obtained in MS medium containing highest concentration of PEG (5%) and SA (80 µM). (Fig. 2a). 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 two weeks of establishment in PGR-free MS medium (Fig. 3b). The results of means comparison analysis revealed the significant adverse effect of PEG on root induction percentage of *in vitro* regenerated shoots of ajowan. The lowest rooting percentage was obtained by the highest degree of drought stress (5% PEG 6000) (Fig. 2b). The greatest rooting percentage was obtained in control treatment. Salicylic acid showed positive effect on rooting percentage of PEG-treated shoots as the greatest mean of rooting percentage under severe drought stress (5% PEG 6000) was obtained by using 40µM of SA (Fig. 2b).

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 shoot induction medium (Table 1). The greatest mean of CAT activity was observed in shoots regenerated in MS medium containing 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 1). The highest levels of PEG 6000 and SA significantly increased the SOD activity (Table 1). The lowest mean of the SOD activity of *in vitro* regenerated shoots of ajowan observed when 1% of PEG 6000 along with 10 µM SA were added to the regeneration medium (Table 1). The highest POX activity was observed at severe artificial drought stress, whereas the lowest activity was observed in control treatment. Salicylic acid under PEG treatment induced a significant increase in POX activity (Table 1). In non-stress condition (0% PEG), SA treatment increased activity of POX, however, there was no significant difference between applied concentrations of SA at the 5% probability level (Table 1).

Table 1

Effect of *in vitro* drought stress and salicylic acid elicitor on the catalase, superoxide dismutase and peroxidase activity in indirect regenerated shoots of ajowan. Induced calli were cultured on MS medium containing 0.25 mg/L NAA and 1.5 mg/L Kin along with combinations of PEG6000 (0, 1, 2, 5 %) and salicylic acid (0, 10, 20, 40, 80 µM) for indirect shoot regeneration.

PEG 6000 concentration (%)	Salicylic acid concentration (µM)	Catalase (unit mg^{-1} protein)	Superoxide dismutase (unit mg^{-1} protein)	Peroxidase (unit mg $protein^{-1}$ min^{-1})
0	0	0.56 ± 0.02^{m}	127.33 ± 0.27^{g}	0.40 ± 0.00^{l}
	10	0.766 ± 0.11^{l}	125.67 ± 1.66^{g}	0.52 ± 0.02^{k}
	20	0.766 ± 0.01^{l}	130.67 ± 0.66^{g}	0.57 ± 0.00^{k}
	40	0.81 ± 0.01^{kl}	133.00 ± 0.57^{g}	0.65 ± 0.01^{j}
	80	1.03 ± 0.03^{ij}	131.67 ± 0.88^{g}	0.71 ± 0.00^{ij}
1	0	0.93 ± 0.03^{jk}	135.33 ± 1.66^{g}	0.74 ± 0.00^{i}
	10	$0.88 \pm 0.01^{j-l}$	97.33 ± 3.66^{h}	0.75 ± 0.00^{hi}
	20	0.93 ± 0.03^{jk}	$138.67 \pm 0.88^{f-g}$	$0.78 \pm 0.00^{g-i}$
	40	1.13 ± 0.03^{i}	$143.00 \pm 1.00^{e-g}$	0.76 ± 0.01^{hi}
	80	1.33 ± 0.12^{h}	$145.67 \pm 0.66^{e-g}$	0.76 ± 0.02^{hi}
2	0	1.46 ± 0.03^{gh}	$148.33 \pm 0.33^{d-g}$	$0.82 \pm 0.00^{f-h}$
	10	1.53 ± 0.03^{g}	$150.67 \pm 0.66^{c-g}$	$0.84 \pm 0.02^{e-g}$
	20	1.60 ± 0.00^{fg}	$153.00 \pm 0.00^{c-g}$	0.87 ± 0.00^{ef}
	40	1.71 ± 0.01^{ef}	$167.00 \pm 0.010^{b-f}$	0.88 ± 0.00^{ef}
	80	1.78 ± 0.01^{de}	$171.00 \pm 1.00^{b-e}$	0.90 ± 0.00^{e}
5	0	1.86 ± 0.03^{cd}	$175.67 \pm 0.33^{b-d}$	1.06 ± 0.03^{d}
	10	1.93 ± 0.03^{bc}	179.33 ± 0.33^{bc}	1.13 ± 0.03^{d}
	20	2.03 ± 0.03^{b}	186.67 ± 1.66^{b}	1.26 ± 0.03^{c}
	40	2.33 ± 0.08^{a}	240.00 ± 0.00^{a}	1.433 ± 0.06^{b}
	80	2.46 ± 0.03^{a}	245.00 ± 2.88^{a}	1.733 ± 0.03^{a}

Values represent the mean + standard error of three biological replicates. Values followed by the same letters are not significantly different at the $P < 0.05$.

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 and SA treated plants with plants regenerated in control medium (Fig. 4a,b). Thymol, γ-terpinene, and *p*-cymene were the main components in the essential oil of *in vitro* regenerated ajowan plants (Table 2). 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 2). The α-thujene percentage of non-treated plants was less than the PEG and SA treated shoots (Table 2).

Table 2

The phytochemical profile of *in vitro* regenerated plants of ajowan under the effects of drought stress and salicylic acid elicitor. Induced calli were cultured on containing 0.25 mg/L NAA and 1.5 mg/L Kin along with combinations of PEG6000 (0, 1, 2, 5 %) and salicylic acid (0, 10, 20, 40, 80 µM) for indirect shoot reg

PEG 6000 concentration (%)	Salicylic acid concentration (µM)	α-Thujene	α-Pinene	Sabinene	β-Pinene	β-Myrcene	α-Terpinene	p-Cymene	β-Phellandrene	γ-Terpinene	Terpinene-4-ol
0	0	0.21 ± 0.00^{k}	0.10 ± 0.00^{b}	0.22 ± 0.01^{a}	1.32 ± 0.00^{k}	0.67 ± 0.01^{h}	0.64 ± 0.00^{l}	16.40 ± 0.00^{l}	0.22 ± 0.00^{n}	27.48 ± 6.65^{b}	0.36 ± 0.00^{l}
	10	0.23 ± 0.00^{ij}	0.13 ± 0.00^{b}	0.18 ± 0.00^{ab}	1.25 ± 0.02^{l}	0.67 ± 0.00^{h}	0.65 ± 0.00^{k}	16.43 ± 0.02^{l}	0.23 ± 0.00^{mn}	34.12 ± 0.06^{a}	0.36 ± 0.00^{l}
	20	0.22 ± 0.01^{jk}	0.52 ± 0.39^{a}	0.22 ± 0.01^{a}	1.36 ± 0.00^{jk}	0.69 ± 0.00^{g}	0.66 ± 0.01^{j}	16.53 ± 0.03^{k}	0.24 ± 0.00^{lm}	34.24 ± 0.02^{a}	0.37 ± 0.00^{kl}
	40	0.24 ± 0.00^{hi}	0.15 ± 0.00^{b}	0.21 ± 0.01^{ab}	1.38 ± 0.00^{ij}	0.71 ± 0.00^{f}	0.68 ± 0.01^{i}	16.62 ± 0.05^{j}	0.24 ± 0.00^{kl}	34.34 ± 0.03^{a}	0.36 ± 0.00^{kl}
	80	0.24 ± 0.00^{hi}	0.15 ± 0.00^{b}	0.19 ± 0.04^{ab}	1.41 ± 0.01^{hi}	0.72 ± 0.00^{f}	0.68 ± 0.00^{i}	16.69 ± 0.01^{ij}	0.25 ± 0.00^{k}	34.44 ± 0.02^{a}	0.36 ± 0.00^{kl}
1	0	0.24 ± 0.00^{h}	0.18 ± 0.00^{b}	0.17 ± 0.01^{bc}	1.56 ± 0.01^{cd}	0.73 ± 0.01^{e}	0.68 ± 0.00^{i}	16.70 ± 0.00^{ij}	0.26 ± 0.00^{j}	35.03 ± 0.03^{a}	0.38 ± 0.00^{ij}
	10	0.25 ± 0.00^{h}	0.18 ± 0.00^{b}	0.17 ± 0.01^{bc}	1.59 ± 0.00^{c}	0.74 ± 0.00^{e}	0.71 ± 0.00^{h}	16.72 ± 0.01^{i}	0.26 ± 0.00^{ij}	35.01 ± 0.01^{a}	0.37 ± 0.00^{jk}
	20	0.26 ± 0.00^{g}	0.19 ± 0.00^{b}	0.13 ± 0.01^{cd}	1.43 ± 0.01^{g-i}	0.73 ± 0.00^{e}	0.71 ± 0.00^{h}	16.82 ± 0.01^{h}	0.27 ± 0.00^{i}	35.05 ± 0.03^{a}	0.38 ± 0.00^{ij}
	40	0.27 ± 0.00^{fg}	0.20 ± 0.00^{b}	0.10 ± 0.03^{d}	1.43 ± 0.01^{g-i}	0.74 ± 0.00^{e}	0.71 ± 0.00^{h}	16.85 ± 0.02^{h}	0.28 ± 0.00^{h}	35.13 ± 0.02^{a}	0.39 ± 0.00^{i}
	80	0.28 ± 0.00^{f}	0.20 ± 0.00^{b}	0.04 ± 0.04^{e}	1.47 ± 0.00^{fg}	0.76 ± 0.00^{d}	0.72 ± 0.00^{h}	16.90 ± 0.00^{h}	0.29 ± 0.00^{h}	35.30 ± 0.07^{a}	0.40 ± 0.00^{h}
2	0	0.31 ± 0.00^{e}	0.25 ± 0.00^{ab}	-	1.43 ± 0.00^{gh}	0.78 ± 0.00^{c}	0.77 ± 0.00^{g}	17.07 ± 0.03^{g}	0.31 ± 0.00^{g}	35.33 ± 0.01^{a}	0.41 ± 0.00^{g}
	10	0.32 ± 0.00^{e}	0.25 ± 0.01^{ab}	-	1.45 ± 0.01^{gh}	0.78 ± 0.01^{c}	0.78 ± 0.00^{f}	17.14 ± 0.01^{fg}	0.32 ± 0.00^{g}	35.51 ± 0.13^{a}	0.41 ± 0.00^{fg}
	20	0.33 ± 0.00^{d}	0.26 ± 0.00^{ab}	-	1.50 ± 0.00^{ef}	0.78 ± 0.00^{c}	0.78 ± 0.00^{f}	17.19 ± 0.00^{ef}	0.33 ± 0.00^{f}	35.92 ± 0.08^{a}	0.42 ± 0.00^{ef}
	40	0.35 ± 0.00^{c}	0.27 ± 0.00^{ab}	-	1.52 ± 0.00^{de}	0.80 ± 0.00^{b}	0.81 ± 0.00^{e}	17.20 ± 0.00^{ef}	0.35 ± 0.01^{e}	36.09 ± 0.04^{a}	0.42 ± 0.00^{ef}
	80	0.36 ± 0.00^{b}	0.28 ± 0.00^{ab}	-	1.55 ± 0.01^{cd}	0.84 ± 0.00^{a}	0.82 ± 0.00^{d}	17.23 ± 0.01^{de}	0.38 ± 0.01^{d}	36.22 ± 0.05^{a}	0.43 ± 0.00^{e}
5	0	0.39 ± 0.00^{a}	0.32 ± 0.00^{ab}	-	1.75 ± 0.03^{a}	0.66 ± 0.00^{h}	0.88 ± 0.00^{c}	17.30 ± 0.00^{d}	0.40 ± 0.00^{c}	36.46 ± 0.01^{a}	0.46 ± 0.00^{d}
	10	0.38 ± 0.01^{a}	0.33 ± 0.00^{ab}	-	1.60 ± 0.00^{c}	0.65 ± 0.00^{i}	0.89 ± 0.00^{bc}	17.43 ± 0.01^{c}	0.40 ± 0.00^{c}	36.57 ± 0.01^{a}	0.47 ± 0.01^{cd}
	20	0.38 ± 0.01^{a}	0.35 ± 0.00^{ab}	-	1.57 ± 0.00^{c}	0.64 ± 0.01^{ij}	0.91 ± 0.00^{a}	18.04 ± 0.03^{b}	0.42 ± 0.00^{b}	37.00 ± 0.00^{a}	0.47 ± 0.00^{c}
	40	0.38 ± 0.00^{a}	0.36 ± 0.00^{ab}	-	1.66 ± 0.04^{b}	0.63 ± 0.00^{jk}	0.89 ± 0.00^{bc}	18.19 ± 0.03^{a}	0.42 ± 0.00^{b}	37.10 ± 0.00^{a}	0.51 ± 0.01^{a}
	80	0.36 ± 0.00^{b}	0.39 ± 0.01^{ab}	-	1.55 ± 0.01^{cd}	0.62 ± 0.01^{k}	0.90 ± 0.00^{ab}	17.47 ± 0.07^{c}	0.44 ± 0.00^{a}	37.46 ± 0.01^{a}	0.49 ± 0.00^{b}

Values represent the mean + standard error of three biological replicates. Values followed by the same letters are not significantly different at the $P < 0.05$.

Based on the results of GC-MS analysis, *p*-cymene of *in vitro* micropropagated plants was increased with increasing levels of PEG and SA (Table 2). 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 2). 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 2). 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 2).

Genetic Stability Assessment

Ten amplified ISSR primers produced 115 scorable bands from *in vitro* regenerated plant of ajowan in control and SA-PEG treated media. An average of 11 bands per primer, ranging from 7 to 13 bands, with only 2.45 % of polymorphism was observed (Fig. 5).

Discussion

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

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 for sustainable production of bioactive compounds of medicinal plants (Koufan et al. 2020). However, undifferentiated calluses 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 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 the morphogenesis and the synthesis and accumulation of SMs in medicinal plants (Li et al. 2020).

The application of biotic and abiotic elicitors in culture medium, which induce defense-related metabolic pathways, is one the creative strategies to change the biochemical profile of medicinal plants and enhance the accumulation of valuable antioxidant bioactive compounds (Liu et al. 2018; Mahendran et al. 2018; Tonk et al. 2016). Elicitors are a group of plant biostimulants, in two biotic and abiotic forms, which their application in *in vitro* cultures at different stages can increase the volume of defensive secondary compounds of medicinal plants (Tonk et al. 2016). Stress hormones (SA, abscisic acid, jasmonic acid, methyl jasmonate), compatible solutes (proline), chitosan, and microbial extracts (bacterial, fungal and yeast) are the most applied biotic elicitors. There are also some abiotic elicitors that can be added to the culture medium for direct stress induction, especially osmotic stress. Polyethylene glycol, NaCl, heavy metal ions and nanoparticles-based metals are examples of abiotic stress-inducing elicitors.

In the present study, the combined effect of a stress-inducing abiotic elicitor and stress signal molecule of SA was assessed on the SMs and enzymatic antioxidants activity of ajowan shoot cultures. Applied PEG 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 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 SA and PEG on SMs content and antioxidants activity of *in vitro* regenerated shoots were evident in the present study. Thymol and *p*-cymene considerably increased under the artificially induced drought stress and SA treatment. 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. 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 *in vitro* methyl jasmonate elicitor on bioactive compounds of *Verbascum sinuatum* 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 gibberelin 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 ($AgNO_3$), as an ethylene inhibitor compound, has been applied to increase bioactive compounds of medicnal plants in different *in*

vitro culture systems (Gonçalves et al. 2019; Yu et al. 2019; Açıkgöz 2020). It is obvious 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 achieving 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. 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; Salehi et al. 2021).

Monomorphic patterns of ISSR primers showed no genetic variation 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). 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 *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, stress tolerance-inducing SA and stress-inducing PEG were used together 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. The co-application of SA and PEG led to the higher activity of enzymatic antioxidants (CAT, POX and SOD) and increased accumulation of valuable thymol and *p*-cymene bioactive compounds. Salicylic acid elicitation under artificially induced drought stress was more efficient than non-stress condition, in terms of *in vitro* production of SMs in ajowan medicinal plant. 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.

Declarations

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Authors' contribution

MN performed *in vitro* and greenhouse experiments and wrote the whole body of 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.

Conflict of interest

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

Ethical standards

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

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Figures

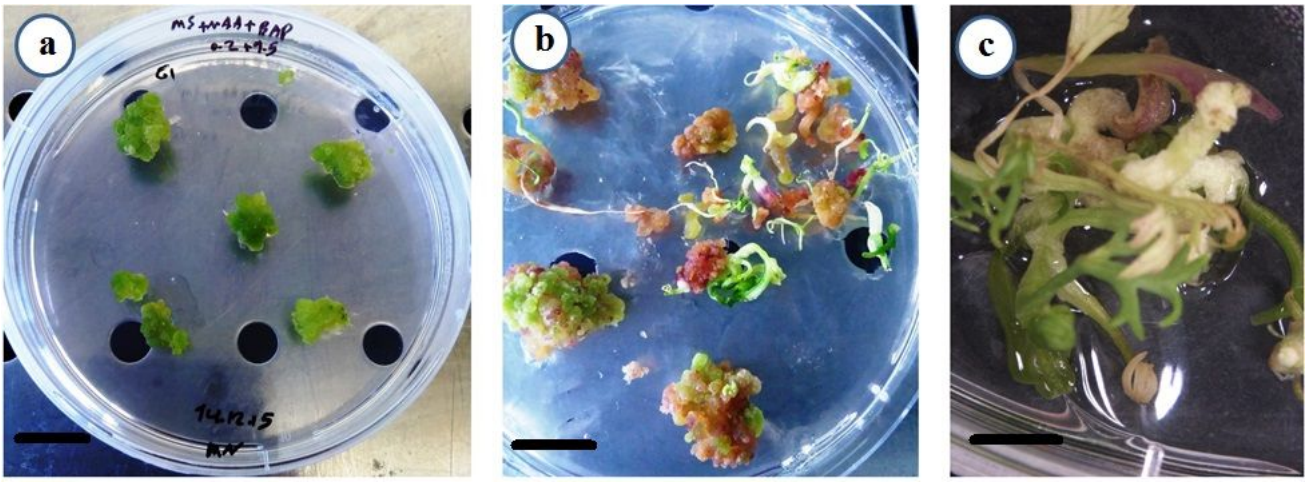

Figure 1

Indirect shoot regeneration of ajowan. (a) Callus induction in MS medium supplemented with 0.2 mg/L naphthalene acetic acid (NAA) and 1.5 mg/L 6-benzylaminopurine (BAP) (bar=2cm). (b) Indirect shoot regeneration from induced calli in MS medium supplemented with 1.5 mg/L of Kin and 0.25 mg/L NAA along with different combinations of PEG (0, 1, 2, 5 %) and salicylic acid (0, 10, 20, 40, 80 µM) (bar=2cm). (c) The symptoms of wilting in shoots regenerated in culture medium containing PEG treatment (bar=4mm).

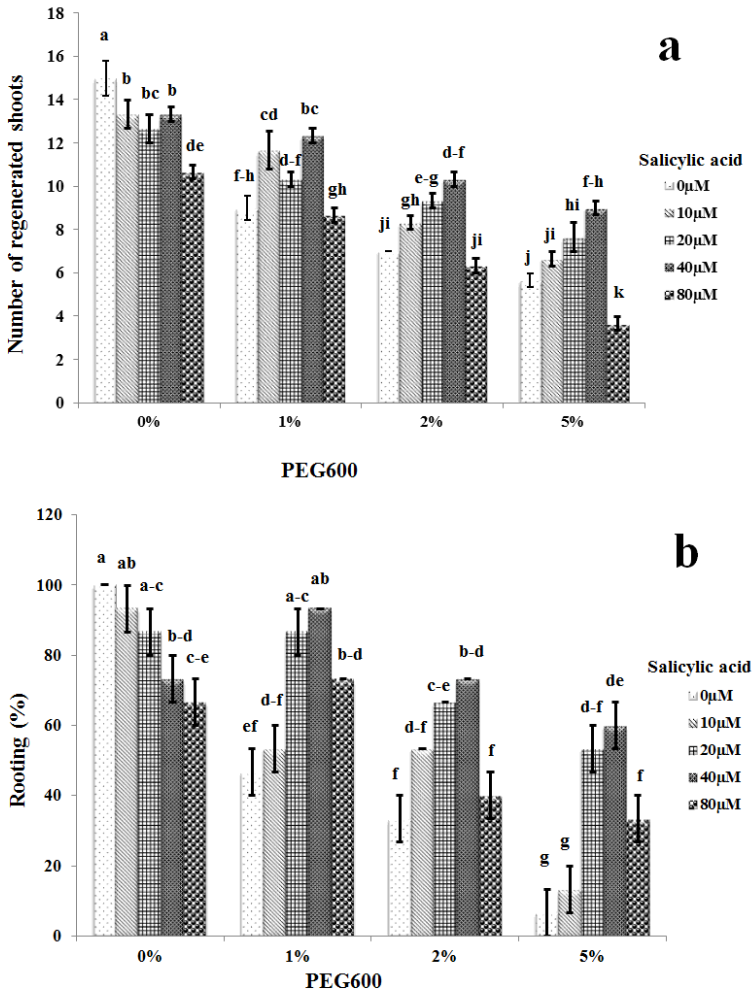

Figure 2

Effect of in vitro drought stress and salicylic acid elicitor on regeneration efficiency of ajowan. (a) The number of regenerated shoots under the effect of PEG and salicylic acid elicitors. (b) Rooting percentage of regenerated shoots under the effect of PEG and salicylic acid elicitors. Different combinations of PEG (0, 1, 2, 5 %) and salicylic acid (0, 10, 20, 40, 80 µM) elicitors were added to the indirect regeneration medium (MS medium supplemented with 1.5 mg/L of Kin and 0.25 mg/L NAA). Values followed by the same letters are not significantly different at the $P < 0.05$.

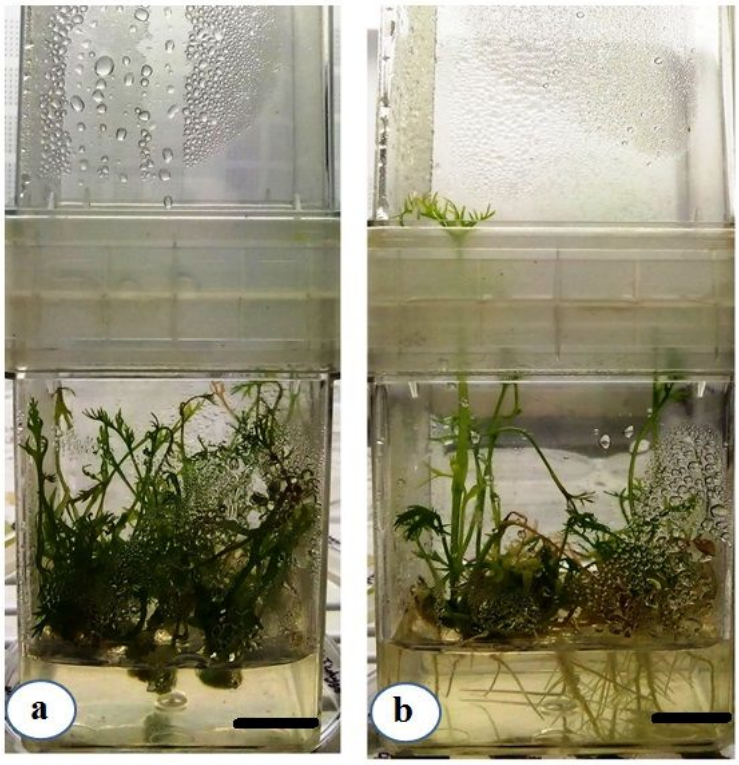

Figure 3

Root induction of in vitro regenerated shoots of ajowan. (a) Establishment of indirect regenerated shoot in half strength PGR-free MS medium (bar=3cm). (b) Well developed roots of indirect regenerated shoots after three weeks settling in root induction medium (bar=3cm).

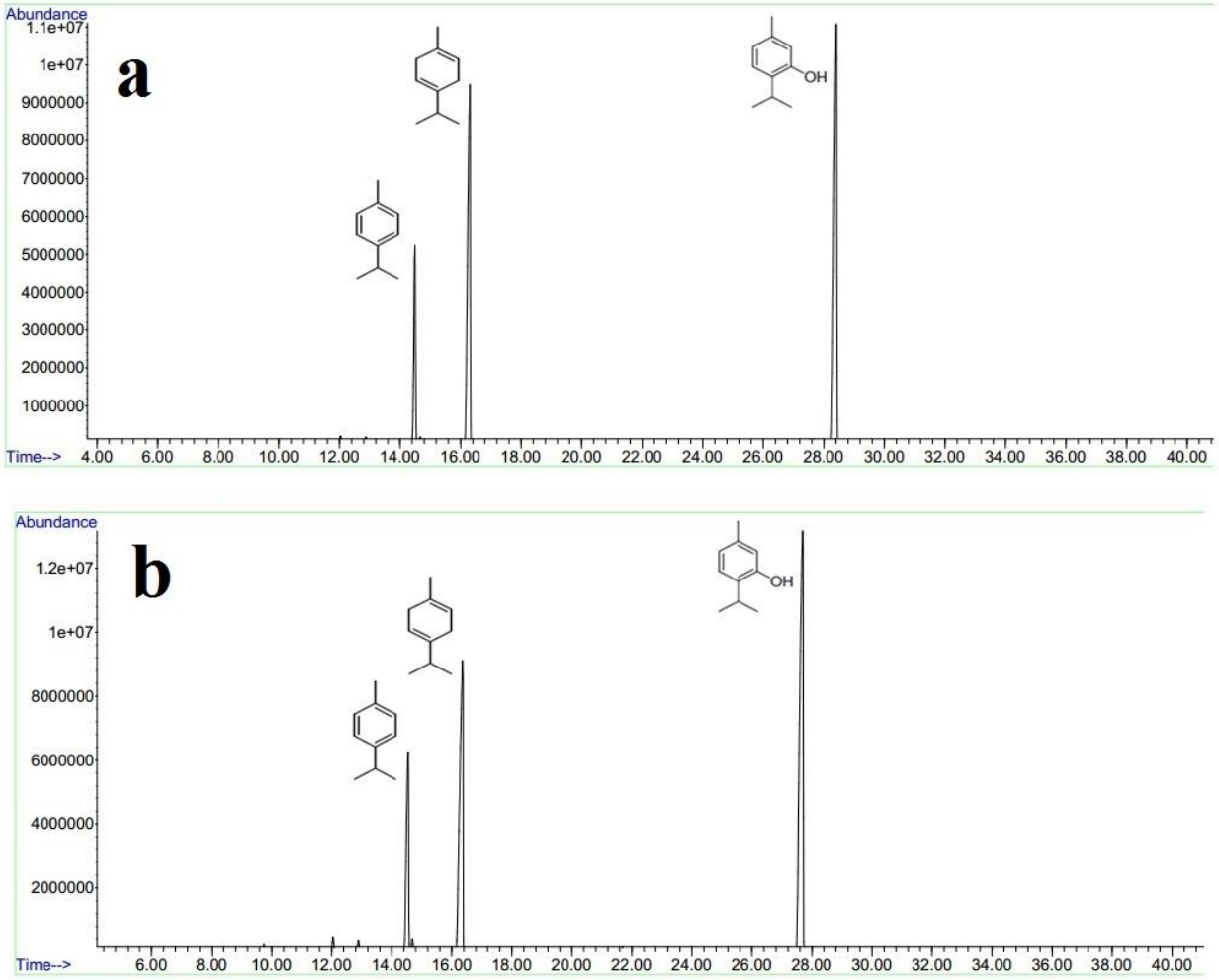

Figure 4

The GC-MS profiling of (a) in vitro regenerated in control and (b) in vitro regenerated under the effect of PEG and SA elicitors.

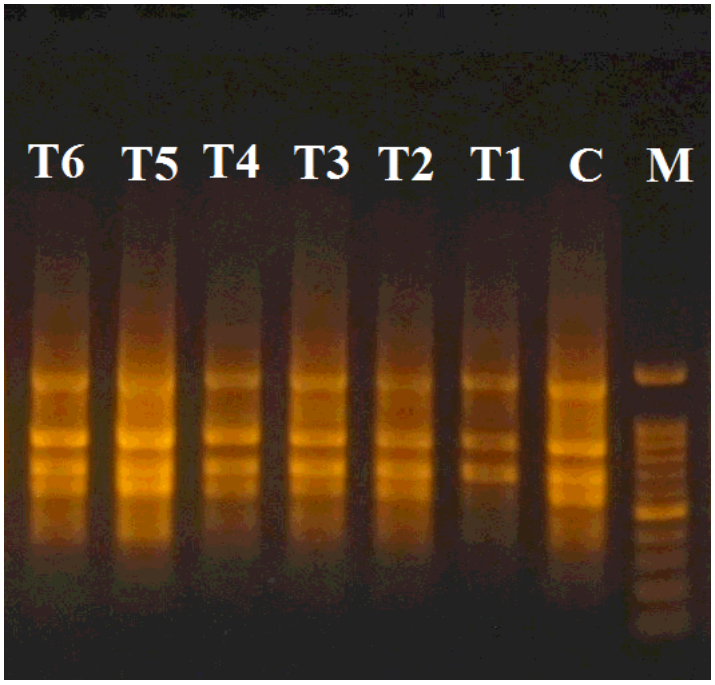

Figure 5

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 PEG and salicylic acid elicitors.