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Genetically stable plants with boosted flavonoids content after *in vitro* regeneration of the endangered *Capparisspinosa* L

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Abstract

Capparisspinosa L. is proven to be a potent hepatoprotective natural remedy. *C. spinosa* (L.) is a rare species which is on its way to extinction. We herein report an efficient *in vitro* propagation and regeneration protocol towards effective conservation of this plant. The use of 2 mg/L Kinetin (Kin.) and 0.4 mg/L 1- naphthalene acetic acid (NAA) on MS basic medium resulted in maximum number of *in vitro* shoot multiplication and adventitious root formation, respectively. Calli were successfully induced using 2 mg/L 2,4- dichloro-phenoxyacetic acid (2,4-D). Direct organogenesis from aerial part explants showed remarkable success using thiadiazurone (TDZ) hormone. Genetic fidelity assessment of the *in vitro* regenerated plants was confirmed by RAPD analysis. Two-fold increase in flavonoids content than estimated in the wild plants was noticed after elicitation with methyl jasmonate 200 µM/L in 6- benzyl aminopurine (BAP) pretreated plants as estimated by HPLC. On the other hand, 200 µM/Lmethyl jasmonate boosted the flavonoids concentration by 1.5 fold in Kin pretreated plants. This work could be considered as a regular platform for conservation of this endangered plant guaranteeing regenerates of genetic high fidelity and improved chemical profile.

Introduction

Capparis spinosa L.F. Capparidaceae is used around the world for many pharmacologicalpurposes.The hepatoprotective effectof *C. spinosa* L. was explained based on its antioxidant activity [1]. Capers are used for treatment of gout by inhibitionthe synthesis of uric acid [1]. Also, they are beneficial for asthmatic patients duetoantagonist effect forsmooth muscle contraction in rat trachea [2]. Many phytochemicals were identified from *C. spinosa* like flavoniods and their glycosides [3]. Quercetin, isoquercetin and rutin flavonoids had anti-H_5N_1 viral activity [4]. *C. spinosa* L. is considered as an endangered plant in North Sinai, Egypt [5]. Despite its medicinal importance, only few studies have been concerned with its propagation [6]. A micropropagation protocolwas introduced for Lebanese *C. spinosa* subsp *Rupestris* using 1 mg/L zeatin, then the shoots rooted with IAA [7]. While, a regeneration protocol was introduced for*C. spinosa* L. subsp. *Rupestris* in Italy from flowerexplant with 13 µM BAP [8]. The main objective of this study was to develop a protocol for an *in vitro* micropropagation; using shoot tip explant and a regeneration system; using leaf, root or stem segments of *C. spinosa* L. from North Sinai with a stablegenetic profile and better bioactive flavonoids yield towards the conservation of this medicinally active species.

Elicitation is considered as an effective strategy for many biomedicinals production. It initiatesor accelerates biosynthetic pathways of secondary metabolites when applied at small concentrations. The plants produce secondary metabolites as a defense mechanism against attack by any pathogens. Biotic elicitors of biological origin and abiotic elicitors were used [9].

To confirm genetic fidelity of regenerate plants to mother plant, Random AmplifiedPolymorphic DNA (RAPD) can be done. RAPD is a useful tool due to its speed and simplicity in addition to a random primer can anneal at many locations of genome to produce its DNA fingerprint [10].

Material and methods

Seed sterilization and germination: Ripe fruits were collected in May 2014, from wild plants of the natural places in St. Katherine Protectorate, Egypt. The seeds were extracted from the fruitsand sterilized according to previously reported sterilization protocol [11]. Surface sterilized seeds were cultured in 10 mL of half strength Murashige and Skoog (MS) basic salts and vitamins medium (Murashige and Skoog, 1962) supplemented with, 3% sucrose and 6.0 g/L agar within a 40 mL capacity jar. The medium pH was adjusted to 5.7 before autoclaving for 20 minutes at 121°C. A total of one hundred jars were inoculated with one seed per jar. The culture jar were incubated in a growth room maintained at 22 ± 2°C, illuminated with Phillips TLM 40W/33RS fluorescent lamp providing 4000 Lux light intensity for 16 hrs a day. The germination percentage was determined 12 weeks from the inoculation date.

Effect of cytokinins on shoot proliferation: Eight weeks old shoot tips(0.5 - 1 cm height) were excised from the *in vitro* seedlings and

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transferred to a 200 mL jar with 30 ml of strength Murashige and Skoog (MS) basic salts and vitamins mediumsupplemented with, 3% (w/v) of sucrose and 6 g/L agar. The pH of the medium was adjusted to 5.7 by 1N of NaOH or HCl and autoclaved as described above. Filter-sterilized benzyl aminopurine (BAP) or 2- isopentyl adenine (2iP) or Kinetin (Kin.) at the concentration of 0.0, 2.0, 4.0 and 6.0 mg/L was added to the media after it was autoclaved.Each treatment consisted of 10 jars each withtwo explants. The incubation conditions were as described for the *in vitro* seedlings above.After 5 weeks of culture, the number of shoots, plant height and plant weight were recorded. Multiple shoots were collected and dried at room temperature, and active compounds were extracted with methanol to perform High Performance Liquid Chromatography (HPLC) analysis.

***In vitro* adventitious root formation:** Seventeen weeks old micro shoots (0.5 - 1 cm height) were sub-cultured onto MS medium supplemented with different concentrations of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or 1- naphthalene acetic acid (NAA) at 0.0, 0.1, 0.2 and 0.4 mg/L. Each treatment consisted of 10 jars and each jar containing two explants. Culture conditions were as described above. The number of roots per initial micro shoot, maximum root length, number of new shoots, plant fresh weight as well as maximum shoot length after 6 weeks. Rooted explants samples were collected, dried at room temperature and extract with methanol to perform HPLC analysis.

Acclimatization: Four well *rooted in vitro* plantlets were removed from the culture medium, washed in tap water to remove agar from cultured media and immersed in 1% of rizolex® fungicide for 10 minutes. The rooted plantlets were transferred to the greenhouse for acclimatization in pots with a moist sand. The plants were irrigated with fine water mist for 3 weeks inside a growth chamber. After 4 weeks, the percentage of the survived plants was recorded. Acclimated plants samples were collected, dried at room temperature and extract with methanol to perform HPLC analysis.

Callus induction: Leaf explants of 0.5 - 1 cm length were placed on a solid agar medium. The culture media consisted of the mineral salts of MS supplemented with 2,4- dichloro-phenoxyacetic acid (2,4-D) at the concentration of 0.5, 1, 2, 4 and 6 mg/L [12]. Each treatment consists of 8 replicates with 4 leaf explants of 0.5 -1 cm length for each sample. The culture conditions were as described above. The callus weight, callus color, number of roots as well as root length were recorded after 4 weeks. The leaf samples from callus were collected, dried at room temperature and extract with methanol to perform HPLC analysis.

Plant regeneration and organogenesis:MS basal medium supplemented with 3% (w/v) sucrose and 6 g/L of agar was prepared and the pH of the medium was adjusted to 5.8 by 1 N NaOH or HCl prior to autoclaving at 121°C and 1.2-1.3 Kg/Cm^2 pressure for 20 minutes. Filter sterilized thiadiazurone (TDZ) at the concentration of 0.0, 110, 220 and 440 mg/Lwere added to the 46°C autoclaved medium temperature of the media. Each treatment consisted of 10 petri dishes (9 cm in diameter) with 30 ml of medium. The aerialpart of 1 gm. weight was cut into very small pieces with 2 ml sterile distilled water by scalpel and cultured on petridishes containing TDZ hormone-incorporated media [13]. The culture conditions were as described above. After 15 days, a successful regeneration was observed and the number of regenerated shoots per explants and also their length were recorded. Different selected lines of the regenerated plants were sub-cultured on MS medium supplemented with 2 mg/L BAP for multiplication. After 4 weeks, the samples of the different lines of regenerated plants were collected, dried at room temperature and extracted with methanol to perform HPLC analysis.Another fresh samples were kept to be examined bythe RAPD analysis.

Statistical analysis for *in vitro* micropropagation protocol: Experiments were set up in completely randomized design. Data were statistically analyzed using CoStat version 6.303 1998-2004 CoHort software 798 Lighthouse Ave PMP 320, Monterey, CA, 93940, USA. Analysis of variance (ANOVA) was performed to compare results. Least significance difference (LSD) test was used to compare means at the 5% significance level.

Genetic assessment

Genomic DNA extraction:DNA was extracted from fresh leaves from four lines of the *in vitro* regenerated plantlets as well as control plants using a CTAB protocol [14] and DNA concentration was determined by measuring OD_{260} at 260 nm wave length using NanoDrop (ND-1000 spectrophotometer).

Random amplified polymeric DNA (RAPD):A set of ten random primers (Table 1) was used in the detection of polymorphism among the regenerated plantlets and control. RAPD-PCR was carried out in 25 µl reaction volume containing 12.5 µL Master Mix (Qiagen), 2 µL primer (20 pmole), 2 µL templates DNA of concentration (20 ng / µL) and 8.5 µL water nuclease free.

Thermo cycling profile and determination of PCR products: PCR amplification was performed in a C1000- Thermo cycler (Master cycler gradient eppendorf) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 30 seconds, an annealing step at 40°C for 30 seconds, and an elongation step at 72°C for 30 seconds. A final extension step ofwas performed at 72°C for 7 min. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TAE buffer (45 mM Tris borate, 1 mM EDTA) at 95 volts. PCR products were visualized on UV light and photographed. Amplified products were visually investigated by presence or absence of the bands.

Capillary electrophoresis: DNA fragments (four lines of the *in vitro* regenerated plantlets as well as control plants) were analyzed in the automatic multicapillary electrophoresis (QIAxcel-QIAGEN). Isolated DNA was placed in the instrument sample tray, 10 ml of the DNA samples were automatically injected into the capillary channel and subjected to electrophoresis according to the protocol AM420 (method separation time: 420 s, method injection time: 10s, method separation voltage: 5.0 kV and method injection voltage: 5.0 kV) of the

Table 1. Sequence of 10 different random primers used for RAPD analysis of control and *in vitro* regenerated plants of *Capparis spinosa* L.

Name	Sequence (5`–3`)	Name	Sequence (5`–3`)
OPA-1	5'CAGGCCCTTC3'	OPk-3	5'CCAGCTTAGG3'
OPA-3	5'AGTCAGCCAC3'	OPk-12	5'TGGCCCTCAC3'
OPA-11	5'CAATCGCCGT3'	OPO-11	5'GACAGGAGGT3'
OPA-15	5'TTCCGAACCC3'	OPO-15	5'TGGCGTCCTT3'
OPk-2	5'GTCTCCGCAA3'	OPO-20	5'ACACACGCTG3'

QIAxcel DNA Screening Kit.

Extraction of phytochemicals from *in vitro* cultured plants: The weighed samples were soaked separately in absolute ethanol for 4 days. Sonication for 30 minutes was done to aid extraction process of the active compounds.The whole process of soaking, sonication and filtration was repeated for 5 times and the supernatant was filtered prior to drying at room temperature. Finally, the weight of viscous ethanolic extract of each sample was determined.

HPLC analysis

Preparation of standard and sample solutions for HPLC: Standard solutions of rutin were prepared in methanol of HPLC grade at a concentration range of 0.01625 to 1 mg/ml for rutin. The plant extract samples were dissolved in methanol HPLC at concentration 1 mg/ml then filtered through a 0.22 µmChrom tech® Nylon syringe filter (Millipore). Triplicate injections were made and a linear regression was generated at wavelength of 257 nm.

Chromatographic method: Chromatographic analysis was carried out in isocratic conditions and at room temperature using reversed phase-C18 column (4.6 mm x 250 mm) packed with 5µm diameter particles and Thermo - Dionex® HPLC Model Ultimate 3000. The mobile phase was methanol-acetonitrile- water (25:15:60, v/v/v) containing 1.0% glacial acetic acid. The mobile phase was filtered through a 0.45 µm Nylon membrane filter and degassed in ultrasonic bath for 15 minutes previous to use. Flow rate and injection volume were 1.0 ml/min and 10 µl, respectively. The chromatographic peaks were identified by comparing their retention time and UV spectra with the reference standard at wave length 257 nm. Quantification was carried out by the integration of each peak using the external standard method.

Elicitation

Chemical feeding (Abiotic elicitors): Four weeks old *in vitro* micro shoots from both Kin and BAP media were sub-cultured into 40 ml capacity jars containing 10 ml MSmedium containing full strength basic salts and vitamins. The medium was supplemented with 3% (w/v) sucrose, 6.0 g/L agar and one of the following chemical elicitors (Table 2), the pH was adjustedto 5.7 prior to autoclaving [15].

Table 2. Chemical feeding with different concentration of sodium chloride, and sodium salicylate.

Abiotic elicitors	Concentration
Sodium chloride	25 mM
Sodium chloride	50 mM
Sodium salicylate	5 mg/L
Sodium salicylate	10 mg/L
Sodium salicylate	20 mg/L

Another abiotic elicitor, methyl jasmonate (MeJA), was tested. MeJA was dissolved in ethanol in order to prepare a stock solution. Filter-sterilized MeJA was added at concentrations of 20, 50, 100 and 200 µM/L to autoclaved MS medium containing full strength basic salts and vitamins, 3% (w/v) sucrose and 6.0 g/L agar prior to dispersion into petridishes of 9 cm in diameter [16]. All elicited samples were collected and extracted to be ready for quantitative determination by HPLC analysis method after 15 days from their culture.

Fungal feeding (biotic elicitors):Two fungal cultureswere used; *Aspergillus niger* NRRL 3 and *Rhizopusstolonifer* NRRL 1472. The fungi were cultured in 500 ml flasks containing 150 ml medium and were shaken at 30°C and 150 rpm on an incubator shaker, Innova® 43 incubator shaker series. The liquid medium composition was as follows in g/L: glucose, 30; ammonium nitrate, 2.5; sodium dihydrogen phosphate, 1; magnesium sulfate. $7H_2O$, 0.25 and zinc sulfate $7H_2O$, 0.05. The cells of *Aspergillus niger* NRRL 3 were harvested after 21 days cultures while *Rhizopusstolonifer* NRRL 1472 after 45 days culture.The harvested fungal cultures werethen, autoclaved, filtered and ground using a mortar. Dry powders were added in concentration of 80 mg/L [15].

Statistical analysis for HPLC assessment and elicitation process: Statistical analysis using SPSS (Statistical package for social science) version 16, software package for data analysis was done. The quantitative data were presented in the form of mean ± SD using *p=0.05*. The test of significance is one way ANOVA was used to compare between the means.

Results and discussion

***In vitro* shoot proliferation:**The MS medium supplemented with 2.0 mg/L kinetinprovided the maximum number for *in vitro* shoot proliferation (5.364 shoots/explant) (Table 3) (Figure 1A). The more the concentrationthan 2 mg/L kinetin, the less the number of the propagated shoots. On the other hand, MS medium supplemented with 4.0 mg/L kinetin provided the longest one of the propagated shoots (2.455 cm). Based on these findings,kinetin is supposed to be the most efficient growth regulator tested for the optimal multiplication of this plant material.Previous studies on Lebanese *C. spinosa* subsp *Rupestris* reported that the maximum shoot number (4.8 shoots/explants) was provided using woody plant medium (WPM) supplemented with1.6 mg/L zeatin [16]. Similar results were reported forother plants. The

Table 3. Effects of 6- benzyl aminopurine (BAP), 2- isopentyladenine (2iP) and kinetin (Kin) concentrations on plant height, fresh weight, number of shoots per explants and callus weight of *Capparis spinosa* L.

Growth regulator (mg/L)			Length of the longest shoot (cm)	Explant's fresh weight (g.)	No. of shoots/explant	Callus weight (g.)
BAP	2iP	Kin.				
0.0	0.0	0.0	2.12^{ab}	0.31^{b}	2.86^{c}	0^{c}
2.0	0.0	0.0	1.36^{c}	$0.53a^{b}$	3.39^{bc}	0.09^{abc}
4.0	0.0	0.0	1.29^{c}	0.59^{a}	4.75^{a}	0.10^{abc}
6.0	0.0	0.0	1.31^{c}	0.69^{a}	4.83^{a}	0.18^{a}
0.0	2.0	0.0	1.91^{abc}	0.56^{ab}	4.50^{ab}	$0.04b^{c}$
0.0	4.0	0.0	1.75^{bc}	0.57^{ab}	4.64^{a}	$0.03b^{c}$
0.0	6.0	0.0	2.36^{ab}	0.69^{a}	4.57^{a}	0.14^{ab}
0.0	0.0	2.0	2.14^{ab}	0.60^{a}	5.36^{a}	0.15^{ab}
0.0	0.0	4.0	2.46^{a}	0.62^{a}	4.46^{ab}	0^{c}
0.0	0.0	6.0	1.28^{c}	0.53^{ab}	3^{c}	0.02^{bc}

(**Note:** Means with the similar letters are not significantly different at 0.05 level of probability using LSD test. n=12).

best shoot propagation and plantlets lengthfor *Gerbera jamesonii* were obtained from medium containing MS supplemented by 2 mg/L Kin [17]. Fotso and coworkers havereported that kinetin was combined with BAPfor best results for micropropagation of *Swieteniamacrophylla* [18].

***In vitro* root formation:** The NAA was found to be more effective than IAA and IBA in robust roots induction fromshoot cultures (Table 4). Of the tested concentrations range, 0.4 mg/L NAA resulted in the maximum number of roots (13.18 roots/explant) (Figure 1B), while the longest one was obtained from MS medium supplemented with 0.2 mg/L NAA.The ascendance in NAA concentrations resulted in more adventitious root formation. Pardo reported that an increase in NAA concentration during the *in vitro* cultivation of *Billbergiarosea* is concordant with increasein the number of roots of *in vitro* cultivated *Billbergiarosea* [19]. Our results are similar to study of *Citrus megaloxycarpa* as the maximum number of roots was produced with 2 mg/LNAA [20].

Acclimatization: Almost 75% of the *in vitro* rooted plants showed remarkable success in accommodation with external field conditions (Figure 1C).

Callus induction: Table 5 discussed the effect of different concentrations of 2,4-D on callus weight when initiated from leaf explants. It also showed that callus initiation was successfully established at all concentrations of that hormone after 4 weeks with no difference between them but neither rooting nor shooting had occurred. The highest weight of brownish white callus was obtained from MS medium supplemented by 2 mg/L 2,4- D (Figure 1D). Many researchers reported that 2,4-D isthe best auxin for callus induction [21]. Three mg/L2,4-Dsucceeded in callus induction in *Solanum tuberosum* L. from leaf and shoot tipexplants [22].

Table 4. Effects of Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA) concentrations on number of roots, root length and rooting percentage of *Capparis spinosa* L.

Growth regulator (mg/L)			Plant's height (cm)	Plant's fresh weight (g.)	No. of roots/ explant	Length of the longest root (cm)
IBA	IAA	NAA				
0.0	0.0	0.0	5.46^{ab}	1.09^{a}	1.55^{b}	0.32^{c}
0.1	0.0	0.0	4.75^{abc}	1.03^{a}	1.60^{b}	1^{bc}
0.2	0.0	0.0	4.54^{bcd}	0.91^{a}	4.67^{b}	0.46^{bc}
0.4	0.0	0.0	3.28^{d}	0.96^{a}	$7.14a^{b}$	1.14^{abc}
0.0	0.1	0.0	4^{cd}	0.97^{a}	4.78^{b}	0.50^{bc}
0.0	0.2	0.0	5.27^{ab}	1.08^{a}	2.27^{b}	0.41^{c}
0.0	0.4	0.0	5.55^{ab}	1.28^{a}	2.36^{b}	0.23^{c}
0.0	0	0.1	5.73^{a}	0.94^{a}	1.73^{b}	1.14^{abc}
0.0	0	0.2	5.25^{abc}	0.88^{a}	$8.38a^{b}$	2.5^{a}
0.0	0	0.4	4.82^{abc}	1.01^{a}	13.18^{a}	1.73^{ab}

(**Note:** Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test. n= 10).

Table 5. Effect of different concentrations of 2,4- dichloro- phenoxy acetic acid (2,4-D) on callus weight from leaf explants of *Capparis spinosa* L.

Concentrations of 2,4-D (mg/L)	Callus weight (g.)	Callus colour
0.5	0.08^{a}	Brownish white
1	0.07^{a}	Brownish white
2	0.11^{a}	Brownish white
4	0.10^{a}	Brownish white
6	0.07^{a}	Brownish white
8	0.06^{a}	Brownish white

(**Note:** Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test).

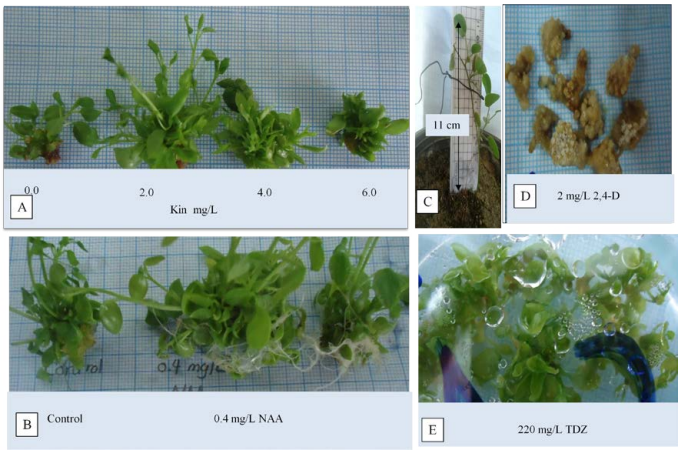

Figure 1. (**A**) shoot proliferation of *Capparis spinosa* L. on MS medium containing 2 mg/L Kin, (**B**) adventitious root formation on MS medium with 0.4 mg/L of NAA, (**C**) *EX vitro* acclimatized plants at external field conditions, (**D**) Effect of MS medium with 2,4-D at 2 mg/L on leaf callus and (**E**) plant organogenesis from mashed aerial part on MS medium with 220 mg/L TDZ.

Plant regeneration and organogenesis: Direct organogenesis from aerial part explants showed remarkable success in *Capparisspinosa* L. There was no difference between different concentrations of TDZ hormon on the number of regenerated plants, while there was signifcant difference between 110 mg/L and both 220 and 440 mg/L of TDZ hormon on the length of regenerated plants (Table 6) (Figure 1E). Direct regeneration is an effective route for production of plantlets with a low risk of any genetic aberration. Other reports proved that TDZ induces shoot induction better than other cytokinins in plant tissue culture [23-24]. Also, Faisal and Anisreported the effectiveness of TDZ at lower concentration for multiple shoot induction in *Rauvolfiatetraphylla* L [25].

RAPD analysis: RAPD was performed to analyze the genetic stability of four *in vitro* regenerated plants of a random selection manner as well as control plant using four random primers. Out of ten random primers, four primers succeeded to develop high amplification (Table 7). Figure 2 (A,B,C and D) showed the similar bandingpatternsbetween control and random *in vitro* regenerated plants that puts an emphasis on that the clonal propagation was developed with low risk of somaclonal variation or any genetic aberration in regenerated plant. Genetic assessment of the *in vitro* regenerated plants by RAPD markers has been reported [26-27]. Bhowmik [28] reported genetic uniformity of *in vitro* micropropagated of *Mantisiaspathulata* using RAPD analysis. Also, all banding profiles from regenerated plants of *Alpiniagalanga* L were similar to the mother plant [29].

HPLC analysis: The rutin standard calibration curve was constructed according to equation of y =25.21 X + 0.061 and regression R^2=0.998 at retention time 3.4 minutes using mobile phase; methanol-acetonitrile- water (25:15:60, v/v/v) containing 1.0% acetic acid glacial at 257 nm (Figure 3A).

HPLC analysis for *in vitro* propagated plants: Peak purity was assessed by comparing the response of total flavonoids content at 257 nm (UV maximum and 275 nm, over time. Only one sharp peak for total flavonoids content appeared on the two wavelengths. The absorbance ratio that expressed by dividing the slice area at wavelength 257 by

Table 6. Effect of different concentrations of thiadiazurone (TDZ) on the number of regenerated plants and their length in *Capparis spinosa* L.

TDZ (mg/L)	No. of regenerate plants	Regenerate Plant's length (cm)
110	16[a]	0.51[b]
220	19[a]	1.50[a]
440	18[a]	2 [a]

(**Note:** Means followed by the same letter within a column are not significantly different at 0.05 level of probability according to L.S.D. test).

Table 7. Sequence of 4 different random primers used for RAPD analysis of control and regenerated plants of *Capparis spinosa* L.

Name	Sequence (5`–3`)	Name	Sequence (5`–3`)
OPA-1	5'CAGGCCCTTC3'	OPA-11	5'CAATCGCCGT3'
OPA-3	5'AGTCAGCCAC3'	OPA-15	5'TTCCGAACCC3'

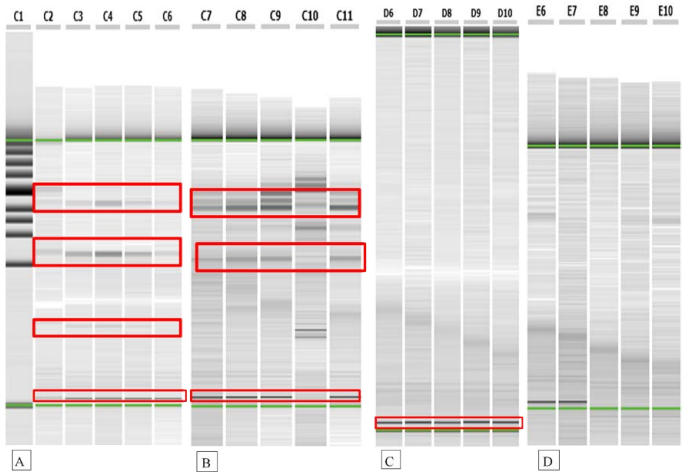

Figure 2 (A, B, C and D). RAPD pattern of control and regenerated plants of *Capparis spinosa* L. C1: Size marker, C2: control, C3-C6: 4 samples for Primer (PA1); C7: control, C8-C11: 4 samples for primer (PA3); D6: control, D7-D10: 4 samples for primer (A11) and E6: control, E7-E10: 4 samples for primer (A15).

the slice area of the other wavelength 275 was constant throughout all corresponding slices of the peaks [30-32]. From our study, wild plants showed the highest total flavonoids content calc. as rutin (3.35 ± 0.15% W/W) (Table 8). All *in vitro* treatments and control groups have shown a decline inthe total flavonoids content. Acclimatization or the transfer of the*in vitro* rooted plantlets from the rooting media to natural external field conditions was shown to increase the total flavonoids content to 2.35 ± 0.07 %W/W. The maximum total flavonoids content as rutin(1.73 ± 0.14%W/W)was obtained from BAP- treated plants among different *in vitro* propagated plants (Figure 3B). Both treatments with highest total flavonoids content (BAP and Kin.) were selected to be exposed to elicitation to improve their total flavonoids content. The previous results confirmed that plant growth regulators play an essential role in phytochemicals production better than control plants [33]. They can enhance flavonoid production, as shown for stimulation of apigenin and luteolin production by BAP application on *Arnica Montana* [34], Also, stimulation of quercetin, catechin and myricetin production was also reported for *Cyperusrotundus* [35].

HPLC analysis for different tested elicitors in*Capparisspinosa L.*samples pretreated with BAP: It is apparent that MeJA (methyl jasmonate) elicitor has a stunning effect on the total flavonoids content (Table 9). The more the concentration of MeJA, the more the flavonoids content in the tested concentration range. The highest flavonoids content was obtained with 200 µM/LMeJA which was twofold that estimated in the wild plants. On the other side, increased concentrations of sodium chloride decreased the flavonoids content. Fungal elicitation, with *Aspergillus niger* NRRL 3, *Rhizopusstolonifer* NRRL 1472,and sodium salicylate chemical elicitor was found to affect negatively on the flavonoids accumulation within the tested range. Also, Ghasemzadeh and Jaafar [36] reported decline in flavonoids concentrations as quercetin in responsetosalicylic acid treatment (10^{-5} M and 10^{-3} M) that inhibits the activity of PAL enzyme, a master key in the flavonoids biosynthesis.

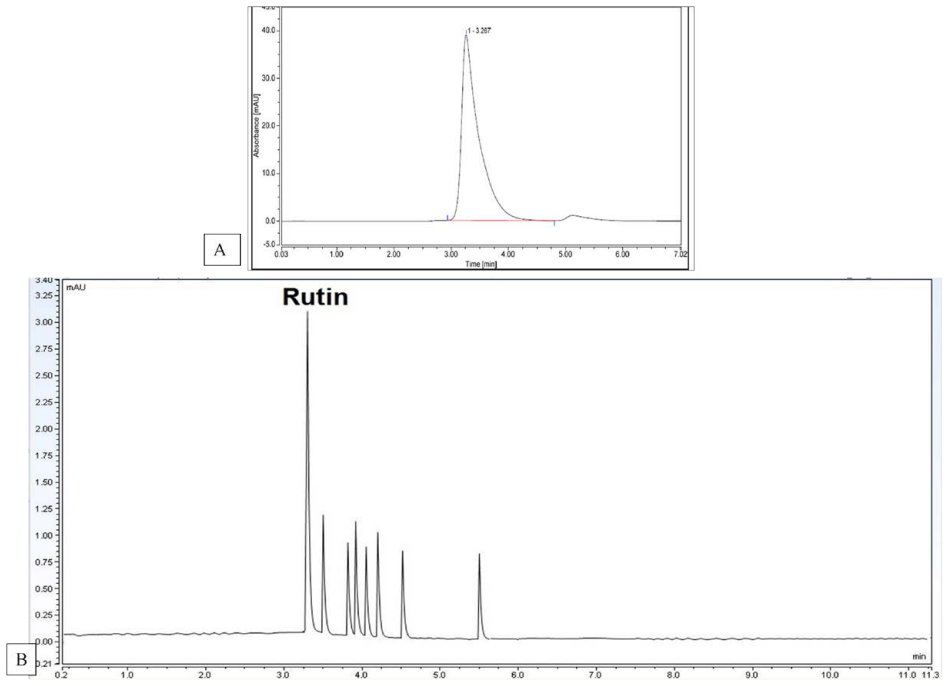

Figure 3. (**A**) HPLC chromatogram of standard rutin and (**B**) HPLC chromatogram of BAP- treated plants at retention time 3.4 minutes using mobile phase methanol-acetonitrile- water (25:15:60, v/v/v) containing 1.0 % acetic acid glacial at 257 nm.

Table 8. The concentration of total flavonoids calculated as rutin corresponding to different *in vitro* media manipulations in *Capparis spinosa* L. cultures.

	Treatments	Total flavonoid (rutin) % (mg/100 mg extract)
1	Wild plant	3.35 ± 0.15
2	Acclimated plantlet	2.35 ± 0.07*
3	Regenertated plantlets	1.53 ± 0.12*
4	2iP- treated plant	0.67 ± 0.04*
5	BAP – treated plant	1.73 ± 0.14*
6	Kin - treated plant	1.57 ± 0.07*
7	Control/multiplication	0.96 ± 0.05*
8	NAA- treated plant	0.73 ± 0.04*
9	IAA- treated plant	1.35 ± 0.03*
10	IBA- treated plant	1.46 ± 0.05*
11	Control/rooting	0.91 ± 0.03*
12	Callus of 2,4-D treatment	0.85 ± 0.03*

(**Note:** Values are expressed by mean ± SD, n=3, *: significantly different from wild plant, BAP:6- benzyl aminopurine ; 2,4-D: 2,4- dichloro- phenoxy acetic acid; IAA: Indole-3-acetic acid ; IBA: Indole-3-butyric acid; 2iP: 2-isopentyl adenine; Kin: Kinetin and NAA: 1- naphthalene acetic acid)

Table 9. Total flavonoids content as rutin in response to different tested elicitors in *Capparis spinosa* L. samples pretreated with 6- benzyl aminopurine (BAP).

	Elicitation treatments	Total flavonoid (rutin) % (mg/100 mg extract)
	Wild plant	3.35 ± 0.15
	BAP treatment	1.73 ± 0.14
1	25 m mole NaCl	1.92 ± 0.03
2	50 m mole NaCl	0.94 ± 0.03
3	5 mg/L sodium salicylate	None
4	10 mg/L sodium salicylat	None
5	20 mg/L sodium salicylate	None
6	20 µM MeJA	3.07 ± 0.12
7	50 µM MeJA	4.16 ± 0.22*
8	100 µM MeJA	5.16 ± 0.09*
9	200 µM MeJA	5.7 ± 0.15*
10	80 mg/L *Aspergillus niger* NRRL 3	1.57 ± 0.21
11	80 mg/L *Rhizopus stolonifer* NRRL 1472	0.62 ± 0.04

(**Note:** BAP: 6-benzyl aminopurine and MeJA: methyljasmonate, Values are expressed by mean ± SD, n=3, *: significantly increase from wild plant)

HPLC analysis for different tested elicitors in *Capparisspinosa* L.samples pretreated with Kin: The different used elicitors were shown to have the same effect on kin. – treated microshoots like BAP – treated microshoots. MeJA elicitor showed a stunning effect on the total flavonoids content (Table 10). The more the concentration of MeJA, the more the flavonoids content in the tested concentration range. The highest flavonoids content was obtained with 200 µM/LMeJA that which was 1.5 fold that estimated in the wild plants. Similar results ofa 2-fold increase in flavonoids, catechin and epicatechin, of *Taxus cuspidate* by using MeJA as an elicitor were documented [37]. Also, other increase in phenolic compounds was reported under elicitation with MeJA [38].

Phenylalanine ammonia lyase (PAL) is the master key enzyme of flavonoids biosynthesis in plants. MeJA induced up-regulation of PAL activity that consequently increased flavonoid production [39].

Conclusion

There is no doubt that the combination of basic media composition and growth hormones intake should indeed be the first consideration

Table 10. Total flavonoids content as rutin in response to different tested elicitors in *Capparis spinosa* L. samples pretreated with kinetin (Kin).

	Elicitation treatments	Total flavonoid (rutin) % (mg/100 mg extract)
	Wild plant	3.35 ± 0.15
	Kin treatment	1.57 ± 0.07
1	25 m mole NaCl	1.58 ± 0.02
2	50 m mole NaCl	0.81 ± 0.03
3	5 mg/L sodium salicylate	None
4	10 mg/L sodium salicylat	None
5	20 mg/L sodium salicylate	None
6	20 µM MeJA	2.02 ± 0.06
7	50 µM MeJA	2.59 ± 0.16
8	100 µM MeJA	4.79 ± 0.04*
9	200 µM MeJA	4.87 ± 0.05*
10	80 mg/L *Aspergillus niger* NRRL 3	1.28 ± 0.06
11	80 mg/L *Rhizopus stolonifer* NRRL 1472	0.77 ± 0.02

(**Note:** Kin: Kinetin and MeJA: methyl jasmonate, Values are expressed by mean ± SD, n=3, *:significantly increase from wild plant)

in optimal growth and secondary metabolites accumulation, but the use of elicitors as adjunct to these two basics have been used with success in this work. This enhancement was performed by using the abiotic elicitor, methyl jasmonate, that provided twofold and 1.5 foldflavonoids content from BAP and Kin -pretreated plantlets, respectively, than flavonoids content estimated in wild plants. We also report a regular platform for conservation of this endangered plant *Capparisspinosa* L. through adjusted regeneration protocol which guarantees regenerates of genetic fidelity as determined by RAPD analysisand a stable chemical profile as estimated by HPLC analysis.

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Conflict of interest

The author declares no competing interests.

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