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In vitro propagation and improving accumulation of coumarin in *Lycium barbarum*, a rare plant in the flora of Egypt

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Abstract

Background: The conservation of natural populations of plants is very important for maintaining biological diversity. *Lycium barbarum* (goji berries) is classified as a very rare plant in the Egyptian flora. Goji berry is a fruit commonly known as 'super-fruit,' due to its beneficial constituents and medicinal properties. The aim of this study was to determine the influence of media formula added with various cytokinins on axillary bud induction of *L. barbarum* from shoot tip. An additional objective of this study was to investigate the effect of 2,4-D, TDZ, zeatin, IBA and NAA on growth and development of the plant through different stages. The present study also aims to improve coumarin accumulation as one of the most active constituents in the plant.

Results: Sterilized shoot tips of *L. barbarum* were cultured without growth regulators on different media formulas: Murashige and Skoog basal medium (MS medium), Gamborg's medium (B5 medium) and Woody plant medium (WP medium). MS medium gave the best shoot induction. The maximum multiplication rate of shoots was recorded on MS medium supplemented with 225.24 µM 6-benzyl amino purine (BAP). The best callus induction rate from in vitro derived young leaves was obtained on MS medium with 552.60 µM thidiazuron (TDZ). At 176.20 µM TDZ, the maximum shoot proliferation ability was obtained through organogenesis from the callus. MS medium with 220.25 µM TDZ produced the highest number (15 buds) and forming rate (96%) of adventitious buds through direct organogenesis. On MS medium with 304.86 µM naphthalene acetic acid (NAA), 100% rooting was obtained. Almost 95% of the in vitro-generated plantlets were successfully acclimatized. Furthermore, suspension culture containing MS medium with 552.60 µM TDZ and cinnamic acid at a concentration of 763 µM increased coumarin synthesis (1.95-fold), compared to control.

Conclusion: The in vitro regeneration of plants via leaf-mediated shoot organogenesis protocol presented in this study is well suited for *L. barbarum* propagation and produced large quantities of identical planting materials for desert revegetation and pharmaceutical industries. A very effective indirect regeneration method via callus culture provides a potential for this ethnomedicinal plant to be improved by genetic transformation procedures, in addition to mass propagation of elite plants. The cell suspension culture medium with 763 µM cinnamic acid had the highest coumarin accumulation.

Keywords: Callus, Coumarin, *Lycium barbarum*, Very rare, Suspension culture, Organogenesis

Background

Egypt's desert flora is the most essential of the country's natural plant life, occupying large swaths of land. In addition to medical, herbal, cosmetic, and culinary uses, these natural plants are used for fruit, animal feed, fuel, fibers, and folk medicine. Overharvesting,

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unproductive agriculture methods, overcrowding, increasing spread of exotic species, and climate variability, high rates of habitat degradation and destruction are all factors that contribute to extinction and loss of plant diversity in Egypt (Shaltout 2019). The conservation of biodiversity is necessary to protect plants from extinction and degradation, and it can provide direct and indirect benefits to humanity. Rare and endangered species are given first priority (Holobiuc et al. 2009). Also, they are gene pools because of their tolerance to the harsh environments in which they survive (Maxted 2013).

Lycium barbarum L. is classified as a very rare plant in the Egyptian flora (Boulos 2009). The fruits have beneficial nutrients and medicinal characteristics (Xin et al. 2013; Fratianni et al. 2018). Fruit extracts are recognized for their therapeutic properties (Lam et al. 2016), such as antioxidant possibility and free radical scavenging, aging prevention, antitumor potential (Kulczyński and Gramza-Michałowska 2016), blood lipid decline (Pai et al. 2013), and glycemia dilution (Ming et al. 2009). The plant fruits and seeds contain bioactive polysaccharides, minerals, vitamins (B1, B6, A, C, and E), amino acids, and important fatty acids (Potterat 2010; Peteros and Uy 2012; Jing and Yin 2010). Goji berry is difficult to propagate normally since seeds have slow germination and low rooting percentages when propagated by cuttings (Silvestri et al. 2018). As a result, this plant must be propagated and preserved by in vitro culture. In vitro culture of *L. barbarum* has been studied in few studies on other countries, but in Egypt no research on *L. barbarum* has been found. Fira and Clapa (2011) employed shoot tip and nodal segments for an efficient micropropagation method, whereas Osman et al. (2013) and Hu et al. (2001) used leaf and axillary buds. Different goji genotypes require distinct growth regulators in culture media. So, developing a highly efficient plant regeneration mechanism for each genotype is critical (Fira et al. 2016). In this respect, the research introduces a new procedure for efficient *L. barbarum* in vitro multiplication, focusing on the role of explant type, mineral nutrition and cytokinin type.

Secondary metabolites in plants serve a variety of functions as signaling molecules and defensive agents (Ncube and Staden 2015). Coumarins are a form of secondary metabolite that found in a wide range of plant cell. Some coumarins are used as anticoagulants and brain cancer medicines, while others have antibacterial, antitumor, and antifungal properties (Mabry and Ulubelen 1980). Nine coumarins have been obtained from the fruits of *L. barbarum* (Zhou et al. 2016; Jarouche et al. 2019). In vitro culture includes callus a culture, suspension culture, elicitation treatment or precursor addition, has become a popular topic for the synthesis of secondary metabolites (Kolewe et al. 2008; Yue et al. 2016; Wilson and Roberts 2012).

Therefore, the aim of this research is to conserve this rare Mediterranean plant by in vitro propagation different approaches (axillary bud formation, direct and indirect organogenesis formation through callus and suspension culture). The production of one of the plant's main active constituents, coumarin, and increasing its level using different treatments was also investigated.

Methods

Plant material and sterilization

Shoots were taken from healthy and wild growing trees at Omayed (in the northwestern coast of Egypt) in 2020. Before being treated with a detergent, the explants were washed for 5 min under running tap water. Shoot tips were disinfected by 30% (v/v) Clorox 5.2% sodium hypochlorite) for 20 min. After that, the explants were thoroughly rinsed five times with sterilized distilled water.

Culture medium and growth conditions

In vitro propagation of *L. barbarum* included three approaches: axillary bud formation, callus culture and direct organogenesis.

Axillary bud formation

In this respect different media formula and various types of cytokinin were used as follows:

Effect of MS, B5 and WPM medium on axillary shoot initiation

Sterilized shoot tip (3–5 cm) explants were cultured without PGRs on Murashige and Skoog (1962) (MS), Gamborg's medium (B5) (Gamborg et al. 1968) and Woody Plant medium (WPM) (Lloyd and McCown 1981) to choose the best formula. For each treatment, at least 10 cultures were raised.

All media formulas were supplemented with 3% (w/v) sucrose and 2.5 g/l phytagel. Before autoclaving at 1.06 kg cm−2 and 121 °C for 15 min., the pH of the media was adjusted to 5.7–5.8.

All cultures were incubated under cool white fluorescent tubes in a culture room at 25 ± 2 °C with a 16-h photoperiod.

The percentage of initiation growth, mean number of shoots/explant, mean shoot length (cm), and mean number of leave/shoot were recorded after 8 weeks of culture.

Effect of BAP, 2iP and Kin on shoot growth and development

MS medium containing 6-benzyl amino purine (BAP), 2-isobentenyl-adenine (2iP), and Kinetin; 6-furfurylaminopurine (Kin) at 0.0, 22.50, 45.00, 90.00, 180.10, 225.24, and 270.28 µM were used to improve shoot

multiplication rate and shoot elongation. The mean number of shoots/explant, mean shoot length (cm), and vitrification rate were calculated after 8 weeks of culture.

(The vitrification rate scored visually according to Pottino (1981) as follows):

1 = No vitrification, 2 = Low vitrification, 3 = Moderate vitrification.

4 = High vitrification, 5 = Severe vitrification.

Callus induction and plant regeneration

In this approach, the following experiment were done.

Effect of 2,4-D and TDZ on callus induction

Young leaves of in vitro produced shoots were used as the initial explants to induce callus culture. Leaves were divided into 1×1.5 cm segments and placed on MS media containing 2,4-dichlorophenoxyacetic acid (2,4-D) or thidiazuron; N-phenyl-N′-1,2,3-thidiazol-5-yl urea (TDZ) at 0.00, 55.26, 110.52, 221.04, 331.56, 442.08, 552.60 and 663.12 µM. After four weeks, the frequency of compact callus induction and callus fresh weight (FW) were measured.

Effect of TDZ and Zeatin on adventitious buds formation

Ten-week-old compact callus weighed (3 g) were cultured on MS medium containing TDZ and 6-(4-Hydroxy-3-methylbut-2-enylamino) purine (Zeatin) at 22.02, 44.05, 88.10, 132.15, 176.20, 220.25, 264.30, and 308.35 µM for adventitious bud induction. The frequency of adventitious buds formation and mean number of adventitious buds/explant were calculated after 6 weeks of culture.

Direct organogenesis formation

Effect of TDZ

In vitro formed leaves were divided into distal segments and cultured with the side down on MS medium added with TDZ at 0.0, 22.02, 44.05, 66.07, 88.10, 110.12, 132.15, 154.17, 176.20, 198.22, 220.25, 242.27, and 264.30 µM) for in vitro induction of direct organogenesis. After 12 weeks, the frequency of shoot regeneration and mean number of adventitious buds/explant were calculated.

Rooting and acclimatization stages

Effect of different concentration of IBA and NAA on root formation

Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) at 20.32, 50.81, 101.62, 203.64, 304.86, and 406.48 µM with activated charcoal (AC) were tested for the root formation. MS medium without PGRs served as control. The average number of roots/shoot, root and plant lengths (cm), and root induction percentage were all measured after 4 weeks.

Acclimatization process:

Rooted shoots were transplanted into polypropylene pots containing mixture of sand and peat moss (1:1 v/v). To maintain high moisture content at the outset of acclimatization, clear plastic bags were used to pack the pots. The plantlets were grown in a greenhouse at 27 ± 2 °C and were fed filtered water daily. After 21 days of adaptation, the bags had been opened and the percentage of plantlets that survived was calculated.

Production of active constituent (coumarin)

Two physical forms of culture media with chitosan and cinnamic acid different applications were used to increase the active constituent (coumarin).

Under total darkness, different concentrations of chitosan as elicitor and cinnamic acid (Alpha Chemika, India) as a precursor at 0.00, 381, 763, and 1526 µM were added to MS solid and liquid callus proliferation medium to improve callus biomass and to enhance the amount of the active constituent (coumarin). Chitosan and cinnamic acid different concentrations were added to the medium after filter sterilization with a Millex syringe operated filter unit (0.22 micron). Both solid and liquid media were supplemented with 552.60 µM TDZ, 3% (w/v) sucrose and 0.01% myo-inositol. Suspension cultures were initiated by inserting friable callus in 250-ml Erlenmeyer flasks containing 100 ml of sterile liquid MS medium sealed with cotton plugs. For gas exchange, flasks were incubated in the dark on a rotary shaker at 110 rpm and 25 ± 2 °C.

Calluses were carefully detached from the culture media after 4 weeks to determine their fresh and dry weights.

Recovering calluses and coumarin extraction

The callus in solid medium and suspension cultures were harvested after 4 weeks, and their fresh weight was registered. After that, the callus cultures were freeze-dried to get their dry weights. Using a kitchen blender, the dried callus cultures were ground into a fine powder.

For each sample, one gram of powdered callus was accurately weighed and ultrasonicated for 15 min., followed by three extractions with 10 ml of ethanol (30 ml total). After filtration, a vacuum rotary evaporator was used to concentrate the mixed ethanolic callus extract, and each residue was balanced to 10 ml using methanol. Each sample was passed through a 0.45 m porosity polytetrafluoroethylene membrane (Nalgene®, New York, USA) before HPLC analysis.

Coumarin quantitative analysis

High-Performance Liquid Chromatography (HPLC) was used to determine the amount of coumarin in *L. barbarum* callus and suspension cultures. Samples of coumarin were injected in HPLC (Ultimate 3000) according to the method of Biswas et al. (2013).

Calculation of coumarin

The peak area and concentration of coumarin in *L. barbarum* samples were measured using a DELL-compatible computer and the Cromelion7 interpretation program by comparing their relative retention time (minutes) with the standard. The concentrations of coumarin were calculated as mg/g fresh weight. By comparing the retention times of the compounds to those of the standards, the compound was identified.

Experimental design and data analysis

Treatments were set up in a completely randomized design; each treatment was reproduced three replicates, with each replicate containing five jars and one explant.

ANOVA was performed using the Costat software program for statistical analysis. Duncan (1955) multiple range tests, as modified by Snedecor and Cochran (1990), were used to examine the significance of differences between the means of all treatments at the 5% level.

Results

Axillary bud formation:

Effect of MS, B5 and WPM media on axillary shoot initiation

Shoots could be initiated from *L. barbarum* explant shoots using three tested media (MS, B5 and WPM). MS medium gave the maximum percentage of shoot initiation 91% in comparison with the other media after 8 weeks of culture (Table 1 and Fig. 1). Moreover, MS medium produced the highest number of shoots (2.2 axillary shoots/explant) and the most leaves (5.1), but only 2.5 cm axillary shoot length. WPM medium produced 1.8 axillary shoots/explants with slightly longer shoot length (3.5 cm).

Table 1 Effect of MS, B5 and WPM on shoot initiation during the establishment stage of *L. barbarum*

Media type	% of growth initiation	Mean number of axillary shoots/explant	Mean length of axillary shoot (cm)	Mean leaf number/shoots
MS	91^{a}	2.2^{a}	2.50^{c}	5.1^{a}
B5	85^{b}	1.8^{b}	3.03^{b}	4.1^{b}
WPM	80^{c}	1.8^{b}	3.50^{a}	3.5^{c}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

Fig. 1 Shoot tip of *L. barbarum* cultured on MS medium

Effect of BAP, 2iP and Kin on the growth and development of axillary shoots

The concentrations of BAP, 2iP, and Kin had a major impact on shoot multiplication. MS medium with

Table 2 Effect of BAP, 2iP and Kin on *L. barbarum* growth and development

Conc. (μM)		Mean number of shoots/explant	Mean shoot length (cm)	Degree of vitrification
Control		2.00^{k}	3.0^{e}	1.0^{h}
BAP	22.50	2.50^{g}	2.8^{g}	1.0^{h}
	45.00	2.80^{e}	2.5^{i}	1.5^{f}
	90.00	3.16^{d}	2.3^{j}	2.0^{d}
	180.10	3.50^{c}	2.1^{k}	2.4^{c}
	225.24	5.10^{a}	2.0^{l}	3.0^{b}
	270.28	3.80^{b}	1.7^{n}	4.0^{a}
2iP	22.50	1.40^{m}	2.9^{f}	1.0^{h}
	45.00	1.80^{l}	2.7^{h}	1.0^{h}
	90.00	2.10^{j}	2.5^{i}	1.0^{h}
	180.10	2.30^{h}	2.3^{j}	1.4^{g}
	225.24	2.60^{f}	2.0^{l}	1.5^{f}
	270.28	2.00^{k}	1.9^{m}	1.6^{e}
Kin	22.50	1.10^{o}	4.0^{a}	1.0^{h}
	45.00	1.30^{n}	3.8^{b}	1.0^{h}
	90.00	1.80^{l}	3.5^{c}	1.0^{h}
	180.10	2.10^{j}	3.1^{d}	1.0^{h}
	225.24	2.20^{i}	3.0^{e}	1.0^{h}
	270.28	2.50^{g}	2.9^{f}	1.0^{h}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

Fig. 2 Multiplication stage of *L. barbarum* on MS medium supplemented with 225.24 µM BAP

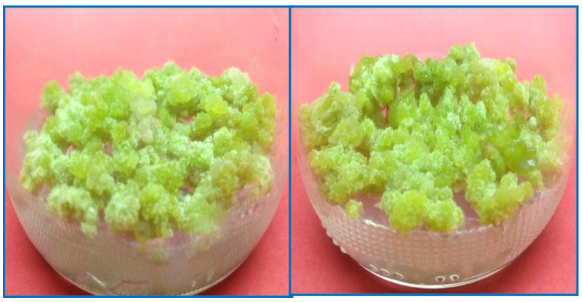

Fig. 3 Callus induction of *L. barbarum* on MS medium containing 552.60 µM TDZ

Table 3 Effects of 2,4 D and TDZ on callus formation from leaf of *L. barbarum* after 4 weeks of culture

Growth regulators concentration (µM)		Mean fresh weight of callus (g/jar)	Frequency of callus induction
Control		0.1^{n}	1^{l}
2,4–D	55.26	0.5^{m}	5^{k}
	110.52	1.0^{l}	10^{j}
	221.04	2.5^{i}	50^{f}
	331.56	4.0^{g}	65^{e}
	442.08	7.0^{e}	80^{d}
	552.60	8.7^{b}	93^{b}
	663.12	7.9^{d}	90^{c}
TDZ	55.26	1.7^{k}	20^{j}
	110.52	2.4^{j}	40^{h}
	221.04	3.3^{h}	100^{a}
	331.56	5.0^{f}	100^{a}
	442.08	8.4^{c}	100^{a}
	552.60	9.5^{a}	90^{c}
	663.12	4.0^{g}	45^{g}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

225.24 µM BAP produced the maximum shoot number (5.1 shoots/explant). MS medium containing 22.50 µM Kin provided the longest shoots of 4.0 cm (Table 2 and Fig. 2). Vitrification was observed in all of the treatments at varying levels. The highest vitrification value was obtained using MS medium containing 270.28 µM BAP (4.0).

Callus induction and regeneration

Effect of different concentrations of 2, 4-D and TDZ on callus induction

Callus was initiated from the cut ends of in vitro derived young leaves after 10 days of inoculation. MS medium contained either 2, 4-D or TDZ; a compact callus was formed. The callus induction frequency was significantly influenced by MS medium supplemented with 221.04, 331.56 and 442.08 µM TDZ (100%). After 4 weeks of culture, MS medium fortified with 552.60 µM TDZ produced the heaviest compact callus (9.5 g/FW) (Table 3 and Fig. 3), while 2,4-D different concentrations gave the low rate of callus induction.

Table 4 Effect of TDZ and Zeatin on adventitious buds formation frequency and number of buds/callus culture

Growth regulators conc. (µM)		Mean number of adventitious buds/explant	Frequency of adventitious buds
Control		0.1^{e}	1^{i}
TDZ	22.02	0.1^{e}	1^{i}
	44.05	1.0^{d}	15^{g}
	88.10	0.1^{e}	26^{d}
	132.15	2.0^{c}	30^{c}
	176.20	4.0^{a}	50^{a}
	220.25	3.0^{b}	40^{b}
	264.30	0.1^{e}	1^{i}
	308.35	0.1^{e}	1^{i}
Zeatin	22.02	0.1^{e}	1^{i}
	44.05	0.1^{e}	1^{i}
	88.10	0.1^{e}	1^{i}
	132.15	1.0^{d}	10^{h}
	176.20	2.0^{c}	25^{e}
	220.25	1.0^{d}	20^{f}
	264.30	0.1^{e}	1^{i}
	308.35	0.1^{e}	1^{i}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

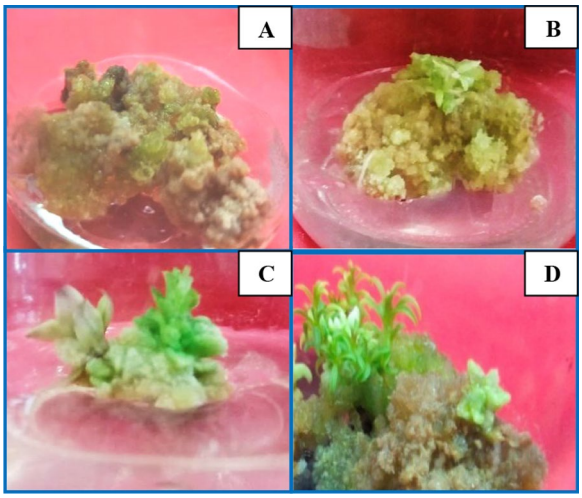

Fig. 4 In vitro regeneration of *L. barbarum* through callus-mediated indirect shoot-organogenic pathway on MS medium containing 176.20 µM TDZ. **a**: Swilling of callus culture. **b**: Organogenesis induction of explants. **c**: New organs began to differentiate on callus surface. **d**: Further production of organs after the second subculture

Table 5 The effect of TDZ concentrations on direct organogenesis formation from *L. barbarum* leaf explants

Treatments (µM)		Mean number of adventitious buds/explant	Frequency of shoot regeneration
Control		2^{j}	1^{l}
TDZ	22.02	2^{j}	5^{k}
	44.05	3^{i}	10^{j}
	66.07	5^{h}	15^{i}
	88.10	6^{g}	20^{h}
	110.12	8^{f}	25^{g}
	132.15	9^{e}	70^{f}
	154.17	10^{d}	80^{e}
	176.20	11^{c}	90^{c}
	198.22	13^{b}	92^{b}
	220.25	15^{a}	96^{a}
	242.27	10^{d}	85^{d}
	264.30	9^{e}	70^{f}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

Effect of TDZ and Zeatin on adventitious buds formation

When the callus was cultured on MS medium supplemented with TDZ and Zeatin at different concentrations, it formed adventitious buds via organogenesis. Within 3–6 weeks, a small number of adventitious buds appeared on the callus tissue's surface, which later developed into shoots. As shown in Table 4 and Fig. 4, the maximum mean number of adventitious buds (4.0) was formed from callus on a medium containing 176.20 µM TDZ with 50% shoot formation. The minimum response was observed on MS medium with Zeatin or without PGRs.

Direct organogenesis formation

Effect of different concentrations of TDZ on direct organogenesis formation

At different concentrations of TDZ, direct organogenesis of *L. barbarum* was observed from leaf explants. With a 96% frequency of shoot regeneration, the presence of 220.25 µM TDZ resulted in the maximum number of shoot buds (15 shoots) that was regenerated directly from leaves (Table 5 and Fig. 5). The rate of shoot regeneration was slowed as the TDZ concentration in the medium was increased further. After about 10 days of cultured on MS medium, the leaf segments swelled. After around 20–30 days of culture, small protuberances appeared along the edges of the leaf explants (Fig. 5). Following that, these protuberances began to grow into adventitious shoot buds. The frequency and number of adventitious shoots induced were dramatically reduced when the concentration of TDZ was increased or decreased. On MS medium without PGRs, the minimum response was recorded.

Rooting and acclimatization stages

Effect of IBA and NAA on the rooting of shoots

The 6.8-cm-long shoots were carefully separated and cultured on MS medium with IBA, NAA, or without auxin. The medium containing 304.86 µM NAA produced the highest root number (22.66 roots/shoot), while the medium containing 304.86 µM IBA produced the longest roots (10.6 cm) and the longest plant (12.1 cm), with 100% root induction for both treatments (Table 6 and Fig. 6). The MS medium lacking PGRs produced the least mean number and length of roots.

Acclimatization process

Acclimatization was successful in about 95% of the in vitro-generated plants (Fig. 7). The well-rooted plantlets produced fresh new leaves after a month of acclimatization.

Active constituent (coumarin)

Table 7 illustrates the effect of different concentrations of chitosan and cinnamic acid on coumarin accumulation in *L. barbarum* callus and suspension cultures. Different concentrations of both compounds were added to MS solid and liquid media supplemented with 552.60 µM

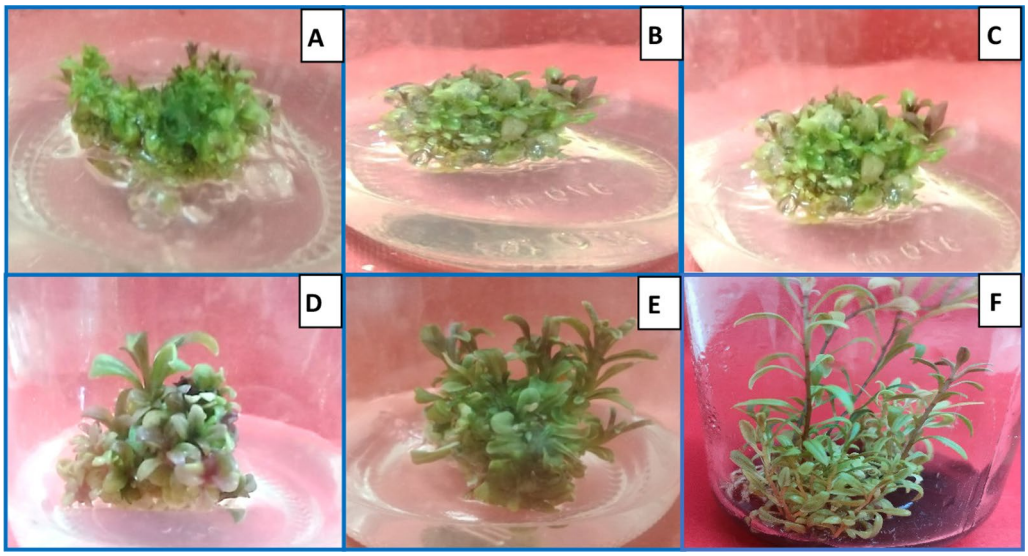

Fig. 5 In vitro regeneration via direct organogenesis from proximal leaf explant of wild *L. barbarum*. **a**: Swilling of leaf buds. **b**: Organogenesis induction of *L. barbarum* explants cultured on MS medium containing 220.25 µM TDZ. **c** and **d** Development of organs during 6 weeks of culture. **e**:Shoot formation. **f**: Further development of shoot formation after the second subculture

Table 6 Effect of IBA and NAA different concentrations on root formation of *L. barbarum*

Auxin conc. (µM)		Mean number of roots/explant	Mean length of roots (cm)	Mean shoot height (cm)	Root induction percentage
Control		3.00^{m}	3.3^{h}	4.5^{m}	15^{j}
IBA	20.32	5.00^{l}	4.3^{f}	10.2^{j}	20^{i}
	50.81	7.00^{k}	6.3^{e}	10.6^{i}	25^{g}
	101.62	10.00^{i}	7.5^{d}	10.8^{h}	80^{e}
	203.64	13.00^{f}	9.4^{b}	11.0^{f}	90^{c}
	304.86	18.00^{d}	10.6^{a}	12.1^{a}	100^{a}
	406.48	12.00^{g}	8.5^{c}	11.5^{c}	95^{b}
NAA	20.32	8.00^{j}	2.2^{m}	9.6^{l}	25^{g}
	50.81	11.00^{h}	2.5^{l}	10.0^{k}	23^{h}
	101.62	15.00^{e}	2.7^{k}	10.9^{g}	76^{f}
	203.64	19.00^{c}	2.9^{j}	11.1^{e}	85^{d}
	304.86	22.66^{a}	3.5^{g}	11.8^{b}	100^{a}
	406.48	20.00^{b}	2.8^{j}	11.3^{d}	95^{b}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

TDZ. The addition of 763 µM cinnamic acid to the suspension culture media was found to be the concentration required to produce the highest coumarin content (Table 7 and Fig. 8), i.e., about 1.95 times as much coumarin was produced as in cultures grown without cinnamic acid (control treatment).

Fig. 6 Rooting of *L. barbarum* adventitious shoots. **a**: MS medium containing 304.86 µM IBA. **b**: MS medium containing 304.86 µM NAA

In addition, callus culture solid medium with 763 µM cinnamic acid produced about 1.67 times as much as cultures without cinnamic acid. Moreover, coumarin reached approximately 0.276 percent (g/100 g fresh weight of callus) after 4 weeks of chitosan application in suspension cultures at a concentration of 763 µM, representing a 1.85-fold improvement over the control. While increasing chitosan over 763 µM, the accumulation of coumarin reduced. The synthesis of coumarin was similarly reduced by increasing the concentration of cinnamic acid to 763 µM.

Discussion

Plant tissue culture media contains macro-elements, microelements, vitamins, and other organic compounds. Murashige and Skoog (MS) is the most commonly used in plant tissue culture. The B5 and N6 (Nitsch and Nitsch 1969) have all been widely used for a variety of plant species (Chu 1978). In addition, in the culture of woody species, the DKW (Driver and Kuniyuki 1984) and WPM medium are used. When selecting a growth medium, both the plant species and the tissue culture medium are taken into account (Gamborg et al. 1995).

Fig. 7 Acclimatized plantlets of *L. barbarum* (**a**: after 8 weeks. **b**: after 16 weeks)

In our investigation, MS medium surpassed B5 and WPM and produced the highest number of *L. barbarum* axillary shoots/explant as well as 91% shoot proliferation This confirms the importance of using MS medium in the establishment stage, which is in harmony with the results obtained by Prudente et al. (2019), who found that throughout axillary bud proliferation, MS medium containing 4 µM BAP resulted in high shoot numbers and regeneration of *L. barbarum*. Murashige and Skoog (MS) is recognized as a standard medium for most plants. It contains a high concentration of ion as particularly nitrogen, potassium, zinc, and chlorine compared to other formulas, (Leifert et al. 1995). Moreover, the results of the current study confirmed the efficiency roles of BAP concentrations on in vitro *L. barbarum* shoot multiplication. In the current study, the greatest shoot number was noticed at 225.24 µM BAP. Our findings are consistent with those of Mangena (2020) who mentioned that BAP regulates cell division, adventitious branch creation, apical dominance, as well as root formation. This is because BAP is more stable than other cytokinins, less susceptible to light oxidation, less expensive and easier to produce. BAP is also more effective because it has the ability to promote the formation of other hormones (Uranbey 2004). Prudente et al. (2019) found that in *L. barbarum*,

Table 7 Effect of different concentrations of chitosan and cinnamic acid on coumarin content (mg/g fresh weight) in callus and suspension cultures of *L. barbarum*

Treatments		Conc (μM)	Conc. Of coumarin (%)	Increase (fold)	Decrease (fold)	Callus fresh weight	Callus dry weight
Mother plant (Grow in open field)			0.04388	0.00	0.00	0.00^{o}	0.0^{k}
Control			0.0268	0.00	0.00	2.00^{n}	0.22^{i}
Callus culture	Chitosan	381	0.00008	0.00	0.003	2.30^{m}	0.21^{j}
		763	0.0328	1.22	0.00	3.10^{l}	0.31^{f}
		1526	0.0296	1.10	0.00	2.90^{k}	0.28^{g}
	Cinnamic acid	381	0.038	1.41	0.00	3.50^{f}	0.35^{d}
		763	0.0448	1.67	0.00	3.90^{c}	0.41^{b}
		1526	0.0424	1.58	0.00	3.30^{h}	0.33^{e}
Suspension culture	Control		0.0356	1.32	0.00	2.50^{l}	0.25^{h}
	Chitosan	381	0.04372	1.63	0.00	3.40^{g}	0.35^{d}
		763	0.0496	1.85	0.00	4.10^{b}	0.41^{b}
		1526	0.042	1.56	0.00	3.20^{i}	0.31^{f}
	Cinnamic acid	381	0.044	1.64	0.00	3.80^{e}	0.39^{c}
		763	0.0524	1.95	0.00	4.40^{a}	0.45^{a}
		1526	0.046	1.71	0.00	3.82^{d}	0.39^{c}

Means followed by the same letter are not significantly different according to Duncan's multiple range test. The mean difference is significant at the 0.05 level. The presence of different letters in the same table indicates a high significance between the numbers. The higher significance between the results obtained, this leads to the numbers taking different letters

using BAP at 11.56 μM led to a rising shoot, more average leaves/plant, and longer shoots.

Hyperhydricity is a physiological problem that affects shoot proliferation and vitality in in vitro culture (Van Den Dries et al. 2013). This occurrence has been attributed to a variety of stress factors, including imbalanced or elevated levels of PGRs in the cultural media (Hazarika 2006). In the current study, the maximum vitrification value was obtained in MS medium containing 270.28 μM BAP. Kataeva et al. (1991) theorized that high levels of cytokinins cause shoot hyperhydricity by causing rapid cell division in meristems in the high humidity of tightly closed vessels. High level of BAP produced hyperhydricity in goji shoots (Ivanova and Staden 2011).

In this investigation, TDZ stimulates callus growth, has beneficial effects on direct and indirect formation of *L. barbarum* organogenesis. Leaf explants were used to stimulate callus growth on an MS medium containing different concentrations of TDZ and 2,4-D. 552.60 μM TDZ, for particular, had a high callus induction rate and produced 9.5 g/FW of callus. The maximum shoot proliferation ability by organogenesis from the callus was obtained in the current investigation at 176.20 μM TDZ.

To investigate the direct organogenesis process in *L. barbarum* in this study, leaf segments were cultured on MS medium with TDZ. The best treatment was MS medium supplemented with 220.25 μM TDZ, which induced 96% of adventitious shoots initially and produced 15 adventitious shoots/explant. These results are close to the results of Karakas (2020) who observed that hypocotyl of *L. barbarum* explants on medium fortifying with 0.25 mg/l TDZ and 0.1 mg/l IAA had the maximum callus diameter. According to Verma et al. (2016), the most effective concentration for callus initiation and shoot differentiation in the crocus species was 4 mg/l TDZ and NAA. TDZ improved purine availability for cellular growth and purine metabolite synthesis (Victor et al. 1999), as well as adenine to adenosin conversion (Capelle et al. 1983).These are critical for the fast processes of cell division and protein synthesis that occur during callus induction and somatic embryo development (Fujimura and Komamine 1980). TDZ can encourage adventitious shoots and somatic embryo at concentrations greater than 1.0 M (Huetteman and Preece 1993). TDZ has been proven to enhance plant shoot development and increase axillary shoot proliferation. Because of their increased cytokinin-like activity and rapid response, resistant woody species have proved good TDZ responders. It aids the initiation of multiple shoots in a variety of hard woody trees (Pai and Desai 2018). In carob (*Ceratonia siliqua*), 4.54 μM TDZ was shown to be the most effective concentration for differentiating adventitious buds and produced the most shoots (Ahmed et al. 2021).

Different types of auxins are commonly used to stimulate rooting in woody plants. In the present study, NAA at 304.86 μM gave 100% root formation and maximum number of roots. Ruta et al. (2020) found that the shoots of *L. barbarum* were applied with 0.3 mg/l IBA had a

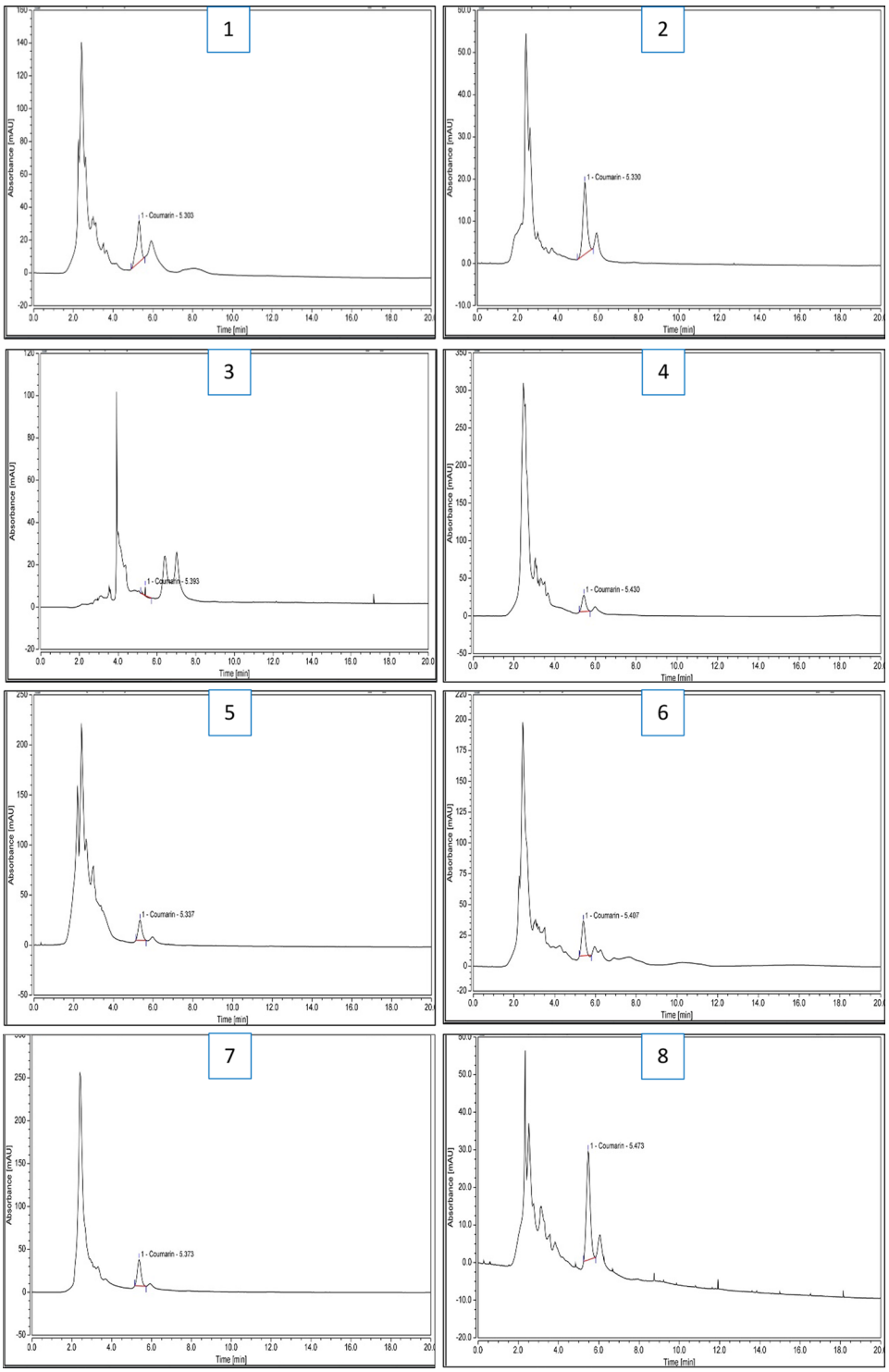

Fig. 8 HPLC chromatograph shows the peak of coumarin from leaf callus of *L. barbarum* cultured on MS callus proliferation medium containing 552.60 µM TDZ under total darkness. 1 = Mother plant (grown in open field). 2 = Callus culture without additives (control). 3 = Callus culture with 381 µM chitosan. 4 = Callus culture with 763 µM chitosan. 5 = Callus culture with 1526 µM chitosan. 6 = Callus culture with 381 µM cinnamic acid. 7 = Callus culture with 763 µM cinnamic acid. 8 = Callus culture with 1526 µM cinnamic acid. 9 = Callus culture without additives (control). 10 = Suspension culture with 381 µM chitosan. 11 = Suspension culture with 763 µM chitosan. 12 = Suspension culture with 1526 µM chitosan. 13 = Suspension culture with 381 µM cinnamic acid. 14 = Suspension culture with 763 µM cinnamic acid. 15 = Suspension culture with 1526 µM cinnamic acid

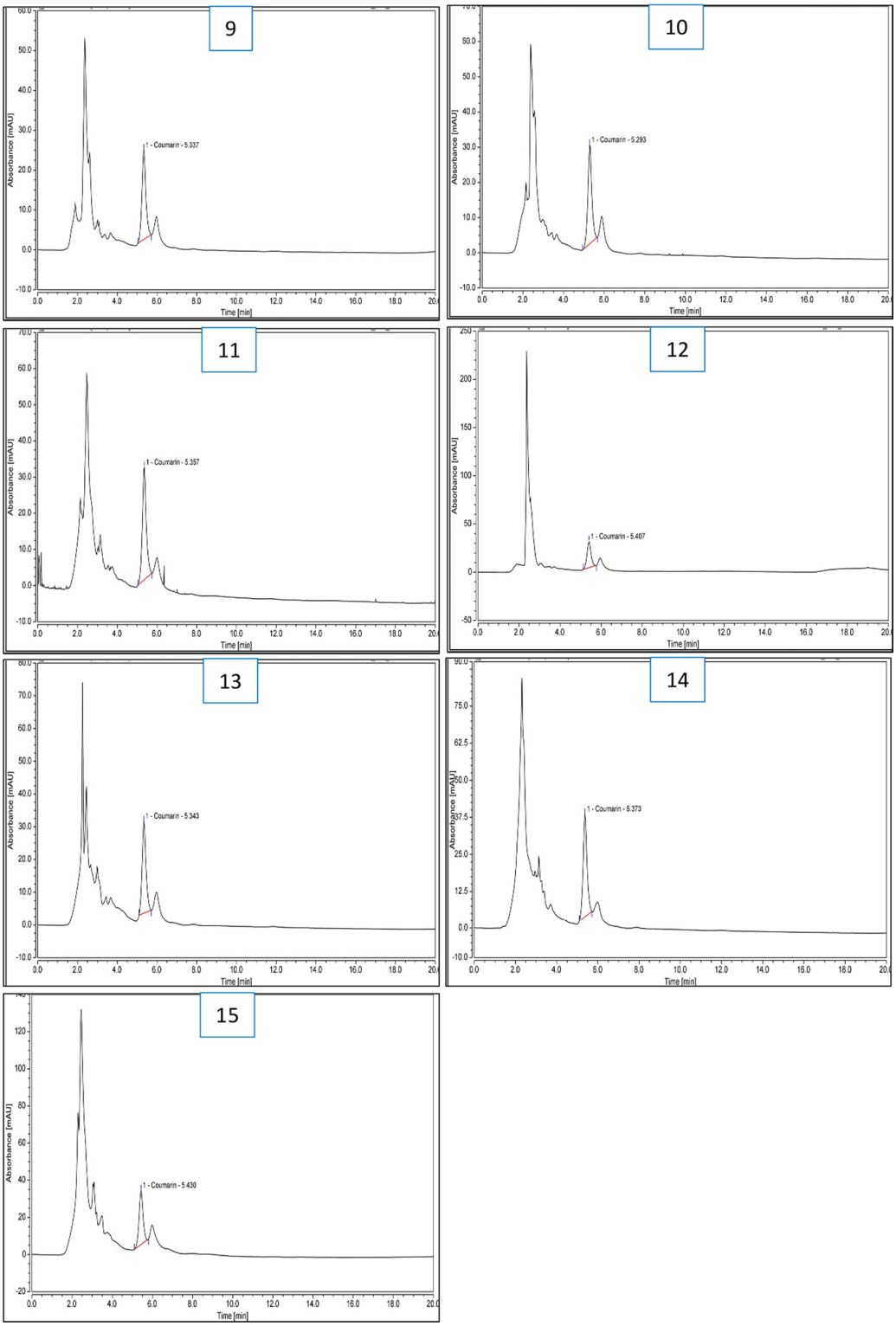

Fig. 8 continued

rooting rate of only 70–82%. NAA is relatively stable in tissue culture medium, according to Hausman (2003).

Secondary metabolites are often produced in vitro by using structures such as undifferentiated calli, cell suspension cultures, or specialized organs like shoots, roots, or somatic embryos. Suspension cultures help plants to produce essential metabolites. The logical initial step in the production of plant secondary metabolites in suspension cultures is to establish a stable and optimal callus culture. Under the right conditions, a moderately friable fraction of the callus transported in liquid medium yields chemical compounds while also eliminating interfering substances seen in field-grown plants (Rajan et al. 2020). Precursors are intermediate chemicals in secondary metabolite production pathways that should be supplied to the culture media at the appropriate stage and concentration (Gueven and Knorr 2011). The use of biosynthetic precursors effectively boosted coumarin synthesis in vitro. Cinnamic acid is a central intermediate in the biosynthesis of coumarin (Brown 1963; Vogt 2010). Chitosan as an elicitor has the ability to be used in in vitro cultures to elicit the formation of secondary metabolites (Orlita et al. 2008).

In this investigation, cinnamic acid and chitosan were added to callus and cell suspension cultures media to induce the endogenous level of coumarin. The highest coumarin content (0.0524%) was found in cell suspension culture medium supplemented with 763 µM cinnamic acid compare with control treatment. Cinnamic acid is a well-known precursor of coumarin in the plant (Brown 1963; Vogt 2010).

Conclusions

The in vitro regeneration of plants via leaf-mediated shoot organogenesis protocol presented in this study is well suited for *L. barbarum* propagation and produced large quantities of identical planting materials for desert revegetation and pharmaceutical industries. A very effective indirect regeneration method via callus culture provides a potential for this ethnomedicinal plant to be improved by genetic transformation procedures, in addition to mass propagation of elite plants. The cell suspension culture medium with 763 µM cinnamic acid had the highest coumarin accumulation.

Abbreviations

ANOVA: Analysis of variance; BA: 6-Benzyl adenine; GA3: Gibberellic acid; IBA: Iindole-3-butyric acid; 2iP: N6-(2-isopentenyl) adenine; IUCN: International Union for Conservation of Nature; Kin: Kinetin; MS: Murashige and Skoog; NAA: α-Naphthaleneacetic acid; PGRs: Plant growth regulators; TDZ: Thidiazuron.

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Author contributions

MEA conceived the study, designed the workflow, did the in vitro propagation of the plant, generated the data, analyzed the data, and wrote the manuscript (including revisions). The author read and approved the final manuscript.

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Availability of data and materials

All the data required for the processing of the conclusions are presented in the results section.

Declarations

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