ORIGINAL RESEARCH

Enhanced somatic embryogenesis, plant regeneration and total phenolic content estimation in *Lycium barbarum* L.: a highly nutritive and medicinal plant

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Abstract

Lycium barbarum (*Solanaceae*), commonly known as Goji (or wolfberry), is popular for its nutritive and medicinal properties and is called a "super fruit" or "super food". Considering the importance of the economic utility, the potential of different explants (hypocotyl, leaf and root) of *L. barbarum* for plant regeneration and somatic embryogenesis has been evaluated in the present study. Two sets of experiments were carried out; the first compared Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D), N^6-benzylaminopurine, thidiazuron (TDZ), kinetin, and zeatin alone, while the second set tested the combinations of TDZ with 2,4-D to induce callus and subsequent shoot or embryo formation, respectively. For callus and subsequent shoot induction (for the first set of the experiment), 2,4-D was the most effective for callus induction (100%), while TDZ at 1 mg/L produced a mean of 5 shoots per callus. For the second set of experiment spontaneous induction of somatic embryos as well as subsequent maximum shoot regeneration was recorded in 0.25 mg/L 2,4-D+ 1 mg/L TDZ supplemented MS medium. Hypocotyl explant proved to be the most responsive organ that induced as many as 6 somatic embryos and subsequent 9 regenerated shoots per callus. Later, the plantlets were successfully acclimatized (100%) and finally transferred to the greenhouse. Total phenolic content was measured from shoot, brown callus and white callus of *L. barbarum* L. grown in MS medium supplemented with auxin alone (for callus) and auxin-cytokinin (for shoots). The highest amount of total phenolic content (640 mg GAE/g DW) was obtained in the shoot. The described protocol provides a simple way to regenerate plants through direct and indirect organogenesis as well as somatic embryogenesis, which would be useful for mass propagation, large-scale production of secondary metabolites, germplasm conservation, and genetic transformation studies in this medicinally important species.

Keywords Acclimatization · Goji berry · Histology · Hypocotyl · Secondary metabolites

Abbreviations

2,4-D	2,4-Dichlorophenoxy acetic acid
BAP	N^6-benzylaminopurine
DW	Dry weight
GAE	Gallic acid equivalent
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
PGRs	Plant growth regulators
TDZ	Thidiazuron

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Introduction

Lycium barbarum L., commonly known as Goji belongs to the family *Solanaceae*. *L. barbarum* is a woody bush, widely distributed in warmer regions of the world particularly China and countries in Europe and around Mediterranean. It is also cultivated in North America and Australia as a hedge plant (Hänsel et al. 1994). Majority of the commercially produced Goji berries come from plantations of *L. barbarum* in the

Ningxia Hui Region in north-central China and the Xinjiang Uyghur Region in western China (Potterat 2010). In addition, the climatic conditions of Ningxia (temperature, altitudes, rainfalls, etc.) are very much similar to the Bolu province of Turkey.

For thousands of years, *L. barbarum* has been utilised as traditional Asian medicine and functional food in dried form, as freshly squeezed fruit juice, or as concentrated beverages (Amagase 2014). *L. barbarum* has been therapeutically used owing to its rich source of polysaccharides, steroid saponins and other bioactive compounds. It is added widely in herbal medicines for its immunodulatory, hepatoprotective and anti-tumor effects as well as its anti-cancer properties. In addition, the genus *Lycium* is the source of a number of steroid saponins and other bioactive compounds that are of medical and pharmaceutical importance. In support of these traditional properties, recent studies indicate that extracts from *L. barbarum* fruit and one of its active compounds, polysaccharides possess a range of biological activities, including effects on aging, neuroprotection, anti-fatigue/endurance, increased metabolism, glucose control in diabetics, glucoma, anti-oxidant properties, immunomodulation, anti-tumor activity and cytoprotection (Amagase and Farnsworth 2011).

Since the beginning of the twenty-first century, *L. barbarum* has a high economical value in the world market. Thus, this plant has been a big target for plant biotechnology for the last few years. However, there are only a few in vitro plant regeneration protocols individually on protoplast culture, callus culture and somatic embryogenesis etc., that have been developed in this species to date (Ratushnyak et al. 1989; Hu et al. 2002, 2008; Osman et al. 2013; Karakas 2020). To date, there is no such all-inclusive report on callus-mediated somatic embryogenesis, subsequent plantlet regeneration and phytochemical assessment in this plant species using different explant sources. In this communication, we describe a simple and suitable protocol for enhanced induction of callus-mediated somatic embryogenesis, plant regeneration and estimation of total phenolics accumulation in different in vitro tissues/organs of *L. barbarum* L.

Materials and methods

Plant material and surface disinfection

Seeds of *L. barbarum* were collected and successively surface-disinfected with freshly prepared 0.1% (w/v) aqueous mercuric chloride solution for 10 min, followed by a quick rinse (10 s) in 70% (v/v) ethanol and 3–4 washings in sterile distilled water. An average of 20–25 seeds were aseptically cultured on Petri plates (90 × 15 mm) containing 30 mL of Murashige and Skoog (MS) semisolid medium (Murashige

and Skoog 1962) and 3% (w/v) sucrose without any plant growth regulators (PGRs). The medium was solidified with 0.8% (w/v) agar and autoclaved at 121 °C and 1.06 kg/cm^2 pressure for 15 min, after adjusting the pH to 5.8 with 0.1 N HCl or 0.1 N NaOH. The cultures were incubated under 16/8-h light/dark photoperiod (using cool-white fluorescent light with an irradiance of 50 μmol/m^2/s) at 25 ± 2 °C.

Callus induction and shoot regeneration

For callus initiation, hypocotyl, leaf and root segments were taken from in vitro germinated seedlings and placed on MS medium, supplemented with different concentrations (0.1, 0.5, 1 and 3 mg/L) of 2,4-dichlorophenoxy acetic acid (2,4-D), N^6-benzylaminopurine (BAP), kinetin, thidiazuron (TDZ) and zeatin, individually. Incubation conditions were the same as described for germination of seed. The induced calli were maintained on the same medium for four weeks. Average number of heart stage somatic embryos were scored after eight weeks of transfer of callus to the proliferation medium. The frequency (%) of explants developing somatic embryos or shoots and mean numbers of somatic embryos or shoots per explant were recorded after eight weeks of culture.

Enhanced callus induction, somatic embryogenesis and shoot regeneration

The hypocotyl, leaf and root segments derived from the first set of experiments were used as explants for enhanced callus induction, somatic embryogenesis and multiple shoot regeneration. Explants were inoculated in Petri plates containing 30 mL of semisolid MS medium fortified with 0.25 mg/L 2,4-D in combination with 0.5, 1 and 3 mg/L TDZ. The frequency (%) of explants developing callus, somatic embryos and multiple shoots from explants were recorded after eight weeks of culture. Average number of heart stage somatic embryos were scored from each of the explants.

Histological studies

For histological studies, the regenerating somatic embryos were fixed in formaldehyde:glacial acetic acid:ethanol (5:5:90; v/v/v) for 24 h, dehydrated through a graded tertiary butyl alcohol series, each for 24 h and then embedded in saturated paraffin wax. Embedded materials were sectioned to 5 μm thickness on a rotary microtome (LEICA rotary microtome model RM 2125 RTS). Paraffin wax was removed by xylene prior to rehydration of the tissues in a graded ethanol series and staining of the tissues was performed using 1% (w/v) safranin. Tissues were briefly washed in water to remove excess stain and then dehydrated in a graded ethanol

series. The photographs were taken by a microscope (Model DM 2500 supplied with a digital camera).

Rooting of shoots

Individual shoots were separated from multiple shoot clumps and inoculated in PGR-free MS medium for root initiation, multiplication and elongation. The cultures were incubated for 4 weeks for complete root development under the same culture condition as stated above.

Acclimatization

Six-week-old plantlets with well-developed roots and shoots were taken out of the culture medium and transferred to the plastic pots containing autoclaved peat moss, compost and soil (1:1:1; v/v/v). Pots were covered with transparent poly-ethylene bags having small pores to maintain a high humidity with required aeration facility and kept in the culture room at 25 °C under 16 h photoperiod (provided by cool-white fluorescent light, irradiance 50 μmol/m^2/s). After three weeks, the plantlets were transferred to larger pots containing peat moss, compost and garden soil (1:1:1; v/v/v) and after another three weeks, they were transferred finally to the greenhouse and then outdoor environment under the sun.

Determination of total phenolic content

The total phenolic content in the extract was determined following a modified Folin–Ciocalteu method (Marigo and Boudet 1979). Briefly, a 20 μL aliquot of the extract was placed in a 2 mL microcentrifuge tube containing 1.58 mL of water and 100 μL of Folin–Ciocalteu reagent (Appli-Chem, Ankara, Turkey). The microcentrifuge tube was allowed to stand for 5 min and then 300 μL 20% Na_2CO_3 (w/v) (Merck, Darmstadt, Germany) was added into the tube. After ~ 20 min at 40 °C, the absorbance of the solution was measured using a spectrophotometer (Hitachi U-1900 UV–Vis, Tokyo, Japan) at $\lambda = 750$ nm. The total phenolic content was expressed as mg gallic acid equivalent/ g dry weight (mg GAE/g DW).

Statistical analysis

The experiments were laid out following a completely randomized design with five replications per treatment taking 10 samples for each replication. The collected data were statistically analyzed using a computer program (SPSS Statistics, version 17.0, SPSS Inc., Chicago, IL, USA). The experimental results were subjected to an analysis of variance (ANOVA) and Duncan's multiple range test. The mean ± standard error (SE) was subjected to Duncan's multiple range test (Duncan 1955) at $P = 0.05$.

Results and discussion

Callus induction and indirect regeneration

Three distinct forms of calli i.e. 'friable', 'nodular' and 'watery' were observed to be induced from root, leaf and hypocotyl. The friable calli were mostly induced from roots (Fig. 1a), watery type calli were mostly observed from leaf explants (Fig. 1b), however, hypocotyl explants chiefly induced nodular, compact and green calli (Fig. 1c). The callus induction was not only found to be dependent on the type of different plant organs used as explants but also the type and concentration of PGRs. Among the five different PGRs, including one auxin and four cytokinins
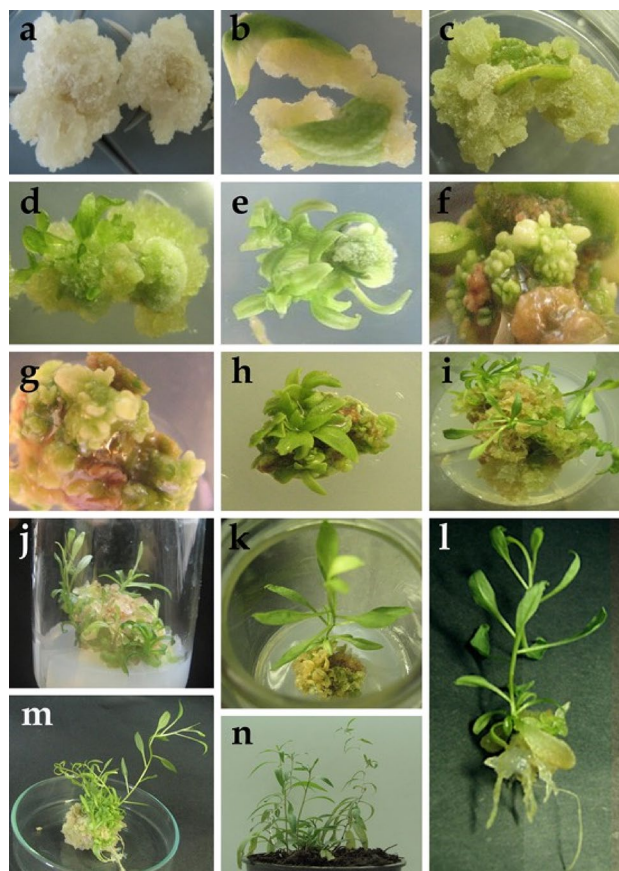

Fig. 1 Plant regeneration from hypocotyls, leaf, and root explants of *Lycium barbarum* L. **a–c** Well-developed callus from **a** root, **b** leaf and **c** hypocotyl explants; **d–e** indirect shoot development from hypocotyl explants on MS medium containing **d** 1 mg/L TDZ alone or **e** in 0.25 mg/L 2,4-D and 3 mg/L TDZ combination; **f–g** somatic embryogenesis from leaf explants culture; on MS medium supplemented with 0.25 mg/L 2,4-D and 1 mg/L TDZ; **h–k** indirect shoot development from leaf-derived somatic embryos on MS medium containing 0.25 mg/L 2,4-D and 1 mg/L TDZ; **l–m** complete plantlet with well-rooted shoots; **n** well-acclimatized plant established in pot under open field conditions

with different concentrations, an exhibition of significantly different response in terms of callus induction from different explants was observed (Fig. 1a–c). Maximum induction of callus (100%) was exhibited by 2,4-D (0.1 mg/L and 0.5 mg/L) (Table 1). In this case, all three explants exhibited highly prolific callus formation. A comparable result of callus development was also found in 0.1 and 0.5 mg/L kinetin from hypocotyl explant only. Interestingly, at the same concentration, the leaf or root did not show any callogenesis. TDZ at 0.5 mg/L concentration results in callus induction from all three different explants but in variable frequencies. Zeatin at the levels of 0.5, 1 and 3 mg/L was competent enough to induce callus from all three explants with a frequency ranging from 23 to 100%. In terms of callus induction efficiency, hypocotyl explants were found to be the most responsive followed by root and leaf explants. This is noteworthy to mention that the control medium (MS without PGR supplementation) neither exhibited callogenesis nor organogenesis. During indirect shoot regeneration study from induced callus, maximum response was recorded in 1 mg/L TDZ wherein as many as 5 shoots per callus were counted (Fig. 1d). In comparison, 0.1 mg/L 2,4-D, 0.1 and 0.5 mg/L kinetin, 3 mg/L zeatin resulted in 3–4 shoots per callus. Maximum indirect shoot regeneration was recorded from hypocotyl-derived calli. On the contrary, there was

leaf- (Fig. 2a) and root-derived calli that exhibited multiple shoot formation only in two and one occasion(s), respectively.
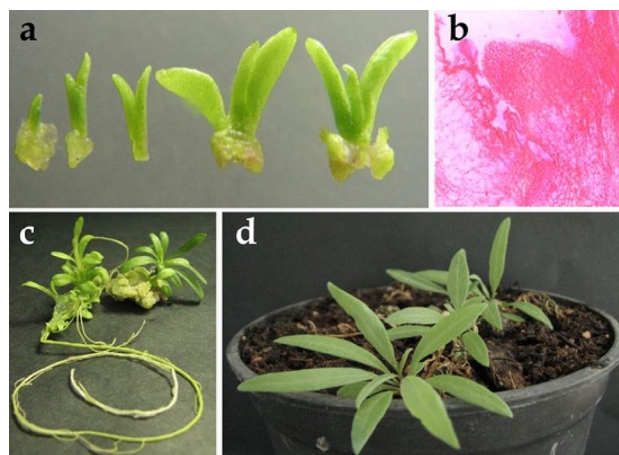
Fig. 2 a In vitro shoot development stages from indirect shoot organogenesis from leaf segment of *Lycium barbarum* L., **b** histological section of emerging shoots from leaf-derived somatic embryos; **c** rooting of the regenerated shoots; **d** regenerated plants in potting mixture [autoclaved peat moss, compost and soil (1:1:1; v/v/v)]

Table 1 Effect of different explants and different concentrations of plant growth regulators (PGRs) on callus formation and shoot regeneration in *Lycium barbarum* L.

PGRs (mg/L)		Percentage of callus formation			Mean number of shoots per callus		
		Hypocotyl	Leaf	Root	Hypocotyl	Leaf	Root
Control		0.0 ± 0.0^j	0.0 ± 0.0^i	0.0 ± 0.0^e	0.0 ± 0.0^e	0.0 ± 0.0^c	0.0 ± 0.0^b
2,4-D	0.1	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	3.4 ± 0.2^c	0.0 ± 0.0^c	2.6 ± 0.2^a
	0.5	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	1.0 ± 0.0^e	0.0 ± 0.0^c	0.0 ± 0.0^b
	1.0	74 ± 1.9^c	80.0 ± 3.6^b	100.0 ± 0.0^a	1.0 ± 0.0^e	0.0 ± 0.0^c	0.0 ± 0.0^b
	3.0	60.0 ± 1.5^e	57.0 ± 1.6^c	90.0 ± 1.6^b	0.0 ± 0.0^f	0.0 ± 0.0^c	0.0 ± 0.0^b
BAP	0.1	28.0 ± 1.8^i	0.0 ± 0.0^i	62.0 ± 2.2^d	2.4 ± 0.2^d	0.0 ± 0.0^c	0.0 ± 0.0^b
	0.5	29.0 ± 2.2^i	0.0 ± 0.0^i	0.0 ± 0.0^e	2.4 ± 0.2^d	0.0 ± 0.0^c	0.0 ± 0.0^b
	1.0	68.0 ± 1.2^d	52.0 ± 2.2^d	0.0 ± 0.0^e	1.4 ± 0.2^e	0.0 ± 0.0^c	0.0 ± 0.0^b
	3.0	64.0 ± 4.2^e	48.0 ± 2.5^e	0.0 ± 0.0^e	0.0 ± 0.0^f	0.0 ± 0.0^c	0.0 ± 0.0^b
Kinetin	0.1	100.0 ± 0.0^a	0.0 ± 0.0^i	0.0 ± 0.0^e	3.6 ± 0.4^c	0.0 ± 0.0^c	0.0 ± 0.0^b
	0.5	100.0 ± 0.0^a	0.0 ± 0.0^i	0.0 ± 0.0^e	4.4 ± 0.6^b	0.0 ± 0.0^c	0.0 ± 0.0^b
	1.0	42.0 ± 0.0^j	0.0 ± 0.0^i	0.0 ± 0.0^e	1.2 ± 0.2^e	0.0 ± 0.0^c	0.0 ± 0.0^b
	3.0	0.0 ± 0.0^j	0.0 ± 0.0^i	0.0 ± 0.0^e	0.0 ± 0.0^f	0.0 ± 0.0^c	0.0 ± 0.0^b
TDZ	0.1	50.0 ± 1.2^g	0.0 ± 0.0^i	0.0 ± 0.0^e	2.2 ± 0.5^d	0.0 ± 0.0^c	0.0 ± 0.0^b
	0.5	100.0 ± 0.0^a	32.0 ± 2.2^g	60.0 ± 1.5^d	0.0 ± 0.0^e	2.4 ± 0.2^a	0.0 ± 0.0^b
	1.0	88.0 ± 1.9^b	0.0 ± 0.0^i	80.0 ± 1.8^c	5.4 ± 0.2^a	1.2 ± 0.2^b	0.0 ± 0.0^b
	3.0	0.0 ± 0.0^j	0.0 ± 0.0^i	0.0 ± 0.0^e	0.0 ± 0.0^e	0.0 ± 0.0^c	0.0 ± 0.0^b
Zeatin	0.1	0.0 ± 0.0^j	0.0 ± 0.0^i	0.0 ± 0.0^e	0.0 ± 0.0^e	0.0 ± 0.0^c	0.0 ± 0.0^b
	0.5	62.0 ± 2.5^e	22.0 ± 1.9^h	100.0 ± 0.0^a	2.4 ± 0.2^d	1.0 ± 0.0^b	0.0 ± 0.0^b
	1.0	52.0 ± 2.2^f	40.0 ± 2.2^f	100.0 ± 0.0^a	2.6 ± 0.4^d	2.4 ± 0.2^a	0.0 ± 0.0^b
	3.0	48.0 ± 5.6^h	41.0 ± 1.6^f	80.0 ± 1.5^c	3.4 ± 0.2^c	0.0 ± 0.0^c	0.0 ± 0.0^b

Data (mean ± SE) with the different letters within columns are significantly different according to Duncan's multiple range test at $P = 0.05$

The efficiency of hypocotyl explant to induce nodular green calli in the present study was found to be similar to that of the earlier report in *Dorem ammoniacum* (Irvani et al. 2009) wherein nodular and proembryonic calli were developed from hypocotyl. On the other hand, in support of the current report, root explants induced friable calli in *Rhodiola imbricate* (Edgew.) (Rattan et al. 2020). Hence, it is evident that type of explants play a major role in inducing variable forms of calli.

In terms of the effects of PGRs, the results of the present study are in comparison with that obtained by Osman et al. (2013), which stated that 0.3 mg/L 2,4-D along with 0.1 or 0.3 mg/L BAP was effective for the maximum callus formation in wolfberry. However, there is a difference in results obtained by Alexandra and Dorica (2020) according to which, a sufficient amount of callus regeneration of this plant occurs only in presence of BAP and gibberellic acid (GA$_3$) (both 0.5 mg/L). In relation to callus regeneration, it has also been reported by Zaman et al. (2021) that the highest amount of alkaloid production (31.07 ± 0.05 μg/mg DW) can be obtained in *Polyalthia bullata* when MS + 2,4-D was used in comparison to other auxins like α-napthalene acetic acid (NAA), picloram, indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA). The most effective PGR required for callus formation, as reported by Amir et al. (2017) was 2,4-D as observed in another member of the *Solanaceae* family, *Solanum nigrum*. In addition, results produced were similar as 0.5 mg/L 2,4-D was capable of producing both maximum fresh weight (3.5 ± 0.08 g) and dry weight (0.40 ± 0.04 g) when coupled with 1.0 mg/L NAA.

Hypocotyl explants produced the highest mean callus diameter (21.40 ± 0.71 mm) when cultured on 0.1 mg/L IAA and 0.25 mg/L TDZ (Karakas 2020). However, these results are in contradiction with the report of Hesami and Daneshvar (2018) where it was shown that hypocotyl-derived explants of *Ficus religiosa* have led to callus formation in presence of 2,4-D and IBA that was further used for shoot regeneration.

Somatic embryogenesis and regeneration

Enhancement of callus formation and subsequent multiple shoot regeneration from callus were recorded in all the combinations of 2,4-D and TDZ. All the three explants (hypocotyl, leaf and root) exhibited cent percent callus induction frequency in all the tested PGR combinations (Table 2). Significantly higher rates of multiple shoot formation from calli were recorded in almost all combinations except in 0.25 mg/L 2,4-D + 3 mg/L TDZ (Fig. 1e). During enhanced indirect shoot regeneration, hypocotyl-derived callus performed best inducing 5–9 shoots. In the case of leaf-derived calli, 3–5 shoots per callus were recorded (Fig. 2a). Root-derived calli were found to be least responsive that induced only 2 shoots per callus in 0.25 mg/L 2,4-D + 1 or 2 mg/L TDZ. Spontaneous induction of somatic embryos was recorded in 0.25 mg/L 2,4-D + 1 mg/L TDZ supplemented medium (Fig. 1f–g). Hypocotyl explant was proved to be the most responsive organ that induced as many as 6 somatic embryos and subsequent shoot regeneration (Fig. 1h–k) per explant followed by leaf explants that induced around 2.5 number of somatic embryos and regenerated shoots from individual explants (Fig. 3). Histological studies confirmed the origin of shoot regeneration from leaf-derived somatic embryos as well (Fig. 2b). On the contrary, root explants produced somatic embryos in the least frequency (< 0.5 number of somatic embryos per explant). All the healthy multiple shoots were successfully rooted with cent percent rooting efficiency in PGR-free MS medium (Fig. 1l–m, 2c). Conventional PGR for root induction (such as IAA or IBA) was not necessary.

As reported by Yu et al. (2020), out of the many types of explants used for somatic embryogenesis, hypocotyl-derived calli proved to be better (92.56%) than root-derived (50.71%) calli, which is in accordance with the results obtained in this study. But on the contrary, the same finding also suggested that higher concentrations of TDZ were successful in inducing calli in higher frequency, which contradicts the finding established in this report. However, Kahia et al. (2016) stated that juvenile leaf explants of cauliflower showed a higher capacity of somatic embryogenesis than hypocotyl

Table 2 Effect of different explants and 2,4-D and TDZ combinations on enhanced callus formation and shoot regeneration of *Lycium barbarum* L.

PGRs (mg/L)		Percentage of callus formation			Mean number of shoots per callus		
		Hypocotyl	Leaf	Root	Hypocotyl	Leaf	Root
Control		0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^d	0.0 ± 0.0^d	0.0 ± 0.0^b
0.25 2,4-D	0.5 TDZ	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	6.6 ± 0.5^b	3.0 ± 0.2^c	0.0 ± 0.0^b
	1.0 TDZ	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	9.6 ± 0.5^a	5.6 ± 0.4^a	2.4 ± 0.2^a
	2.0 TDZ	10.0 ± 0.00^a	100.0 ± 0.0^a	100.0 ± 0.0^a	5.0 ± 0.4^c	4.0 ± 0.4^b	2.4 ± 0.2^a
	3.0 TDZ	100.0 ± 0.0^a	100.0 ± 0.0^a	100.0 ± 0.0^a	0.0 ± 0.0^d	0.0 ± 0.0^d	0.0 ± 0.0^b

Data (mean ± SE) with the different letters within columns are significantly different according to Duncan's multiple range test at $P = 0.05$

Fig. 3 Development of indirect somatic embryos from hypocotyl, leaf and root explants *Lycium barbarum* L. cultured on MS medium containing 2,4-D and TDZ

Explants
MS + 2,4-D(0.25 mg/l) + TDZ (1.0 mg/l)
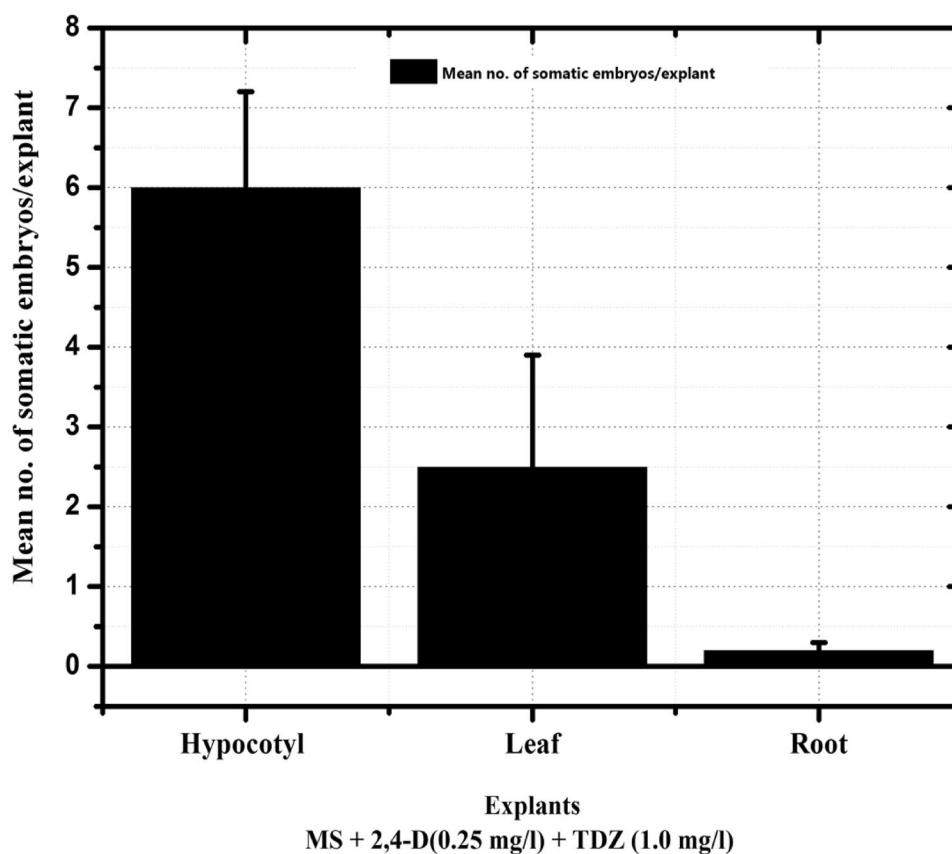
explants. As far as responses observed in hypocotyl-derived explants are concerned, the H$^+$-ATPase plays a crucial role. When there is an auxin added to the hypocotyl, it is credited with the activation of the H$^+$-ATPase by phosphorylation of the penultimate Threonine in the C-terminus (Gallemí and Martínez-García 2016). This might hint towards a probable mechanism for the active response of hypocotyl-derived explants over other types.

The result obtained here is concurrent with the report by Galán-Ávila et al. (2020), which suggests that hypocotyl gives a better response than any other explant in terms of shoot regeneration. Out of a number of explants used, 49.45% of hypocotyl explants responded for shoot regeneration, followed by 4.7% cotyledon and 0.425% of true leaves. To dive further, when a series of explants like half hypocotyl, complete hypocotyl and cotyledonary node were competitively analysed for their respective responses in soybean, cotyledonary node had a higher activity over the others in terms of shoot induction potential or regeneration rate. This might be alluded to the higher number of actively dividing cells in the portion of cotyledonary node than in hypocotyl (Raza et al. 2017).

Instead of a combination of TDZ and 2,4-D, the auxin-cytokinin combination of BA and 2,4-D, both 2 mg/L proved to be the best in callus proliferation among 4 potato varieties

as reported by Al-Hussaini et al. (2015). It also reported that shoot regeneration was observed in a combination of 0.22 mg/L TDZ and 0.49 mg/L NAA, which is in contradiction with that reported in this study. Another contradictory result was obtained by the combination of 0.5 mg/L 2,4-D and 0.5 mg/L BA, as observed in leaf and hypocotyl explants of *Cannabis sativa* L. (Movahedi et al. 2016). However, in the case of strawberry it has been reported that the combination of 2.27 μM TDZ and 2.27 μM 2,4-D, out of the combinations of a large number of other cytokinins and auxins showed the highest rate of callus induction (100%). This is in accordance with the report in the study but the only point of difference is instead of 0.25 mg/L 2,4-D and 1 mg/L TDZ performing the best in both callus regeneration and mean number of shoots, shoot regeneration ability was best in 0.44 μM BA as reported by Chung and Ouyang (2021). The same combination, 4.5 μM 2,4-D and 0.45 μM TDZ has also been favourable for inducing embryogenic calli from tepal explants of *Tricyrtis* spp. (Nakano et al. 2004). The probable reason for this PGR combination to be the most effective which might allude to the discovery of signal transduction pathways of 2,4-D mimicking an auxin, leading to the cross-talks between hormones at a molecular level. Presence of benzyl ring along with –CH$_2$COO$^-$ group can be credited with the molecular biochemistry of 2,4-D as an

auxin. Though the exact mechanism of 2,4-D auxin activity is not known, the general understanding of the auxin signaling pathway that leads to cell-to-cell auxin transport is well known. Gene expression of auxin response genes secreted by the plant is controlled by 2,4-D by degrading Aux/IAA repressor proteins (Song 2014). Along with this, there are few mechanisms linked with the activity of TDZ as well as cytokinins which might be a probable factor for its efficiency in the case of wolfberry too. Probable metabolism of TDZ by plant cells, oligomerisation in solutions by TDZ, increased utilisation and catabolism of 5C and 6C sugars by TDZ in the media are a few of the many possible reasons for its superior performance of TDZ out of the remaining combinations (Erland et al. 2020).

Eventually, the plants were rooted spontaneously on PGR-free medium and showed high survival rates. This finding is in good agreement with that previously reported for *Allium ascalonicum*, in which IBA is not needed for rooting (Tubić et al. 2014).

Acclimatization

Cent percent survival rates were recorded during acclimatization of in vitro regenerated plantlets in peat moss, compost and soil mixture after 6 weeks, in the present study (Fig. 1n, 2d). Previous report by El-Naggar et al. (2020) supports the utility of peat moss as a substratum as a ratio of 40% peat moss and 60% has led to production of high-quality *Gazania splendens* plantlets. On assessing the performance of biofiltration in the laboratory, it was found that the oxidation rate was the highest in the case of commercial compost added with straw (1.68 μg CH_4/gdw/h) (Fedrizzi et al. 2018). In the case of date palm plantlet micropropagation, vegetative growth parameters show an optimum value when peat moss: perlite (2:1; v/v) was used in a shaded nursery and clay: perlite: compost (1:1:1; v/v) was used in a sunlight nursery (Hassan 2017). However, it was reported that tomato waste can be used as an alternative to vermicompost and peat moss on the basis of higher quality vegetable transplant production in the case of nurseries, by the former substratum (Abdel-Razzak et al. 2019). Due to the increased expense of peat moss, biochar also has been seen to function as a replacement to peat for soil-free substrates. Its high cation exchange capacity and porosity along with low density have made biochar a potent substitute as reported by Margenot et al. (2018).

Total phenol production

Phenol production is an important criterion required in plants because they provide protection against stress, playing fundamental activities in maintaining structural integrity. On performing the present experiment, it was found that the phenolic content was maximum in shoots, followed by brown callus and white callus. The approximate phenolic contents of the respective explants proved to be around 640, 390, and 120 mg GAE/g DW (Fig. 4). Three types of in vitro samples were used for testing where shoots were inoculated on auxin and cytokinin, and calli on cytokinin alone. The assessment of phenol production has earlier been done in black goji berry using accelerated solvent extraction by He et al. (2018). Using 70% ethanol concentration, the maximum phenolics produced were 17.92 mg GAE/g, being an excellent source of nutraceuticals. In addition, Abuduaibifu

Fig. 4 Total phenolic content in the shoot, brown callus and white callus of *Lycium barbarum* L. grown on MS medium supplemented with auxin alone (for callus) and auxin with cytokinin (for shoots)
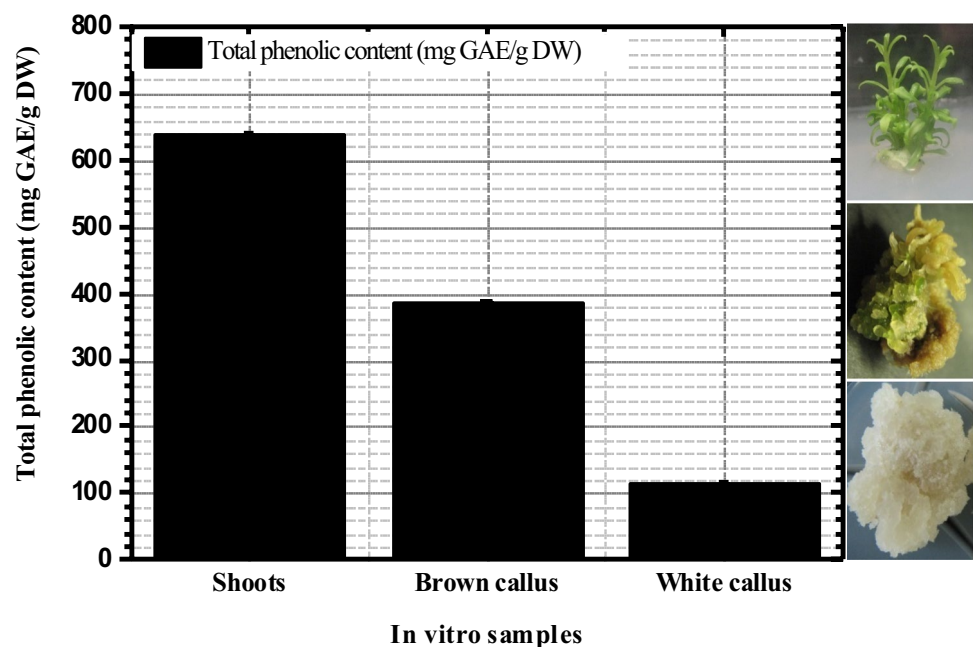
and Tamer (2019) also reported that when in vitro total phenolic content was checked for red and black goji berry, the earlier substrate possessed more phenolic content and antioxidant capacity than the latter one. Depending on the type of extracts as prepared for total phenol estimation, the chloroform: methanol (1:1; v/v) extract of rhizome showed a higher total phenolic content as compared to that of callus in *Zingiber officinale* Rosc. This comparison of phenol estimation in rhizome (60.34 ± 0.43 mg GAE/g) and callus (33.6 ± 0.07 mg GAE/g) was reported by Ali et al. (2018). Further, when shoots obtained from callus were tested for total phenolic content estimation by Kikowska et al. (2020), biomass from methanolic extracts of *Eryngium alpinum* L. shoots revealed that the range was from 272.52 to 458.38 mg/100 g DW, which is 19.59–32.95 times higher than phenolic acids in general and this is in favour of the findings that have been reported in this study. It is supported by the detailed comparison of total phenolic acid estimation between shoots and calli by Szopa et al. (2018), which states that callus showed 1.7 times phenolic acid content in *A. arbutifolia* and 2.2 times in *A. prunifolia*, while shoot extracts exhibited 3.2 times and 2.7 times phenolic acid respectively in the two species. This concurs with the fact that the overall production of bioactive depsides is higher in shoot extracts as compared to that in callus culture.

Conclusion

To summarise, we have successfully developed a new, effective and reproducible in vitro system for *L. barbarum*. Somatic embryos, shoot organogensis and plant regeneration could be obtained through callus culture using hypocotyl and leaf as explants. The present investigation elucidates that the cultures of *L. barbarum* can serve as a potential source of secondary metabolites under suitable conditions. Further investigations will be needed to modify this protocol for mass propagation and gene transformation of this species.

Declarations

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