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REVIEW ARTICLE

Plant tissue culture-mediated biotechnological approaches in *Lycium barbarum* L. (Red goji or wolfberry)

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Abstract
Plant tissue culture has long evolved since its first successful attempt by Gottlieb Haberlandt in 1902, and has then emerged as a powerful alternative approach in the breeding and conservation of various plant species. Extensive utilization of plant tissue culture has been documented in efforts to improve plant production, as well as to conserve and breed endangered and hard-to-propagate plant species through plant biotechnology. Restrictions associated with certain growth requirements, such as geographic, seasonal, and climatic limitations, could be overcome through plant tissue culture, hence enabling the mass propagation of plant species all year round. In this article, studies on various plant biotechnological approaches to breed *Lycium barbarum* L. (red goji) through plant tissue culture are reviewed to highlight the efficiency and usefulness of the plant tissue culture technique on red goji, summarizing the importance of biotechnology in plant studies.

Keywords *Lycium barbarum* · Micropropagation · Plant cell and tissue cultures · Red goji (wolfberry), traditional Chinese Medicine

1 Introduction

In 1838, Schleiden and Schwann proposed that each plant cell has the autonomous capability to regenerate into a whole plant. Due to this concept, plant cell and tissue culture experiments exploit the totipotent characteristic in micropropagation and production of plant bioactive compound (Mustafa et al. 2011). The wide applicability of plant tissue culture has led to more research in trait improvement, tolerance enhancement, and important metabolite production (Thorpe 2007).

Human interaction with plants has been immemorially established. Plant biotechnology has added diverse growing dimensions to these interactions by applying various techniques to improve quality and overcome limitations associated with plant resources, which can guarantee total independence of external factors (Alvarez et al., 2020). Over the years, the demand and use of plants have continuously increased. Not only preferred as food ingredients, plants are also highly favoured for therapeutic purposes in which the global preference towards plant-derived medicines and herbal treatments is still growing and increasing (Ekor 2014; Islahudin et al. 2017). The upsurge in market growth for herbal medicinal products is driven by multiple factors among which are the skyrocketing cost of pharmaceutical drugs and the public preference towards naturally-derived products. According to a report featured by Reuters Plus (2018) on the global herbal medicine market, the increasing demand for herbal remedies as a global healthcare preference is expected to reach $111 billion by the end of 2023.

However, widespread adulteration of herbal products has invaded the global market. The quality of herbal medicines, which are perceived to be cheaper and a safer

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Fig. 1 The dried red goji berries for consumption and used in cooking

alternative, has been compromised and could take a toll on consumer health (Ekor, 2014). Scientifically named *Lycium barbarum* L., red goji, popularly known as a superfruit, is an example of a herbal medicine frequently adulterated with active pharmaceutical ingredients such as steroids and sex stimulants (National Pharmaceutical Regulatory Agency, 2018). Thus, the application of plant biotechnology is undeniably important to ensure sustainable access to raw medicinal plants.

The purpose of this review is to highlight and summarize the plant biotechnology studies carried out on *L. barbarum* or commonly known as red goji, with the main focus on plant tissue culture application. Goji is a deciduous woody perennial plant that can grow up to 1 to 3 m high. The wild *L. barbarum* berries are commonly gathered from the hills in the Ningxia region of China as well as remote areas of central China near inner Mongolia. The berries are exported in their dried form to other regions (Fig. 1). Also known as wolfberry, red goji is highly regarded as an upper-class medicinal plant species in Traditional Chinese Medicine or Chinese pharmacopoeia due to its remarkable health benefits (Bucheli et al. 2011). Due to its popularity, imported red goji is not only adulterated in its processed form but is also subjected to pesticide adulterants which is considered non-compliance with Good Agricultural Practice (Mebdoua, 2018). Therefore, the step to efficiently mass propagate red goji irrespective of geographical and seasonal limitations is worthwhile through plant biotechnology application.

By adopting the appropriate protocols, red goji can be micropropagated for mass breeding and the production of disease-free and pest-resistant somaclones of red goji can be made available. Large-scale commercial production through plant tissue culture has been attempted by Agri-Starts Inc. in the US and has revealed fruitful outcomes. It has been reported that the tissue-cultured liners are sold nationwide to cater to production demands throughout the US and Canada (Chen et al. 2018).

Due to the numerous medicinal benefits of red goji, most of the studies done on this species are directed toward their pharmacological activities and phytochemical constituents. Recently, increased attempts to propagate red goji by utilizing plant biotechnology through plant tissue culture have been witnessed in various regions worldwide. In this article, the review on plant biotechnology intervention through plant tissue culture works on *L. barbarum* within the past three decades was conducted. In the following sections, multiple plant biotechnology approaches on *L. barbarum* are reviewed covering four major aspects of plant tissue culture studies encompassing in vitro regeneration and acclimatization studies, callus induction, and somatic embryogenesis. In addition, the molecular studies that were conducted during somatic embryogenesis and organogenesis are also discussed and reviewed. The present article is essential in providing a properly documented review of the topic and may confer benefits in providing potential areas to be ventured into for the development of future research in plant biotechnology of medicinal plant species.

2 Micropropagation and regeneration studies in *L. barbarum*

2.1 Studies on the importance of plant growth regulators (PGR) and explant selection on regeneration potency in *L. barbarum*

Recently, there has been an increased global preference for propagation attempts of red goji in various regions around the globe. In this section, the works related to regeneration studies through plant tissue culture of *L. barbarum* are summarized in Table 1.

From the present review, efforts to regenerate *L. barbarum* through plant tissue culture have escalated, with most studies on regeneration protocol development established within this decade (2013 onwards). It has also been documented to widely expand out of their countries of origin. Over the years, most of the research related to regeneration studies dealt with the optimization of hormonal treatments by using different concentrations and combinations of Plant Growth Regulators (PGR). This is also associated with the appropriate selection of explants for the optimum production of clonal plantlets.

The earliest documentation of regeneration protocol in red goji was reported by Ratushnyak et al. (1990) in which organogenesis and acclimatization of *L. barbarum* were successfully attained by optimizing explant materials

Table 1 Key information on plant tissue culture interventions involving protocols in regeneration, callus induction and somatic embryogenesis studies in *L. barbarum*

Explant / culture	Optimum Media Treatment	Findings	Reference
Leaf segment, callus culture and protoplast	Leaf-derived: 1.5 mg/l BAP AND 0.5 mg/l NAA. Callus-derived: culture transfer to TM-4 Protoplast-derived: 0.1 mg/l NAA	Leaf segments regenerated shooting response in optimally-treated media. Organogenesis was also promoted in callus culture. The shoots were also successfully rooted from protoplast and the acclimatized clonal plantlets had normal morphological traits.	Ratushnyak et al. (1990)
Hypocotyl protoplast	0.3 mg/l 2,4-D and 0.3 mg/l BA in KM8P liquid medium and 0.1 mg/l BA and 0.2 mg/l IBA in half strength MS medium	Whole plantlets regenerated from hypocotyl protoplasts. Regeneration of whole plantlets was preceded by callus culture.	Tian et al. (1993)
Somatic embryos	Root	Low plantlets regeneration identified from somatic embryos (10%). All randomly selected acclimatized plantlets in the greenhouse had survived and phenotypic comparison was found to be similar to that of zygotic seedlings.	Hu et al. (2008)
Leaf and nodal segment	Leaf: 0.5 mg/l NAA and BAP Node: 0.1 mg/l NAA and 1.0 mg/l BAP	Optimally treated leaves regenerated shoot with 7.60 ± 2.80 leaves per plantlet, 1.69 ± 0.75 cm height and profuse rooting. Optimally treated nodes produced 32.90 ± 7.27 leaves per plantlet and 2.60 ± 0.99 cm high.	Osman et al. (2013b)
Leaf and stem	1.0 mg/l NAA and 1.0 mg/l BAP	Optimally regenerated plantlets achieved 63 ± 1.5% and 50 ± 1.3% survival rate when they were acclimatized on black soil and mixed soil (2:1 ratio of black soil to red soil) respectively.	Taha et al. (2015)
Shoot	0.3 mg/l BAP	The best planting media for acclimatization was the media which used sterilized peat moss and perlite (1:1) which produced 22.67 ± 0.33 cm median height of clonal plantlets and 48.00 ± 0.58 number of leaves.	Yusoff (2016)
Leaf and nodal segment	1 mg/l IBA	The nodal explant was more responsive to form adventitious buds. A high in vitro regeneration rate (89 to 95%) achieved in morphogenetic culture and 100% rooting. A high survival rate (up to 90%) of acclimatized plantlets grown in peat substrate mixed with sand.	Danaila-Guidea et al. (2015)
Micro cutting	1.33, 2.22 µM BA	Optimum in vitro rooting achieved on wheat starch-solidified MS hormone-free media. The acclimatization accomplished by using float hydroculture and floating perlite.	Fira et al. (2016)
Node	0.5 mg/l BAP in DKW medium	The optimum treatment showed significant formation of multiple shoots through direct organogenesis with reduced hyperhydricity.	Silvestri et al. (2018)
Regenerated shoots from the initially cultured nodal explants	Different BAP concentrations optimally required to exert specific effects in plantlet regeneration	It was estimated that BAP at 11.56 µM under red/blue LED lamp produced the highest shoot number (8.15) and at 12.42 µM BAP, longer shoots (12.34 cm) were regenerated. 5 µM BAP produced 9.2 leaves per plant and 100% survival of acclimatized *ex vitro* plants was recorded.	Prudente et al. (2019)
Node and hypocotyl	Nodes: 0.5 mg/l BA and NAA (shoot induction), 0.25 mg/l TDZ (shoot proliferation) Hypocotyl: 0.5 mg/l BA and NAA (shoot proliferation)	Nodes: Optimum shoot induction recorded 23.33 ± 1.86 mean number of shoots with 100% shooting response and 4.20 ± 0.57 shoot proliferation. Hypocotyl: Optimum shooting proliferation scored 8.20 ± 0.49 shoots per explant.	Karakas et al. (2020)
Axillary bud	0.1 mg/l BAP	Optimum shoot induction produced 4.8 mean number of shoots/explant and an average of 4.6 cm shoots. Optimum immersion cycle of 6 min every 24-hour produced healthy shoots with the greatest length (4.0 cm), minimal hyperhydricity (10%) and high percentage of rooted shoots (80%).	Ruta et al. (2020)
Callus Induction			

Table 1 (continued)

Explant / culture	Optimum Media Treatment	Findings	Reference
Leaf and internode	Initiation: 0.4 mg/l 2,4-D Subculture: 1 mg/l 2,4-D and 0.2 mg/l NAA	Initiation of callus was obtained from the culture of explants on MS medium treated with 0.4 mg/l 2,4-D. The formed callus was further subcultured successfully on the same culture medium with different PGR concentration consisted of 1 mg/l 2,4-D and 0.2 mg/l NAA. Isolation of protoplast from callus tissue was performed through enzymatic method and produced cell colonies, minicallus and organogenesis.	Ratushnyak et al. (1990)
Root	0.2 mg/dm^3 2,4-D	After initiation of callus phase on MS culture medium supplemented with optimum PGR treatment, three subcultures were carried out onto the same medium and induction of somatic embryo formation was further conducted.	Hu et al. (2008)
Leaf and node	Leaf: 0.3 mg/l 2,4-D and 0.1 mg/l or 0.3 mg/l BAP Node: 0.1 mg/l BAP with either 0.3 mg/l or 0.5 mg/l 2,4-D	Callus was optimally induced at the greatest percentage (100%) in both leaf and nodal explants with friable morphology.	Osman et al. (2013a)
Leaf and stem	2.0 mg/l NAA and 0.5 mg/l BAP	Callus induction was optimally produced in the culture treated with optimum PGR in MS medium. The calli formed were further used for in vitro regeneration study.	Taha et al. (2015)
Hypocotyl explant	0.25 mg/l TDZ and 0.1 mg/l IAA	Optimum callogenesis with 100% induction and greatest mean diameter of callus (21.40 ± 0.71 mm) was produced.	Karakas et al. (2020)
Somatic Embryogenesis			
Root explants	500 mg/dm^3 lactalbumin hydrolysate	Induction of somatic embryogenesis was achieved by transferring the previously formed callus onto the MS medium supplemented with 500 mg dm^{-3} lactalbumin hydrolysate. About 60 somatic embryos per 0.25 g fresh mass were successfully formed from this treatment.	Hu et al. (2008)
Leaf explants	1.0 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BAP	A series of sequential callus subculture on solid MS basal media with a reduced amount of sucrose (20 g/l) promoted the occurrence of somatic embryogenesis.	Osman et al. (2013a)

from various sources; for instance, from leaf segments, protoplast, callus cultures, and using appropriate treatment of PGR. Upon morphological analysis, the acclimatized plantlets that have attained maturity were found to carry normal morphological traits. In 1993, another protocol was developed by Tian et al. (1993) in which they obtained regenerated whole plantlets from hypocotyl protoplasts which were isolated in an enzyme mixture solution of 1% cellulase and 1% pectinase. The optimum PGR combination was noted by the procedure of transferring plant calli to a half-strength MS medium of 0.1 mg/l BA and 0.2 mg/l IBA before the whole plantlets regenerated efficiently.

The important relationship between optimum PGR and explant has been manifested by numerous studies that highlighted the success of regenerating red goji plantlets by using the plant tissue culture method. Thus, more studies investigating the importance of PGR treatment and explant combinations have been conducted from 2013 onwards. For instance, Osman et al. (2013b) reported that plantlets' optimal shoot growth and rooting were achieved from an optimum NAA (α –naphtalene acetic acid) and BAP (6-benzylaminpopurine) combination in leaf-derived plantlets (7.60 ± 2.80 leaves/plantlet). Meanwhile, profuse shoot growth and large number of leaves (32.90 ± 7.27 leaves/plantlet) were found in nodal-derived plantlets, but the rooting response was quite poor. This finding indicated that the response to PGR treatment is explant-specific and nodal explants favoured the regeneration of more clonal leaves as compared to leaf explants. Meanwhile, Taha et al. (2015) and Yusof et al. (2016) also conducted plant regeneration studies that incorporated the research on PGR and explant selection for obtaining healthy plantlets. Interestingly, in addition to that, they also extended the protocol to include acclimatization work. The findings related to acclimatization protocols will be further discussed in this article.

In line with the findings highlighted by Osman et al. (2013b), Danaila-Guidea et al. (2015) found a greater response due to the rapid formation of adventitious bud in nodal-derived in vitro seedlings compared to their leaf-derived counterparts. Another interesting micropropagation work was carried out by Silvestri et al. (2018) on the commercial cultivar of *L. barbarum* (Nixia 1); whereby they focused on the effectiveness of cytokinins on shoot organogenesis in which they employed hormonal modulation by using different types of cytokinins at different concentrations which include BAP (0, 0.5, 1, 2 mg/L), N6-(2- isopentenyl) adenine (2-iP; 0, 0.5, 1, 2 mg/L) and Zeatin (ZR; 0, 0.5, 1, 2 mg/L). Silvestri et al. (2018) discovered the ineffectiveness of 2-iP and ZR in promoting

direct organogenesis as they negatively affected the response by producing lower-than-control regeneration scores, and no multiple shoot formation was observed in the 2-iP and ZR-treated cultures. Concerning the rooting response, it was found that optimal in vitro rooting was attained upon the addition of polyamine (putrescine) at 160 mg/l when combined with a low concentration of auxin.

The importance of cytokinin in the regeneration of *L. barbarum* was also investigated and documented by Prudente et al. (2019). Three types of cytokinins were used in their study (BAP, kinetin, and thidiazuron). They found that BAP promoted a higher regeneration percentage and shoot length of lateral buds as compared to kinetin and TDZ. As for the number of leaves which was individually evaluated in each treatment, it was determined that the most leaves (9.2 leaves) formed in the cultures treated with 5 µM BAP.

Karakas (2020) also discovered and verified the importance of optimum PGR and explant pairing in influencing shoot production. Karakas (2020) found that the regeneration capacity was associated with the appropriate type and concentration of cytokinins and the explants used. For instance, in hypocotyl explants, shoot regeneration was only observed when they were treated with BA and NAA combinations. Optimum shoot proliferation (8.20 ± 0.49 shoots per explant) was identified in those treated with an equal concentration of BA and NAA at 0.5 mg/l each.

2.2 Studies on the importance of growth medium and external factors on regeneration of *L. barbarum*

Several documented studies focused on the impact of different types of growth medium and their composition to successfully regenerate *L. barbarum*. Fira et al. (2016) used natural sources as an alternative instead of the commercially available solidifying agent in a growth medium such as agar to investigate the effectiveness of wheat starch in *L. barbarum*. The study concluded that the use of wheat starch was efficient in the multiplication stage and also managed to produce a great rooting response in the micropropagation of *L. barbarum.*

Another noteworthy finding on growth medium in the regeneration studies of *L. barbarum* was discovered by Silvestri et al. (2018). Various plant tissue culture media were used to test their effectiveness through axillary shoot proliferation. The regenerative potential of *L. barbarum* was tested on different culture media, including Murashige and Skoog (MS) media (Murashige and Skoog 1962), Quoirin and Lepoivre (QL) media (Quoirin and Lepoivre 1977), Driver and Kuniyuki (DKW) media (1984), and Woody Plant (WPM) media (Lloyd and McCown 1980). Direct regeneration of multiple shoots was produced from nodal explants treated with optimum hormonal treatment in the DKW medium. Besides promoting optimum shoot multiplication, the DKW medium was found to help reduce the occurrence of detrimental effects following the use of the MS medium characterized by a drastic growth reduction and hyperhydricity. The findings inferred that the in vitro regenerated *L. barbarum* favours low ion concentrations since the MS medium is composed of a greater amount of NH_{4+}. Due to the unfavourable ratio of NH_4^+ and NO_3^- in the MS medium (Ivanova and Van Staden 2009), the regeneration response in *L. barbarum* was found to be less effective as compared to the DKW medium.

Even though *L. barbarum* is regarded as a halophyte, the requirement of in vitro regenerated plantlets towards minerals composition might differ from the in vivo wild plants. In addition, the repetitive subcultures in the in vitro culture also contributed to the incidence of hyperhydricity (Ivanova and Van Staden 2009; Silvestri et al. 2018); hence the certain ratio of NH_4^+ and NO_3^- in the culture media need to be met for optimal growth and formation to overcome the occurrence of ammonium stress which may negatively affect plant growth (Sarasketa et al. 2016).

Apart from using the conventional plant tissue culture method, Ruta et al. (2020) adopted a large-scale plant production in liquid culture by using a temporary immersion system (TIS). Different immersion cycles were used to analyse the impact of shoot quality in *L. barbarum* plantlets. It was found that the shortest daily immersion (6 min every 24 h) was identified to promote the optimum growth and multiplication rate with minimum signs of hyperhydricity (10%). Through the application of TIS in this study, the quality of plantlet regeneration was found to be inversely related to the immersion time, which affected the shoot length, hyperhydricity percentage, and rooting response.

It is imperative to have a good explant to produce healthy plantlets. Besides, the position of the explant cultured on the growth media also influences the success of plant regeneration. Fira et al. (2016) reported that explants placed vertically yielded superior growth with a higher multiplication rate than their horizontally-placed counterparts, and the impact of the explant position factor was analysed to be statistically significant. The plantlets regenerated from the vertically cultured explant were taller, more vigorous, and produced many axillary buds. In addition, the plant biomass, height, proliferation, and multiplication rates were found to be greater than those of plantlets derived from horizontally placed micro cuttings.

Light is one of the important external factors which significantly influences plant growth. One study published by Prudente et al. (2019) highlighted the impact of light sources on the in vitro multiplication of red goji.

Conventionally, a white fluorescent lamp is used for the micropropagation of plants. However, there are attempts to improve the light sources for a more determined spectral composition to promote optimal plant growth. Hence, the use of light-emitting diodes (LED) lamps as an alternative to white fluorescent light for in vitro plant regeneration has been explored. Red-blue (RB) LED was proven to promote better growth of plantlets with increased shoot and leaf numbers and greater shoot length compared to those grown under the white fluorescent lamp (Prudente et al. 2019).

2.3 Acclimatization studies in *L. barbarum*

Attempts to acclimatize in vitro regenerated plantlets had been carried out previously. In a study by Hu et al. (2008), a protocol had been developed to regenerate plantlets of *L. barbarum* from somatic embryos of the species which were induced from root-derived calli. Despite the low regeneration percentage (10%), the survival of the plantlets after being acclimatized in the greenhouse was discovered to be successful as all of the plantlets that were randomly selected survived. Upon phenotypic observation, the plantlets were found to be phenotypically similar to the zygotic seedlings.

In 2015, in addition to establishing an efficient in vitro regenerative system for organogenesis, Taha et al. (2015) extended the protocol to include acclimatization work. The survival rate of the acclimatized plantlets was found to be considerably good as it was recorded to achieve 63 ± 1.5% and 50 ± 1.3% when they were acclimatized on black soil and mixed soil (2:1 ratio of black soil to red soil), respectively. Another acclimatization work on *L. barbarum* was reported by Yusof et al. (2016) in Malaysia; whereby, the best planting media for acclimatization of clonal plantlets was found in media utilizing a sterilized peat moss and perlite (1:1) combination, which productively produced clonal plantlets. The median height of acclimatized plantlets was recorded to be 22.67 ± 0.33 cm with a 48.00 ± 0.58 number of leaves.

High survival percentage of acclimatized *L. barbarum* had been reported in different studies. For instance, Danaila-Guidea et al. (2015) found a 90–98% average survival percentage in acclimatized plantlets grown in peat substrate mixed with sand. This successfully formed healthy leaves, new branches of roots, and multiple axillary shoots over a period of one month after being transferred to ex vitro conditions. Meanwhile, different methods of acclimatization had been studied by Fira et al. (2016). In their study, the acclimatization was carried out by using float hydroculture in floating cell trays, floating perlite, and Jiffy7 pellets. After successfully attained in vitro regeneration, the plantlets were transplanted into the potting mix and high survival percentages were recorded in all three methods. A 100% survival was recorded in plants acclimatized in Jiffy7 pellets, meanwhile considerably high survival percentages were recorded in hydroculture floating cell trays (94.07%) and floating perlite (74.73%).

A study to observe the impact of lighting sources on acclimatization was conducted by Prudente et al. (2019). The findings showed the improved development of shoot growth could be achieved by using red/blue LED light as the illumination source as compared to the white fluorescent light and the effectiveness of BAP in the in vitro multiplication of *L. barbarum*.

All of the reported studies discussed in this chapter manifest the positive potential for this species to be grown under ex vitro conditions with a proper supply of optimal growth requirements. The proven successful attempts suggest the possibility for this medicinal species to be propagated out of its country of origin by using plant biotechnology through tissue culture techniques. Nevertheless, studies in plant acclimatization in *L. barbarum* should be further explored to understand the mechanism of plant acclimatory responses. This is pertinent to ensure its survival and yield enhancement, hence guaranteeing the relevancy of plant tissue culture for sustainability in view of global climatic changes.

3 Callus induction and somatic embryogenesis in *L. barbarum*

3.1 Callogenesis and callus culture of *L. barbarum*

A callus is an amorphous mass of unorganized thin-walled parenchyma cells (Yadav and Tyagi 2006). Callus formation has been regarded as an important step in plant regeneration which may involve organogenesis and somatic embryogenesis pathways. Upon the culture of selected explants under suitable conditions, the production of a proliferating mass of undifferentiated cells, known as callus, may be produced (Vannini et al. 2012). The formation of callus is also described as a plant response to wounding. Callus formation is considered to be among the most important subjects in micropropagation technologies. This is owing to the reasons that it can be further multiplied hence producing numerous new clones of certain species and being used in suspension cultures specifically prepared and modified to harvest certain desirable plant products to be extracted (Mineo 1990).

The earliest documented study in *L. barbarum* which involved the use of callus was reported by Ratushnyak et al. (1990) in which the regeneration originated from callus culture. *L. barbarum* plant regeneration was assessed from

leaf, callus, and callus protoplasts. In their study, they managed to induce callogenesis from leaf and internode tissues cultured on MS medium (Murashige and Skoog 1962) supplemented with 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and further sub-cultured onto media treated with 1 mg/l 2,4-D and 0.2 mg/l α-naphthaleneacetic acid (NAA).

Hu et al. (2001) studied the effects of polyamines on organogenesis and somatic embryogenesis in the calli of *L. barbarum*. Polyamines are low molecular weight aliphatic nitrogenous bases containing two or more amino groups. They are known to have a significant role in their functions as plant biostimulants and thus play important roles in the growth and developmental processes of plants (Chen et al. 2018). In the study conducted by Hu et al. (2001), they found that the content of Putrescine (Put) accumulated quickly in the initial stages of calli differentiation and then decreased afterward.

In 2008, another study that involve the use of callus culture was documented in which the researchers studied the micropropagation process in *L. barbarum* and induced the formation of callus from the root as the starting material (Hu et al. 2008). Callus formation was successfully induced from root explants cultured on MS medium with the addition of 0.2 mg/dm3 2,4-D. The successfully induced calli were then used in further micropropagation studies to induce somatic embryos.

Apart from the aforementioned studies, another research was carried out by Osman et al. (2013a) whereby they studied the induction of callus in *L. barbarum* from leaf and nodal explants. It was found that callus was optimally induced (100% callus induction) from leaf explant treated with 0.3 mg/l 2,4-D and 0.1 mg/l or 0.3 mg/l BAP in MS media. In addition, the calli produced from such treatments were morphologically friable and greenish. Meanwhile, the induction of callus from nodal explant was optimally achieved in the treatment of 0.1 mg/l BAP with either 0.3 mg/l or 0.5 mg/l 2,4-D in MS media. The percentage of callus inductions was recorded to be 100% in both of the treatments with the profuse formation of calli. Nevertheless, the calli formed from those treatments produced calli that were morphologically different. Those treated with 0.1 mg/l BAP and 0.3 mg/l 2,4-D were yellowish-green and compact. On the other hand, the calli was yellowish, greenish, watery, and friable in those treated with 0/1 mg/l BAP and 0.5 mg/l 2,4-D.

Another attempt to mass-propagate *L. barbarum* was further documented in a report by Taha et al. (2015). In their study, the researchers aimed at establishing a highly efficient regeneration system for the mass production and commercialization of red goji. They discovered that leaf and stem explants produced good responses and the optimum hormonal regime for callus induction was the treatment combination of 2.0 mg/l NAA and 0.5 mg/l BAP.

The formation of callus induced by various types of explants (leaf, petiole, stem, root, and hypocotyl) was also studied previously (Karakas 2020). A total of 28 different concentrations and combinations of plant growth regulators, which were thidiazuron (TDZ), NAA, benzyl adenine (BA), and indole-3-acetic acid (IAA), were used in this study. According to Karakas (2020), the best callogenesis was observed in hypocotyl explant, in which the highest percentage of callus induction was achieved (100%), and the mean diameter of callus formed peaked at 21.40 ± 0.71 mm. The optimum PGR combinations that produced optimal callogenesis were identified in the treatment consisting of 0.25 mg/l TDZ and 0.1 mg/l IAA. Another treatment that produced a 100% callus induction percentage was observed in those treated with 0.25 mg/l BA and 0.25 mg/l NAA. Nevertheless, the mean diameter scored by the treatment was slightly lower (21.00 ± 0.29 mm).

Meanwhile, the most recent study on this species had shown the superiority of 2,4-D over the other PGRs (BAP, TDZ, Kinetin and Zeatin), resulting in 100% response for callus induction. It had also been reported in the study that the supplementation of TDZ at 1 mg/l optimally regenerated five shoots per callus (Verma et al. 2022).

Studies in callus cultures have great potential to be applied in multi-faceted areas of plant biotechnology. Considering limited findings and studies in bioengineering application in callus cultures of *L. barbarum*, this area of research is undeniably important to be discovered due to its capacity to be applied in commercial applications. Successful production of secondary metabolites and therapeutic antibodies are potentially attained by optimum manipulation of callus cultures in biotechnology studies. In addition, the callus culture-mediated approach could benefit agricultural and horticultural points of view by producing optimum yield in bioreactors and large-scale vessels for commercial mass production.

3.2 Somatic embryogenesis and molecular studies of *L. barbarum*

As one of the biotechnological approaches in the agricultural field, somatic embryogenesis offers a myriad of advantages. Through somatic embryogenesis, the multiplication rates can be significantly increased, and this can be scaled-up through liquid suspension culture, hence enabling the handling of many embryos at one time (Vannini et al. 2012). The development of somatic embryos is very much comparable to that of zygotic embryos. The similarities are presented from the globular stage through the torpedo stage. Unlike zygotic embryogenesis, which requires undergoing a

dormancy process before germination and growth (Bentsink and Koornneef 2008), somatic embryogenesis, on the other hand, gives rise to plantlet regeneration from a somatic cell to an embryo cell, yielding further regeneration of the whole new plant (Guan et al. 2016).

In 1998, the first recorded work on somatic embryogenesis in *L. barbarum* was documented by Cui et al. (1998). Another study was documented afterward by Hu et al. (1998) and both studies worked on the changes involved at the molecular level in *L. barbarum* during somatic embryogenesis. Cui et al. (1998) analysed the content of abscisic acid (ABA) during the stages of somatic embryogenesis in *L. barbarum* by using the Enzyme-Linked Immunosorbent Assay (ELISA) method. They found that there was a specific protein which preferentially accumulated in embryogenic callus, and it was attributed to the role of ABA during the somatic embryogenesis process. From their findings, Cui et al. (1998) suggested that the expression of specific genes and embryogenic protein could be synthesized in *L. barbarum* due to ABA promotion. Maximum content of ABA occurred twice during somatic embryogenesis and it was claimed to be significant in the globular stage of somatic embryogenesis in *L. barbarum*. Apart from that, the supply of exogenous ABA in the culture was found to also enhance the potential of somatic embryo formation in *L. barbarum*.

Meanwhile, in the study conducted by Hu et al. (1998), a comparative analysis had been carried out by the researchers in which the synthetic activities of DNA, RNA, and protein in somatic embryogenesis were studied. Hu et al. (1998) found that the synthesis of RNA was first initiated before meristemoid and embryogenic cells were formed. This was followed by an increase in DNA and protein synthesis. A rapid increase in the DNA synthesis rate was observed during the globular embryo formation stage in the somatic embryogenesis process and subsequently, the maximum syntheses of RNA and protein occurred during this stage. As for the components of soluble protein, they were observed to change regularly, and during the initiation stage of somatic embryogenesis, a peptide (153.6kD) was identified.

Another study on somatic embryogenesis at the molecular level was carried out by Hu et al. (2001) in which they examined the level of polyamines during somatic embryo formation. Spermidine was identified as the prominent form of endogenous free polyamines during somatic embryogenesis in *L. barbarum*. The maximum level of spermidine was observed to increase after day 1 of the culture in embryogenic calli and peaked at day 10. Hu et al. (2001) also studied the effects of exogenous supplies of polyamines on somatic embryogenesis and found that putrescine positively affected the process. In their findings as well, they found that a specific inhibitor of S-adenosyl methionine decarboxylases, known as MGBG (Methylglyoxal bis-[guanylhydrazone]), reduced the number of somatic embryos in *L. barbarum*.

The capacity of metal ions absorption during somatic embryogenesis in *L. barbarum* was investigated by Li et al. (2001), whereby the absorption rate of trace metal ions following different concentrations of silver nitrate ($AgNO_3$) was examined. They concluded that silver ion coordinated the absorption of metal ions and influenced the resistance or competition of metal ions absorption. The impact of $AgNO_3$ on somatic embryogenesis capacity was also studied by Li et al. (2001) and they managed to deduce that silver ion promoted somatic embryo formation in which at optimum concentration, cell differentiation and somatic embryogenesis were promoted. Following an increase in $AgNO_3$ concentration, the frequency of somatic embryogenesis was observed to increase up to 50 mg/l. Nevertheless, as the level is increased beyond the optimum level, the impact was found to be remarkably inhibitory and toxic in nature.

The effects of hydrogen peroxide on somatic embryogenesis was studied by Kairong et al. (1999). In their study, they found that the maximum somatic embryogenesis frequency was attained after being treated with 200 µM exogenous hydrogen peroxide for 15 days. However, a higher concentration of hydrogen peroxide at 300 µM inhibited somatic embryogenesis. They also noted that higher intracellular levels of hydrogen peroxide were identified in the embryogenesis of cells rather than in calluses. Indeed, the relationship between hydrogen peroxide and the differentiation of embryogenic cells was evident in this study. Subsequently, Kairong et al. (2002) conducted research on the effects of hydrogen peroxide on protein synthesis during somatic embryogenesis in *L. barbarum* and found that embryogenic callus cultured in MS medium supplied with 200 µmol/l hydrogen peroxide synthesized new protein. From their study, they suggested that the exogenous addition of hydrogen peroxide-induced gene expression during somatic embryogenesis was due to the presence of several new in vitro translated proteins in embryogenic callus upon hydrogen peroxide treatment.

Induction of somatic embryogenesis by hormonal and protein manipulations was previously carried out in several studies. For instance, Hu et al. (2008) induced the formation of somatic embryos from root explants with the addition of lactalbumin hydrolysate, a type of protein portion of milk whey that has been enzymatically hydrolysed. After a series of subcultures, the calli which formed from the starting material of root explants were transferred to the somatic embryogenesis induction media, which was composed of 500 mg/dm3 lactalbumin hydrolysate. The inductive intervention utilizing lactalbumin hydrolysate was found

to be successful. The importance of optimum hormonal combination in producing somatic embryos in red goji has been highlighted in a recent finding. It was found that the optimum combination of 2,4-D (0.25 mg/l) and TDZ (1 mg/L TDZ) in MS medium had resulted in the spontaneous induction of somatic embryos. Explant-wise, the induction from hypocotyl was reported to be highly responsive with six somatic embryos and subsequent regeneration of 9 shoots per callus (Verma et al. 2022).

Another attempt to induce somatic embryos was reported by Osman et al. (2013a). Differently conducted from that of Hu et al. (2008), Osman et al. (2013a) adopted hormonal manipulations and sucrose-induced stress approaches to induce somatic embryogenesis. Two types of starting materials were used i.e., leaf and node, in which those from leaf explants were found to be optimum in producing somatic embryos. This study found that the treatment of 1.0 mg L-1 2,4-D and 0.1 mg L-1 BAP in MS media during the callus induction phase in leaf explant was the optimum treatment to produce somatic embryos. After a substantial amount of calli was obtained, a series of sequential subcultures on solid MS basal media with a reduced amount of sucrose (20 g/l) was carried out. The findings from this study highlighted the effectiveness of sucrose-induced stress in *L. barbarum* calli which may enhance embryogenic potential and hence promote the occurrence of somatic embryogenesis.

The integration of the molecular approach in somatic embryogenesis study is important not only for molecular identification purposes but also for the development and improvement of the understanding of the molecular mechanism in *L. barbarum*. Research in somatic embryogenesis in *L. barbarum* could be possibly extended and enhanced for the potential development of synthetic seeds of *L. barbarum*. Analysis of survival competency upon desiccation and feasibility of cryopreservation of somatic embryos are among the areas that can be explored in the future.

4 Genetic transformation and transgenic regeneration in *L. barbarum*

An attempt to apply genetic transformation was documented in the early '90s by Du et al. (1994) in which they employed the use of Agrobacterium tumafaciens C58 C1 (harbouring the plasmid pGV 3850::neo1103), which infected young stem segments of *L. barbarum*. The explant which had shown the rapid formation of callus was identified to favour the transformation process. The transformed calli successfully regenerated buds upon being cultured on a differentiation medium that contained 25 mcg/ml of kanamycin. Morphologically, it has been determined that 30% of the regenerated buds were phenotypically normal and developed into whole plantlets after being transferred to the root induction medium. The success of genetic transformation and transgenic regeneration was highlighted in this study, whereby the researchers found that the rate of plants regenerating from transgenic calli was higher than those that did not undergo the transgenic process. The confirmation of in vitro transformation analysis was carried out to verify the integration of foreign genes into the genome of *L. barbarum*. Upon the nopaline detection test, NPT-II enzyme activity assay and Southern blotting hybridization, the transformation process was confirmed to have occurred and expressed in the plant.

Another protocol for transgenic regeneration of *L. barbarum* through the genetic transformation that could be regarded as a high-frequency system had been developed by using somatic embryogenesis (Hu et al. 2002). The work was initiated by co-cultivating leaf segments of *L. barbarum* with *Agrobacterium tumafaciens* EHA101 (pIG121Hm) which carried the neomycin phosphotransferase II gene as a selectable marker and an intron-β-glucuronidase (GUS) gene as a reporter marker. Calli produced from the callus induction medium containing 50 mg/l kanamycin had shown kanamycin-resistant traits in 60% of the cultured explants. Somatic embryos were profusely formed (166 g/fresh weight callus) after the sub-cultured transformed calli were cultured on a somatic embryogenesis induction medium containing 25 mg/l kanamycin, which then successfully regenerated into plantlets. The success of transformation and transgenic regeneration of this study had been analysed using plant molecular biology analyses. Upon β-Glucuronidase (GUS) staining and polymerase chain reaction (PCR) and Southern blot analyses, the genetic transformation process was confirmed by verifying the integration of T-DNA into the *L. barbarum* genome.

Optimization work was further carried out involving factors affecting the transformation efficiency. The optimization study encompassed aspects of the pre-culture period, age of leaf explant, use of acetosyringone, strains and density of Agrobacterium, and temperature of co-cultivation (Hu et al. 2006). They found that a higher regeneration frequency of transformed calli could be achieved at a lower temperature (24 °C) during co-cultivation. Hu et al. (2006) reported that the genetic transformation could be optimized to achieve up to 65% success rate of producing transformed calli when the leaf explants (from three-week-old seedling) were pre-cultured for three days in callus induction medium inoculated with Agrobacterium strain EHA101 (pIG121Hm) at the density of OD600 = 0.2, followed by co-cultivation at 24 °C for three days. Regeneration protocol was further developed by the successful attainment of somatic embryos, thus transgenic plant regeneration was

successfully established. Stable integration and expression in *L. barbarum* were confirmed by PCR analysis and the presence of strong GUS activity was also detected in callus-derived somatic embryos and *L. barbarum* plants.

Another recent breakthrough in plant breeding technology is the application of CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9). From an agricultural point of view, this technology may confer benefits on horticultural crops by enhancing the production, quality, and nutritional values of plant species (Bhatta and Malla 2020; Xu et al. 2020). In recent years, a number of studies on fruit improvement have been conducted to regulate various desirable phenotypic properties such as fruit colour, ripening and firmness (Wang et al. 2019), extended shelf life (Yu et al. 2017), and bioactive compounds composition such as lycopene content (Li et al. 2018). A substantial number of studies had been carried out on tomatoes, a species that falls under the same family as *L. barbarum* (Solanaceae). This promising gene-editing technology can be potentially applied in the future improvement of fruit production.

Rao et al. (2020) recently discovered that polyploidization may affect stress tolerance traits in goji. It was found that superior drought tolerance could be attained through chromosome doubling in black goji (*Lycium ruthenicum*). Due to the properties of *L. ruthenicum*, which possesses an extremely high abiotic stress tolerance trait, the study on polyploidization in black goji was conducted to investigate the strengthening factor which may enhance its stress-tolerance capacity. Rao et al. (2019) discovered the efficiency of colchicine treatment in *L. ruthenicum* leaves in vitro to induce high-frequency polyploidy (31.4%). Treatment of colchicine in diploid plants was observed to produce autotetraploid individuals in which chromosome doubling exhibited an increase in abscisic acid content (78.4%) under natural conditions. In this study, Rao et al. (2020) demonstrated the superior drought stress resistance in tetraploids compared to diploids, owing to the increase in ABRE binding factor 5-like (ABF5-like) gene, which promoted the activation of ABA signaling pathway. Another significant discovery highlighted in this study is the strong induction of osmotic protein expression which resulted in increased stress tolerance at the translational level.

In a more recent study on the genetic transformation of *L. ruthenicum*, Wang et al. (2021) studied the genetic background that controls the fruit size of *L. ruthenicum* for breeding efficiency. In this study, fw2.2 is the gene of interest in determining the fruit weight which represents the major quantitative trait locus that regulates fruit size and weight. Wang et al. (2019) utilized CRISPR/Cas9-mediated gene editing technology and proved the efficiency of the system for generating target mutations in black goji. In this study, the researchers have successfully established an efficient regeneration and genetic transformation system in this species through which the target sites for fw2.2 were validated. From this recent breakthrough, they established a high editing efficiency whereby 95.45% of the transgenic lines containing mutations were identified in the fw2.2 target site. In addition, four homozygous mutations and nine biallelic mutations were obtained in this gene. Owing to these findings, they found that there might be an association between fw2.2 and regulation and/or repression of fruit cell division. Nevertheless, further morphological analyses and transcriptome sequencing are required to verify the impact of genome editing on fruit development in black goji.

Despite the breakthroughs reported in *L. ruthenicum*, there has not been any documented studies on the application of CRISPR-Cas9 in *L. barbarum*. Hence, this signifies the potential for an interesting area of research to be conducted in the future to produce a high-quality yield of red goji.

5 Conclusion & future perspective

The application of plant biotechnology on red goji has great potential to be explored extensively. Attributable to its medicinal properties, sole dependency on wild-type or in vivo grown plant species might be insufficient to meet the increasing demand. The various benefits of biotechnology-based applications could be harnessed efficiently not only to fulfill the needs in propagation, but also to enhance the production of bioactive constituents and fruit quality. Thus, research in plant biotechnology through plant cell and tissue cultures offers continuous benefits if continuously explored. Limitations and challenges in plant tissue culture generally associated with operational costs of labour, greater usage of energy and human resources, and the emergence of somaclonal variations could be overcome and minimized with the improvement in operational efficiency, automated system approach, proper selection of donor plants as well as optimization and appropriate conduct of culture procedures.

This review attempts to provide insight on the proven effectiveness of plant biotechnology in red goji production. Due to the efficiency of plant biotechnology to produce multiple copies of high-quality plantlets, the plant tissue culture-mediated approach serves as an important tool to overcome limitations in production capacity. This will ensure continuous access to a fresh supply of crops regardless of geographical and seasonal restrictions. Hence, further comprehensive studies are indeed required to warrant optimum efficiency and promising quality in red goji production through plant biotechnology.

The studies to integrate the use of genomic and gene-editing tools in plant tissue culture technology applied to

L. barbarum are seen as interesting with great potential in research areas to be discovered. The breeding of desirable phenotypic properties with enhanced tolerance is direly needed in facing the challenges associated with climate change and this could be possibly addressed through extensive research in plant biotechnology. This documented review of plant biotechnology, which focuses on the plant tissue culture-mediated approach, serves as a useful reference for future exploratory and improvement studies to be further conducted on *L. barbarum*.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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