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Original Article

Efficient *in Vitro* Callus Induction, Regeneration and Shoot Multiplication Protocols for *Stachys schtschegleevii* L.; A Rare Medicinal Plant

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ABSTARCT

Stachys schtschegleevii is an important medicinal plant, containing highly valuable compounds such as verbascoside, pinene, limonene, and myrcene. In this study, we report efficient *in vitro* callus induction and shoot regeneration protocols for *S. stchschegleevii* from two different explant sources of leaf and internode. The highest callus induction rate was observed in internode explants using MS medium complemented with 0.3 mg/l TDZ and 0.7 mg/l 2,4-D. The resulting calli were embryogenic with the ability to grow and maintain their structure on MS medium containing 1.5 mg/l BAP at high efficiencies (90%). The β-gulucoronidase (gus) gene was then successfully transferred to the internode explants by the particle gun method, demonstrating their potential to express foreign genes. In conclusion, here we report efficient protocols for callus induction and regeneration of *S. schtschegleevii* that can be used in germplasm maintenance, production of secondary metabolites in *in vitro* cultures, and molecular breeding through genetic engineering.
Keyword: *Stachys schtschegleevii*; *In vitro* regeneration; Micro-propagation; *Lamiaceae*; Shoot multiplication

INTRODUSCTION

Stachys is one of the largest genera in the *Lamiaceae* family with more than 300 different species which have been mainly used as therapeutic agents in traditional medicine [1]. Some members of this genus are also used as ornamental plants and edible food sources [2]. In ethno-medicine, generally, the aerial parts of the plant and roots are used in the form of extracts or decoction [3]. The emerging high demand for herbal plants in traditional medicine merits the use of plant tissue culture techniques for the cost-effective production of plant organs. Plant tissue culture systems are commonly used in both basic- (e.g., to understand gene function and plant evolution) and applied- (for example in germplasm conservation and commercial propagation) research [4-6]. An efficient tissue culture system is also crucial for successful molecular breeding of plants through genetic engineering [7]. Iran is known as the diversity hub of *Stachys* species with 35 species of this genus, 13 of which are endemic [8]. According to the Euro+Med PlantBase database, the *Stachys schtschegleevii* L. species (also called Hedge nettle) is native to the South Caucasus and the Arasbaran region of Iran (https://www.europlusmed.org/) and is distributed in Mazandaran, Azarbaijan, and Semnan provinces of Iran [9]. In general, most species of this genus grow in mountainous environments with low temperatures, river banks, and forests. Climate change, which has mostly been towards global warming [10, 11], can affect the phenology and distribution of medicinal plants and put them at risk of extinction [12]. Hedge nettle contains several compounds with antimicrobial, antioxidant, and anti-cancer activities, and therefore, has different therapeutic, flavouring, and preservative applications in herbal medicines [13]. Despite the widespread use of Hedge nettle in traditional medicine, little efforts have been made to develop tissue culture and regeneration systems in this plant; However there are a few reports on *in vitro* tissue culture of some plant species belonging to genus *Stachys*, such as *S. sieboldii* [14-16] and *S. leucoglossa* [17], no reports have been published concerning *in vitro* tissue culture and regeneration in *S. schtschegleevii* so far. Establishing an efficient tissue culture and regeneration protocol for Hedge nettle, in addition to providing requirements for molecular breeding of this plant through genetic engineering, will provide a highly valuable alternative method for organ production for medicinal uses, alleviating its over-harvesting pressure from natural habitats [16].

In this study, by intensive evaluation of different ratios of selected auxin and cytokinin hormones, callus induction and shoot regeneration response of Hedge nettle from two explant sources including leaf and internode, was investigated. Also, since the genetic transformation systems have not been reported for this plant so far, we evaluated the potential of responsive explants to gene transfer by transient transformation and expression of the GUS reporter gene using the particle bombardment method.

MATERIAL AND METHODS

Seed Sterilization and Establishment of Sterile Plants

Seeds were surface-sterilized by a novel method by adding 70% alcohol followed by shaking well for one minute. After a brief wash with sterile distilled water, seeds were sown in 5% sodium hypochlorite solution for 15 min at room temperature. After 3 times washing with sterile double-distilled water, seeds were cultured in glass jam containers containing 50 ml of MS basic medium. The cultures were then placed in a growth chamber with a diurnal cycle of 16 hours of light (50 µE) and 8 hours of darkness at 25 °C for germination and growth.

Explant Preparation and Culture

Internode and leaf explants were prepared from five- to six-week-old sterile plants. To this end, internodes were cut in about 3 mm fragments using a sharp scalpel, and leaf explants were prepared in the size of about 3 mm^2. Six to seven pieces of each explant type were cultured at equal distances in a 10 cm in diameter petri dishes containing about 35 ml of MS medium [18] containing different concentrations of hormones. The cultures were then placed in the growth chamber with above-mentioned conditions. After 5-6 weeks of culture, callus induction and shoot regeneration was evaluated in explants.

Root Induction and Acclimatization of Regenerated Plants

Root induction from regenerated plants was performed on the MS basal medium [18]. For acclimatization, rooted plantlets were thoroughly washed with tap water and cultured in a mixture of soil and perlite (1:1 ratio) followed by transferring to the greenhouse for adaptation and growth.

Transient Transformation of the GUS Gene in Internode Explants

Transient expression of the *GUS* reporter gene was used to evaluate the efficacy of gene transfer to internode explants [19], by the gene gun method. Briefly, gold particles of 1 µm in diameter were coated with the pFF19G vector [20], which contains the *GUS* gene under the control of the cauliflower mosaic virus (CaMV) 35S gene promoter and terminator. Young internode segments of 5-week-old sterile plants were cut into 0.3 cm pieces and placed in the centre of a petri dish containing MS medium. The pFF19G vector was coated on the gold particles according to the standard protocols, followed by bombardment on the explants using a Bio-Rad Helium-driven PDS-1000/He particle gun (Bio-Rad, USA).

Histochemical GUS Staining

For histochemical GUS staining, bombarded leaves, after 24 h incubation at 28 °C, were incubated overnight in GUS staining solution [including 1 mM EDTA (pH=8), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 200 mM potassium phosphate (pH=7.0), 1% Triton-X100, 1 mg/ml X-Gluc], at 37 °C [19]. To remove plant pigments, stained internodes were incubated for one hour in 50% ethanol followed by incubation in pure ethanol for several hours at room temperature.

Statistical Data Analysis

Statistical analysis of data was performed using MSTAT-C software, and mean comparison was performed with Duncan's multiple range test.

Results

Seed Germination and Establishment of Sterile Cultures

To produce sterile Hedge nettle mother plants, disinfected seeds were germinated and grown on basal MS medium. The seeds were germinated after about 3 to 4 weeks with around 50% efficiency. The seedlings were then grown on the same medium to produce sterile plants for tissue culture experiments.

Callus Induction and Shoot Regeneration from Cultured Explants on Different Media

Our primary experiment using MS basal medium complemented with different concentrations of two hormones, IAA (0, 0.02, 0.2, and 1.5 mg/l) and BAP (0, 0.02, 0.2, and 1.5 mg/l) showed that the hormonal combination of 1.5 mg/l BAP and 1.5 mg/l IAA results in higher callus induction rate for both explants (data not shown). However, the callus induction rate for the internode explant was significantly higher than leaf explant (Fig. 1 and Fig. 2A). As indicated in Figure 2A, the callus induction rate from internode explants on this medium was more than 70%, but no regeneration was observed. Therefore, we tested other hormonal combinations to achieve shoot regeneration. While keeping the BAP concentration at 1.5 mg/l, when we reduced the amount of IAA to 0.5 or 1 mg/l, more than 35% of induced calli displayed shoot regeneration (Fig. 1 and Fig. 2B). Similar results were observed for leaf-derived calli. Interestingly, at a lower IAA concentration (0.5 mg/l), regeneration mainly occurred through organogenesis (Fig, 1D, E and Fig. 2B), whereas, at moderate IAA concentrations (1 mg/l), somatic embryogenesis appeared to be dominant (Fig. 1F, G and Fig. 2B). It should be noted that these media were not suitable for primary callus induction from internode explant (Fig. 2B). These results show that for successful and efficient plant regeneration from Hedge nettle tissues, a two-step protocol containing different levels of IAA and BAP is required.

The response for *in vitro* regeneration can also be affected by different auxin and cytokinin types [21]. Therefore, we tested some combinations of other auxin and cytokinin including 2,4.D and TDZ for callus induction and regeneration from internode explants. Efficient callus induction was observed in tested levels (Fig. 1C and Fig. 2C), however, the callus structure was very different (Fig. 1C) and we could not regenerate plants from this callus type. Therefore, it seems that 2,4.D and TDZ combination is not suitable for regeneration-competent callus production in Hedge nettle.

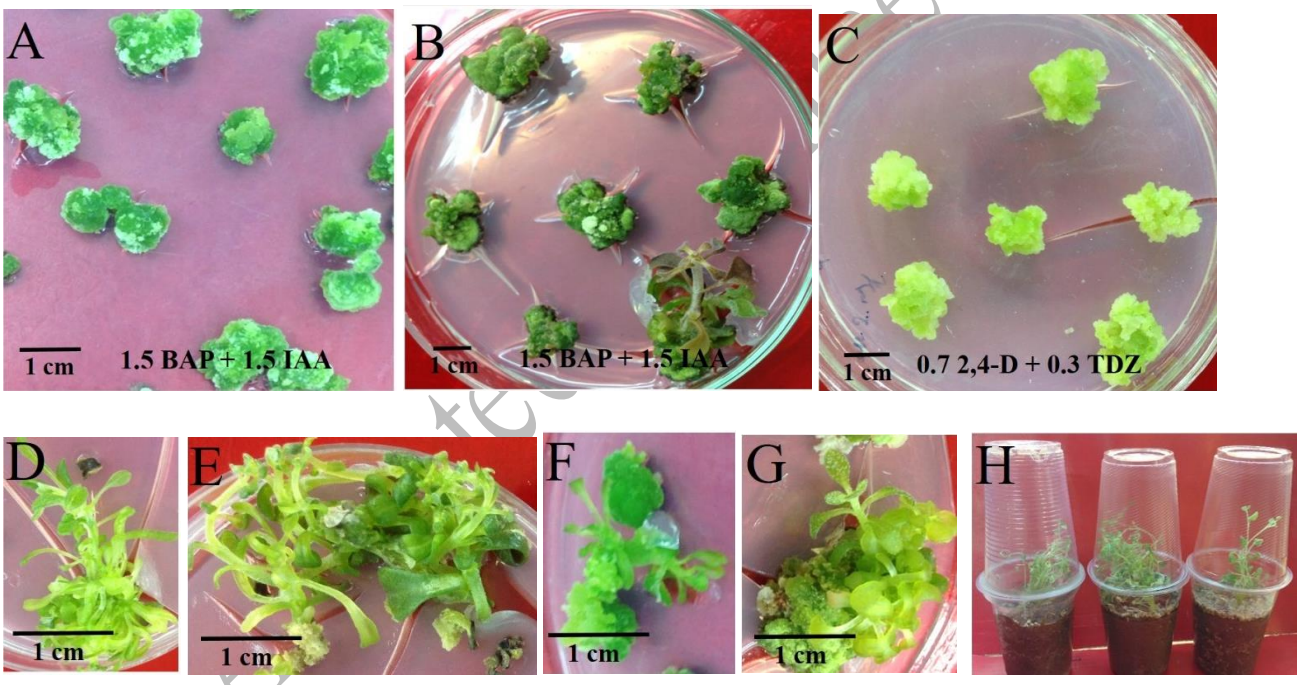


Fig. 1 Samples of different callus types induced on MS medium containing various hormone combinations from internode (A and C) and leaf (B) tissues of *S. schtschegleevii*. (D-G) Close-up pictures of several calli showing indirect shoot regeneration via organogenesis (D, E) and embryogenesis (F, G). (H) Transferring rooted plants in soil for acclimatization and further growth. The hormone levels are stated as mg/l.

Regenerated plants could be rooted on basal MS medium and successfully acclimatized into the greenhouse condition (Fig. 1H). the addition of 0.8% activated charcoal (AC) into the medium, significantly increased the root induction rate (Fig. 3A-D).

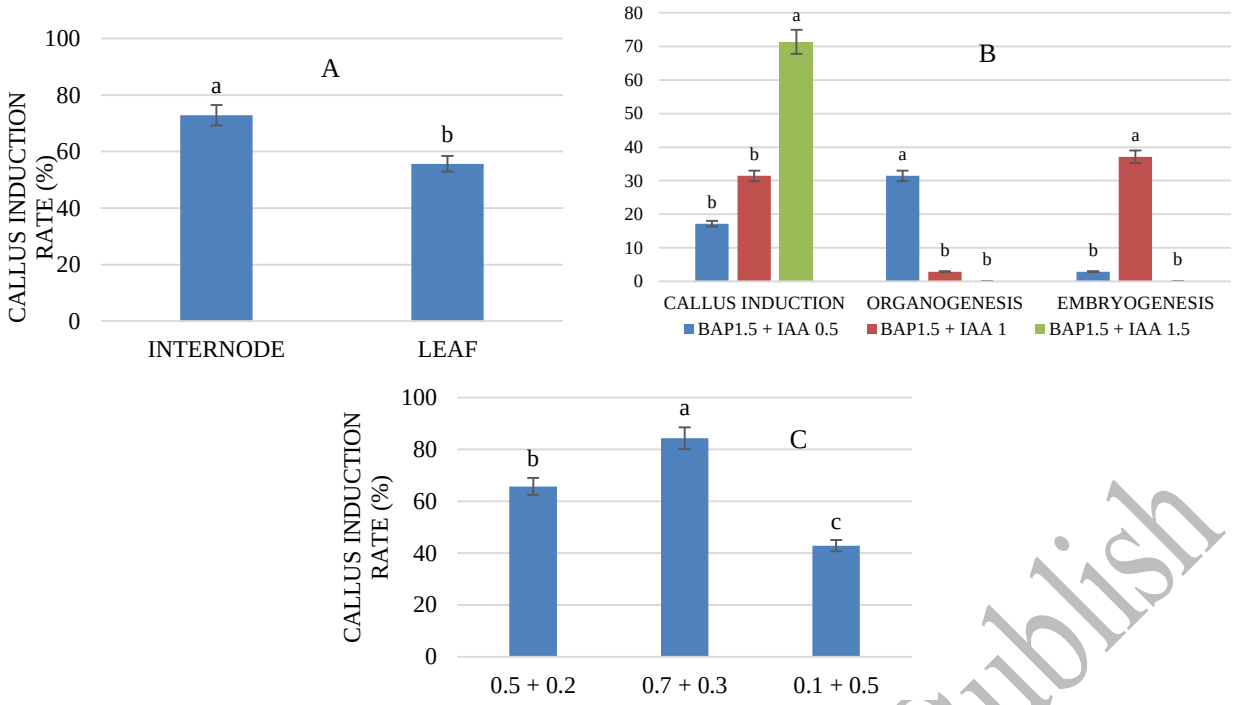
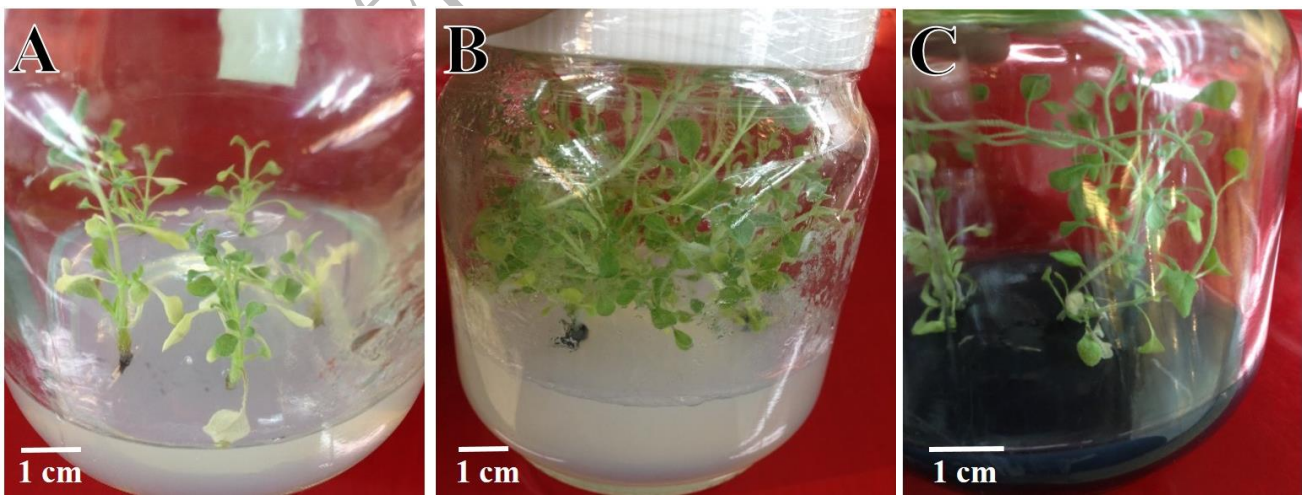


Fig. 2 Diagrams of mean comparison for callus induction and shoot regeneration from two different explants of *S. schtschegleevii* on MS medium complemented with different hormone combinations. (A) Mean comparison of callus induction from leaf and internode explants on MS medium containing 1.5 mg/l BAP and 1.5 mg/l IAA. (B) Callus induction and different types of regeneration from internode-derived calli on 3 different combinations of BAP and IAA. (C) Callus induction from internode explants on 3 different combinations of 2,4-D and TDZ hormones. The two values used to label columns, show 2,4-D and TDZ concentrations, respectively. Different letters on columns show significant differences based on Duncan's multiple range test for the corresponding features.

Transient Transformation and Expression of GUS Reporter Gene in Internode Explants

We used the pFF19G vector [20] containing the GUS reporter gene to investigate the potential of internode explants for transformation and expression of foreign genes by the biolistic bombardment method (Fig. 3E). More than 60% of bombarded internode segments successfully expressed the GUS reporter gene (Fig. 3F), indicating the rational potential of internode explant for foreign gene transfer by the biolistic gun system. In this study, 1 µm in diameter gold particles, 1100 psi rupture disk pressure, 10 cm sample distance from the stopping screen, -0.8 bar vacuum level, and mono adapter were used. The results showed that these parameters are suitable for Hedge nettle transformation by the biolistic method.



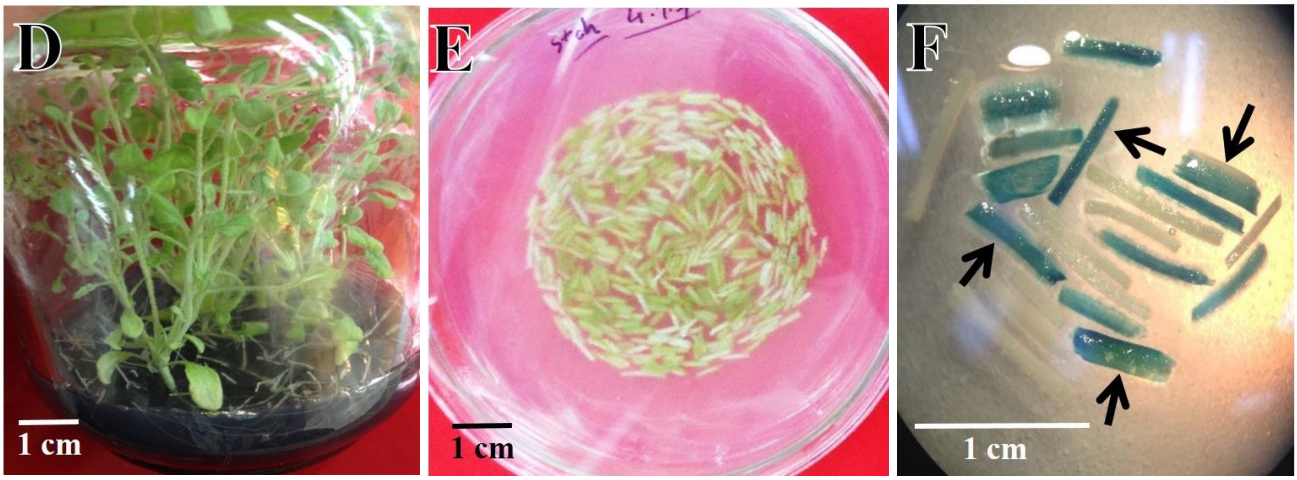


Fig. 3 Shoot elongation and multiplication from regenerated plants on different media, including MS basal medium (A), MS medium complemented with 1 mg/l BAP (B), MS medium containing 0.8% activated charcoal (C), and MS medium complemented with 1 mg/l BAP and 0.8% activated charcoal (D). (E) Preparation of internode explants for bombardment with GUS-containing-plasmid-coated particles. (F) Examples of the results obtained from the transient transformation and expression of the GUS gene in internode tissues using the biolistic transformation system. Some internode segments demonstrating GUS expression are indicated by black arrows.

DISCUSSION

The *Lamiaceae* family is one of the most important herbal families, incorporating a wide variety of medical plants and aromatic spices [22]. *In vitro* tissue culture and micro-propagation protocols have been reported for several members of this family including Thyme [23], Mint [24], Oregano [25], Basil [26], Sage [27], Savoury [28], Rosemary [29], Self-heal [30], Hyssop [31], and Lemon balm [32]. Leaf and internode explants are two commonly used plant explants in tissue culture experiments due to their easy preparation from young sterile plants [33]. In this study, we evaluated the potential of these two explants for callus induction and shoot regeneration in Hedge nettle (*S. schtschegleevii*), a rare plant that thrives in some of the world's most challenging environments. We developed efficient protocols for callus induction and shoot regeneration from leaf and internode explants of this plant. The highest callus induction rate was observed on MS medium containing 1.5 mg/l IAA and 1.5 mg/l BAP for internode explant (Fig. 1,2). Induced calli showed a reasonable shoot regeneration rate on a similar medium with reduced IAA levels. At low IAA levels (0.5 mg/l), shoot induction occurred mainly through organogenesis, while somatic embryogenesis seemed to be the dominant regeneration type at moderate IAA concentrations (1 mg/l) (Fig. 2B).

Auxin and cytokinin are two plant hormones with major effect on callus induction and regeneration in tissue culture experiments [34, 35]. Both hormones contribute to cell division and meristem establishment in a ratio-dependent manner [34, 36]. In general, a higher cytokinin-to-auxin ratio will induce shoot organogenesis. This process can be achieved by two systems, including direct and indirect-callus-mediated pathways [37]. In Hedge nettle, similar to Arabidopsis, it appears that indirect organ regeneration occurs by a two-step process, each requiring a different hormonal combination. Note that the endogenous levels of two hormones in different tissues can affect suitable exogenous levels of both hormones for the best results. This can support the different responses of two examined tissues (leaf and internode) on similar media (Fig. 2). Although other tested auxin and cytokinin including 2,4-D and TDZ could also produce callus structures (Fig. 2C), however, these calli did not appear to be regeneration-competent. The regenerated shoots showed rapid growth and shoot multiplication on MS medium containing 1 mg/l BAP (Fig. 3).

Activated charcoal is characterized by a high adsorptive capacity for inhibitory substances such as phenolic compounds which might be produced by cultured tissues, and products of sucrose breakdown, produced by autoclaving [38]. In this study, root growth and development in regenerated plants were significantly promoted by the addition of 0.8% activated charcoal (AC) (Fig. 3). Also, in an AC-containing medium, the application of 1 mg/l BAP resulted in a highly significant positive effect on shoot proliferation (Fig. 3A-D). These results can be of high importance for the rapid *in vitro* propagation of Hedge nettle. These outcomes can be used for rapid

propagation of Hedge nettle *in vitro*. It has been shown that AC can selectively affect the accumulation of secondary metabolites in *in vitro* culture [39]. How this compound affects the secondary metabolites of the Hedge nettle plant *in vitro* remains to be investigated.

In addition, we demonstrated the potential of internode segments for transfer and expression of foreign genes by transient expression of the GUS reporter gene (Fig. 3F). The results can be used in breeding programs of Hedge nettle by genetic engineering methods.

Briefly, the workflow for the complete procedure of the optimized callus induction and plant regeneration protocol can be summarized as follows (about 15-20 weeks):

- Seed sterilization by 70% EtOH for 1 min, followed by 5% NaOCl for 15 min
- Seed culture on hormone-free MS basal medium for germination (3-4 weeks)
- Plant growth in *in vitro* conditions to provide sterile mother plants (2-3 weeks)
- Preparation of 3 mm long internode segments from sterile plants
- Culture 6-7 explants with equal distances in Petri dishes (10 cm in diameter) containing 35 ml of callus induction medium (CIM: MS medium complemented with 1.5 mg/l BAP and 1.5 mg/l IAA)
- Incubate cultures in growth chambers with a diurnal cycle of 16 h light (50 µE) and 8 h darkness, constant temperature of 25 °C, and cabin humidity of 70%, for 4 weeks
- Transfer induced calli on regeneration medium (For organogenesis: MS + 1.5 mg/l BAP and 1 mg/l IAA; and for embryogenesis: MS + 1.5 mg/l BAP and 0.5 mg/l IAA) for 3-4 weeks
- Transfer regenerated shoots on hormone-free MS medium for shoot elongation and root formation (2-3 weeks)
- Transfer rooted plants into the soil (1:1 ratio mixture of pot soil and perlite) for acclimatization (1-2 weeks)

The capacity of different plant species for *in vitro* regeneration varies considerably. Some plant families such as *Solanaceae, Asteraceae, Gesneriaceae, Liliaceae,* and *Cruciferae* have a relatively high regeneration capacity. However, regeneration in some other plant families such as *Malvaceae* and *Chenopodiaceae* is challenging [40]. Our results show that the Hedge nettle belonging to *Lamiaceae*, can be placed in the second category.

Declaration of Conflicts of Interest

The authors have no conflicts of interest to declare.

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