Available Online

JOURNAL OF SCIENTIFIC RESEARCH

J. Sci. Res. **15** (1), 215-224 (2023)

www.banglajol.info/index.php/JSR

In vitro Micropropagation of *Bergenia ligulata* (Hook. f. & Thomson) Engl. through Leaf Explant

S. Rani*, R. Puri, A. Qasim, P. Boora, D. Angmo

Department of Botany, Panjab University, Chandigarh (160014), India

Received 22 April 2022, accepted in final revised form 2 October 2022

Abstract

Berginia ligulata is medicinally significant and endangered plant of the western Himalayan Region. This plant is used to treat many diseases such as urinary problems and heart problems. By using leaf explant, an efficient and rapid micropropagation protocol was developed. In *in-vitro* propagation, MS medium was supplemented with benzyl amino purine (BAP), 1-naphthalene acetic acid (NAA) and indol-3-acetic acid (IAA) to induce multiple shoots from leaf explants. The most positive response for the formation of calluses was observed on MS media supplemented with MS medium + BAP(1 mg/L) + NAA(1 mg/L) followed by MS medium + BAP (0.5 mg/L) + NAA (1 mg/L) after 3 weeks. Maximum number of shoots were obtained on MS medium + BAP (2 mg/L) +IAA (2 mg/L). Maximum % shoot response was obtained on MS medium + Kn (2.5 mg/L) concentration. The maximum number of roots was observed on MS medium without growth regulators. These well-rooted plants were established in pots and acclimatized in the lab and transferred to field conditions. This process is useful to produce endangered plants on large scale.

Keywords: Endangered medicinal plants; Micropropagation; MS medium; Plant growth regulators.

doi: http://dx.doi.org/10.3329/jsr.v15i1.59313 J. Sci. Res. **15** (1), 215-224 (2023)

1. Introduction

Bergenia ligulata (Hook. f. & Thomson) Engl. [family Saxifragaceae], known locally as 'Pashanbheda, is a perennial herbaceous medicinal plant and in the western Himalayan region, it is an endangered plant species according to the IUCN. In the past, this plant's rhizomes were used for treating diabetics, for dissolving kidney stones, and for dissolving gall bladder stones, pulmonary infection, cough and cold [1,2]. The methanolic extract *B. ligulata* was evaluated for phytochemical screening using standardized methods. At present, approximately 152 volatile and non-volatile compounds are isolated from Bergenia genus which contained a wide range of bioactive compounds of therapeutic value [3]. The phytochemical analysis showed that the plant extract is a rich source of secondary metabolites such as alkaloids, coumarins, phenol, flavonoids, benzenoids,

* *Corresponding author*: sushilarani992@gmail.com

tannin, saponins, lactones and terpenoids which make this plant a valuable medicinal herb with broad spectrum pharmacological activities like antioxidant, antiviral, antibacterial, diuretic, antipyretic, anti-inflammatory, antioxaluria and antidiabetic activities [3,4]. Wild medicinal plants are harvested as a raw material for medicine, which is the main cause of genetic diversity loss and degradation of habitat.

In India, the majority of population utilizes plant-derived medicine to meet their medical and health care demands. Even though herbal medicines have been used for various ailments since ancient times, large quantities of medicinally important plants are still collected from their natural environment [5]. Due to lack of control over the over-harvesting of these medicinal plants, tradesmen, local people and vaidaya harvest them extensively, resulting in the extinction or near-extinction of several species. Natural medicines are becoming increasingly popular because they are viewed as green medicine that is always assumed to be non-toxic. The health care system will turn out to be more expensive, therefore, there is an urgent need to develop technologies to be essentially introduced in the health care system and also preserve the natural habitat of medicinal plants. Thus, utilizing biotechnological methods, especially *in-vitro* micropropagation possesses an incredible potential for the regeneration of rare and endangered medicinal plants that can be reintroduced into their natural environment on a huge scale for the production of high- value medicinal products [6]. Therefore, *in vitro micropropagation* plays an important role in producing high-quality plant-based medicines.

At the end of 1960s, a number of cellular culture techniques were introduced as a tool for study and production secondary metabolites [7]. It is essential to develop a reliable *in-vitro* micropropagation protocol for the commercially important medicinal plant species for rapid regeneration and production of high-quality plant chemicals (secondary metabolites) so that there should be a continuous supply of healthy plant material to pharmaceutical industry for the production of medicinally important drugs sustainably [8]. A growing interest in secondary metabolism has been triggered by increasing commercial relevance of secondary metabolites especially in the possibility of customizing production of bioactive compounds using the tissue culture technique.

The *in-vitro* micropropagation, techniques are successfully developed for conservation of genetic material/ germplasm of various medicinally important plant species throughout the Himalayas including *Aconitum violaceum* [9], *Origanum vulgare* [10], *Berberis aristata* [11], *Aconitum ferox* [12], *Nardostachys jatamansi* [13]. The *in-vitro* propagation for several endangered medicinal plants has been already established. These *include* such as *Thymus persicus* [14], *Siphonochilus aethiopicus* [15] and *Celastrus paniculatus* [16]. There are a number of difficulties that can arise during the process of tissue culture like discoloration of media and browning of explant. In the present study, we developed simple and effective technique for in *vitro* micropropagation of *B. ligulata* using leaf disc explants for mass multiplication.

2. Experimental

2.1. *Collection and Identification of plant*

The plants were collected in July from the Shimla district of Himachal Pradesh and authenticated from the Herbarium of Botanical Survey of India North West Circle, Dehradun (BSI), under accession no 468.

2.2. *Explant sterilization*

After collecting and washing the leaf explants with detergent under running tap water for 30 minutes, soil particles were removed. The leaf explants were trimmed until they ranged in size from 3.0 to 4.0 cm. To complete the process, wash the explant with mild detergent (1-2 drops of Teepol) solution for about 10 minutes and followed by five repeating washing with distilled water. Under aseptic conditions, the explant was immersed in the solution containing systemic fungicide (Bavistin 0.25 %) for 5 to 7 minutes afterward rinse with sterilized distilled water. After fungicide treatment, 0.1 % mercuric chloride solution was used to remove surface contamination. Then, the explant was washed with sterilized distilled water. Finally, to prevent contamination, the surface- sterilized explants were dried on filter papers in the laminar flow air chamber just before inoculation on media.

2.3. *Media and culture condition*

Surface-sterilized leaf explants were aseptically inoculated on basal MS media (Murashige and Skoog). This basal medium was supplemented with various plant growth regulators like BAP, IAA, and NAA in different concentrations. Using 1 N HCl and 1 N NaOH, the pH was adjusted to 5.4 to 5.6. The test tubes (25 mL in each) were dispensed with MS media and plugged them with non-absorbent cotton. The media was autoclaved at 121 ℃ for about 20 min. Finally, the culture was incubated at 25± 2 ℃ under 8 h of photoperiod at an irradiance of 40 µmoles m^{-2} s^{-1} from cool white fluorescent tubes. Total four replicates were used in the present study and subculturing was carried out every six and eight weeks. Each culture was checked regularly and morphological changes were observed. The generalized protocol for micropropagation of *Berginia ligulata* was explained in Fig. 1.

2.4. *Induction of shoot, subculturing and elongation of shoot*

The leaf explants germinated *in vitro* were used for the induction and multiplication of shoots. For the proliferation of multiple shoots, different plant growth regulators, such as BAP, Kinetin were used at different concentrations. The surface-sterilized leaf explant was inoculated on the growth medium containing different concentrations of plant growth

regulators. Sub-culturing was done using an appropriate growth regulator which produces maximum shoots. Multiple shoot clusters were transferred to the elongation medium.

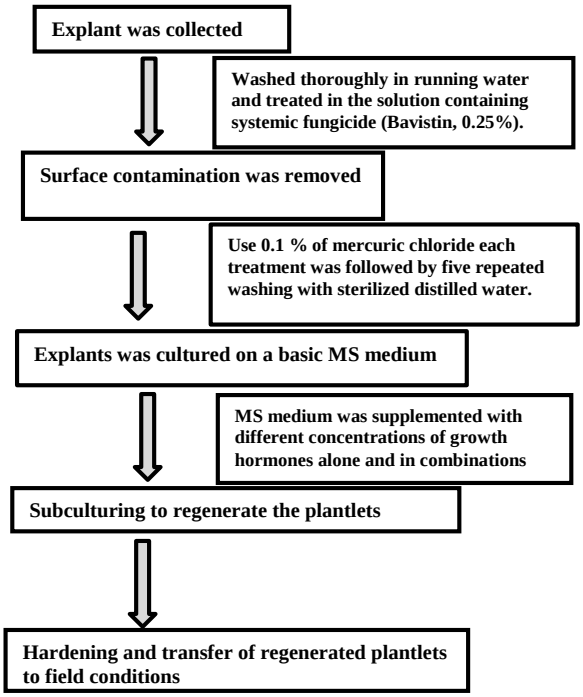

Fig. 1. General method of sterilization of leaf explant of *Berginia ligulata.*

2.5. *In vitro rooting*

Individual shoots were aseptically inoculated on MS medium supplemented with various growth regulators for *in-vitro* rooting. Different growth regulators were tested for the effectiveness of *in vitro* rooting in MS medium.

2.6. *Hardening and acclimatization*

In-vitro rooted plantlets removed from culture media, were washed with sterilized distilled water to remove the excess agar medium and transferred to sterilize soil containing pots. To ensure the excessive humidity around the plants, polythene sheets were used to cover pots. As soon as the plantlets were hardened, they were transferred to the greenhouse condition and watered twice a week. After acclimatization to field conditions, the plantlets were transferred in the field.

2.7. *Statistical analysis*

The results were analyzed statistically using one- way analysis of variance (ANOVA) followed by comparing mean values using post hoc Duncan's Multiple Range Test at $p<0.05$. The data was then, expressed as mean ±SEM of three replicates using SPSS software program.

3. Results and Discussion

Plant material plays an important role in efficacy and success in tissue culture [16]. Several endogenous and exogenous factors contribute to the process of *in-vitro* micropropagation of plants. There are three major factors involved to determine plant growth such as physiological condition of explants, concentration of plant growth regulators and their interaction in culture media [18,19].

3.1. *Callusing*

The progress of *in-vitro* micropropagation is depends on type and concentration of plant growth regulators used in tissue culture medium. In our present study we obtained best response when the leaf explant was grown on MS media supplemented with BAP (1 mg/L) + NAA (1 mg/L) followed by MS media with BAP (0.5 mg/L) + NAA (1 mg/L) after three weeks (Table 1) (Fig. 2). It was observed that callus was induced when the nodal explants of *Catharanthus roseus* were placed on Murashige and Skoog medium supplemented with 0.2 mg/L NAA and 2 mg/L KN [20]. During *in vitro* micropropagation induction of callus depends on plant explant used, concentration and type of plant growth regulators and interaction of explant with plant regulators added to the MS medium. It was also observed that MS media supplemented with BAP (1.5 mg/L) and NAA (1mg/L) was best medium for growing *Berginia ciliate* callus [21].

Table 1. The effect of plant growth regulators on the formation of callus from leaf explant of *Berginia ligulata*.

BAP	NAA	Nature of Callus	Callus formation intensity
0.2	0.2	-	-
0.4	0.4	-	-
0.5	1	RWC	++
1	1	LGSC	++++
1.5	1	LGSC	+++++

RWC radish white callus LGSC Light green soft callus

3.2. *Shoot induction and shoot elongation*

Some shoot buds developed directly from the leaf explants and in some cases, the leaf explant first formed the callus, which developed shoots on further subculturing. Callus induction from leaf depends on stage and meristematic activity of the leaf cell. MS

medium supplemented with different concentrations of BAP and Kinetin was tried for best response. Some selected leaf explants were cultured on MS media supplemented with various conc. of BAP (1.0, 1.5, 2.0, 2.5 mg/L) and Kn (1.0, 1.5, 2.0, 2.5 mg/L) (Table 2). Without considering explant type, best shoot proliferation was observed on BAP than Kn. Maximum % response was obtained on MS+ BAP(2.5mg/l). High shoot regeneration efficiency was reported with MS media supplemented with BAP as compared to Kn in *Solanum surattense* [22] and also the same result was reported in *Lens culinaris* [23], which is similar to the present research work. The number of shoots per explant increased with increase in concentration of BAP. The height of shoot (cm), the number of shoots per explant and % response were recorded after 4 weeks of culture. The shoot-lets obtained were very minute so they were sub-cultured for proliferation on high strength MS medium. After that, shoot growth was increased significantly. After shoot proliferation, best-grown shoots were transferred to MS medium supplemented with BAP (2.5 mg/L) for shoot elongation. If the shoots were not sub-cultured and elongation continued then, hyperhydricity symptoms appeared. According to some researchers, plantlets of *Primula* that were not subcultured to new medium had a higher probability of developing hyperhydricity and abnormal growth [24]. The *in vitro* micropropagation of *Piper crocatum* on the MS medium supplemented with BAP (5.0 mg/L) + 2,4-D (0.5 mg/L) + charcoal produced most appropriate number of shoots and has less problem of browning of media [25].

Table 2. Formation of shoots from in vitro inoculated leaf explant of *Bergenia ligulata* on MS medium supplemented with growth regulators.

Phytohormones(mg/L)	Shoot Number (mean± SE)	Shoot Length (mean± SE)	% Response (mean± SE)
Control	-	-	-
Kn(1.0)	0.5 ± 0.28^{a}	0.25 ± 0.14^{a}	8.7 ± 5.15^{a}
Kn(1.5)	1.5 ± 0.29^{ab}	0.87 ± 0.23^{ab}	50 ± 2.04^{bcd}
Kn(2.0)	2.0 ± 0.40^{bc}	1.56 ± 0.42^{bc}	52 ± 3.14^{bcd}
Kn(2.5)	2.7 ± 0.47^{cd}	1.93 ± 0.21^{cd}	58 ± 3.14^{bcd}
BAP(1.0)	3.5 ± 0.64^{dc}	2.1 ± 0.27^{cd}	65 ± 4.56^{cd}
BAP(1.5)	3.7 ± 0.28^{de}	3.6 ± 0.23^{e}	50 ± 1.47^{bcd}
BAP(2.0)	4.0 ± 0.40^{e}	2.7 ± 0.47^{d}	50 ± 1.42^{bcd}
BAP(2.5)	5.2 ± 0.25^{f}	2.6 ± 0.23^{d}	71 ± 4.26^{d}
BAP(2)+IAA(2)	8.2 ± 0.26^{g}	3.8 ± 2.87^{e}	50 ± 11.5^{bcd}
BAP(1.5)+IAA(1)	5.7 ± 0.47^{f}	2.0 ± 0.2^{cd}	$42.11.0^{bc}$
BAP(1)+IAA(1)	7.7 ± 0.48^{g}	4.6 ± 0.25^{f}	45 ± 11.0^{bcd}
BAP(1)+IAA(1.5)	2.5 ± 0.28^{bcd}	1.6 ± 0.23^{bc}	37 ± 3.17^{b}

Data recorded after 4 weeks

Values are mean± SE of four replicates

Means followed by the different letters were not significantly different at $P \leq 0.05$(Duncan's Multiple Range Test)

3.3. *Rooting of shoot*

In the present study, *in vitro* raised elongated shoots were transferred to the rooting media containing rooting hormones. Plant growth regulators were used in various conc. range from 1.0, 1.5, 2.0, 2.5, mg/L for best result (Table 3). After four weeks of inoculation of elongated shoots to rooting media the number of roots, length of roots and % response were recorded. IBA alone did not show any result in this range of concentration. However, IAA in combination with NAA in the same or different concentrations shows less rooting. Highest number of roots were obtained on MS media without growth regulators whereas, very thing roots or less number of roots were obtained on MS media supplemented with rooting hormones (IAA, IBA and NAA). The similar result where MS media without growth regulators produced maximum roots were also observed in *Stevia rebaudiana, Curculigo latifolia* [26], *Artemisia vulgaris* [27] *Ocimum sanctum* [28] and *Echinacea purpurea* [29]. These observations also supported the present research work. It was reported that shoot developed from the callus MS media supplemented with BA (2.5 mg/L) and NAA (0.1 mg/L) was best for root formation in *Pelagonium graveolens* [30]. It was also observed that MS medium supplemented with IBA (1.0 mg/L) and IAA (0.5 mg/L) induced long, thick and high number of roots in planntlets of *Baliospermum montanum* [31].

Table 3. Formation of roots from in vitro inoculated leaf explant of *Bergenia ligulata* on MS Medium supplemented with growth regulators.

Phytohormones (mg/L)	Root Number (Mean ±SE)	Root Length(cm) (Mean ± SE)	% Response (Mean±SE)
Control	12.8 ± 0.42^{h}	9.2 ± 0.11^{i}	87.2 ± 0.85^{f}
IAA(1.0)	12.2 ± 0.75^{h}	8.6 ± 0.19^{hi}	83.5 ± 3.60^{ef}
IAA (1.5)	12.0 ± 0.40^{h}	8.6 ± 0.20^{hi}	77.25 ± 3.70^{def}
IAA (2.0)	11.0 ± 0.57^{gh}	8.4 ± 0.14^{h}	75.0 ± 6.45^{def}
IAA(2.5)	9.7 ± 0.47^{gh}	7.0 ± 0.16^{g}	77.5 ± 4.78^{def}
IBA(1.0)	8.7 ± 0.25^{ef}	6.9 ± 0.19^{g}	75.0 ± 2.88^{def}
IBA(1.5)	7.7 ± 0.47^{de}	5.7 ± 0.32^{f}	67.5 ± 2.50^{cde}
IBA(2.0)	6.0 ± 0.91^{cd}	4.9 ± 0.21^{e}	68.2 ± 3.11^{cde}
IBA(2.5)	5.0 ± 1.08^{bc}	3.6 ± 0.23^{d}	65.0 ± 2.85^{cd}
IAA(1)+ IBA(1.0)	4.0 ± 0.91^{b}	2.5 ± 0.21^{c}	48.7 ± 3.10^{b}
IAA(1)+ IBA(1.5)	1.75 ± 0.47^{a}	1.8 ± 0.42^{c}	53.7 ± 5.54^{bc}
IAA(1.5)+NAA(1.0)	0.5 ± 0.28^{a}	1.0 ± 0.20^{b}	45.0 ± 9.57^{b}
IAA(1)+NAA(1.5)	7.5 ± 0.64^{de}	0.62 ± 0.37^{a}	12.5 ± 7.50^{a}

Data recorded after 4 weeks
Values are mean± SE of four replicates
Means followed by the different letters were not significantly different at $P \leq 0.05$(Duncan's Multiple Range Test).

4. Acclimatization and Transfer of Plantlets in Soil

Well-rooted and acclimatized plantlets were transferred to the sterilized soil contained plastic pot under excessive humidity at a temperature of 25 °C for acclimatization. After

Hardening, plantlets were transplanted in large size pots and progressively acclimatized to their natural environment. Successful acclimatization of the *in vitro* regenerated plantlets was reported in *Peganum harmala* [32] and also well-rooted micropropagated plantlets of *Albizia lebbeck* L. have been acclimatized and established in sterilized soil and sand-containing pots [33].

Fig. 2. In vitro micropropagation of Bergenia ligulata (a) inoculation of leaf explant on MS medium (b-f) callus formation (g-k) shoot formation from callus and direct leaf explant after 4weeks. (l-m) multiplication elongation of the shoot after 4 weeks (n-p) direct rooting after 4 weeks (q) formation of complete plantlets (r) transferred to field after 3 weeks.

5. Conclusion

In this present work, we established an efficient and effective method of *in vitro* micropropagation protocol by using various concentration of growth regulators for production of maximum plantlets of *Berginia ligulata*. The germination of leaf explants in culture media is influenced by life cycle of *Berginia ligulata*. This technique may be useful for commercial growers. As a result of this protocol rare and superior genotypes of medicinal plants can be propagated and released back into their natural habitat as well as made available to cultivators for cultivating in agriculture practices. *In vitro* micropropagation has enormous potential for the generation of quality plant-based therapies. Therefore, *Invitro* micropropagation of endangered medicinal has become more popular in recent years, possibly because of better understanding of secondary phytoconstituents present in commercially useful medicinal plants. The advancement of plant tissue culture may allow cost- effective commercial production of rare and endangered medicinal plants, their cells, and the substances they produce. In general, developed countries are making good progress in optimizing medicinal plant protocols. However, in developing and undeveloped countries there is an urgent need for research in this particular area for the conservation of medicinally important plants so that the use of medicinal plants can be maximized.

Acknowledgment

The authors are thankful to chairperson of Botany Department for providing laboratory facilities and to the CSIR for providing fund for the research.

References

1. S. Srivastava and A. K. S. Rawat, J. Sci. Ind. Res. **67**, 65 (2008).
2. M. Singh, N. Pandey, V. Agnihotri, K. K. Singh, and A. Pandey, J. Trdit. Compl. Med. **7**, 152 (2017). https://doi.org/10.1016/j.jtcme.2016.04.002
3. B. Koul, A. Kumar, D. Yadav, and O. J. Jin, Molecule **25**, 5555 (2020). https://doi.org/10.3390/molecules25235555
4. A. Gohain, A. Sharma, H. J. Gogoi, R. Cooper, R. Kaur, G. A. Nayik, A. M. Shaikh, B. Kovács, F. O. Areche, and M. J. Ansari, Plants **11**, 1129 (2022). https://doi.org/10.3390/plants11091129
5. R. Kumari, Indian J. Sci. Res. **10**, 39 (2020). https://doi.org/10.32606/IJSR.V10.I2.00006
6. K. Yadav, N. Singh, and S. Verma, J. Agri. Tech. **8**, 303 (2012).
7. L. Tripathi and J. N. Tripathi, Trop. J. Pharm. Res. **2**, 243 (2003).
8. Y. Sahoo, S.K. Pattnaik, and P.K, Chand, In Vitro Cell. Dev. Biol. – Plant **33**, 293 (1997). https://doi.org/10.1007/s11627-997-0053-3
9. J. M. Rawat, B. Rawat, R. K. Agnihotri, A. Chandra, and S. Nautiyal, Acta Physiol. Plant. **35**, 2589 (2013).
10. A. Pandey, T. Belwal, S. Tamta, I.D. Bhatt and R.S. Rawal, Mole. Biol. Rep. **46**, 2231 (2019). https://doi.org/10.1007/s11033-019-04678-x
11. L. Brijwal, A. Pandey, and S. Tamta, In Vitro Cell. Dev. Biol. -Plant. **51**, 637 (2015). https://doi.org/10.1007/s11627-015-9716-7
12. M. Singh, A. Chettri, A. Pandey, S. Sinha, K. K. Singh, and H. K. Badola, Proc. Nat. Acad. Sci., India Sect. B: Biol. Sci. **90**, 313 (2020). https://doi.org/10.1007/s40011-019-01104-x
13. B. Bose, S. Kumaria, H. Choudhury, and P. Tandon, Plant Cell, Tissue Organ Culture (PCTOC) **124**, 331 (2016). https://doi.org/10.1007/s11240-015-0897-x
14. Z. Bakhtiar, M. H. Mirjalili, A. Sonboli, M. M. Farimani, and M. Ayyari, Biologia **69**, 594 (2014). https://doi.org/10.2478/s11756-014-0346-z
15. G. L. Ngwenya, N. Moodley, M. E. Nemutanzhela, and B.G. Crampton, S. Afr. J. Bot. **2**, 414 (2010). https://doi.org/10.1016/j.sajb.2010.02.078
16. S. Senapati, S. Aparajita, and G. Rout, Biologia **68**, 627 (2013). https://doi.org/10.2478/s11756-013-0187-1
17. B. Tisserat, Embryogenesis, Organogenesis, and Plant Regeneration, Plant Cell Culture. A Practical Approach (1985) pp. 79-126.
18. R.E. Litz, R.S. Witjaksono, D. Efendi, F. Pliego-Alfaro and A. Barceló-Muñoz, Persea americana avocado. Biotechnology of fruit and nut crops. CABI Publishing, Wallingford, 326-347(2005). https://doi.org/10.1079/9780851996622.0326
19. K. Palanisamy and P. Kumar, For. Ecol. and Manag. **98**, 277 (1997). https://doi.org/10.1016/S0378-1127(97)00116-3
20. M. Ikeuchi, K. Sugimoto, and A. Iwase, The plant cell. **25**, 3159 (2013). https://doi.org/10.1105/tpc.113.116053
21. U. K. Shrestha and B. Pant, Botanica Orientalis: J. Plant Sci. **8**, 40 (2011). https://doi.org/10.3126/botor.v8i0.5557
22. M. Rahman, M. Amin, M. Islam, and R. Sultana, Acta Agri. Slov. **97**, 11 (2011). https://doi.org/10.2478/v10014-011-0002-z
23. G. Ye, D. L. McNeil, A. J. Conner, and G. D. Hill, N. Z. J. Crop Hortic. Sci. **30**, 1 (2002). https://doi.org/10.1080/01140671.2002.9514193
24. E. E. Benson, J. E. Danaher, I. M. Pimbley, C. T. Anderson, J. E. Wake, S. Daley, and L. K.

Adams, Biodivers. Conser. **9**, 711 (2000). https://doi.org/10.1023/A:1008941726419
25. A. Zuraida, E. Sabrina, M. Shukri, M. Razali, and H. Norma, Inter. J. Pure Appl. Biosci. **3**, 10 (2015).
26. N. Babaei, N. A. P. Abdullah, G. Saleh, and T. L. Abdullah, Sci. World J. **2014** (2014). https://doi.org/10.1155/2014/275028
27. G. Sujatha and B. R. Kumari, Plant Biotech. Reports **1**, 149 (2007). https://doi.org/10.1007/s11816-007-0028-1
28. N. K. Singh and C. B. Sehgal, Plant Growth regul. **29**, 161 (1999). https://doi.org/10.1023/A:1006201631824
29. A. Koroch, H. R. Juliani, J. Kapteyn, and J. E. Simon, Plant Cell, Tissue Organ Cul. **69**, 79 (2002). https://doi.org/10.1023/A:1015042032091
30. R. Gupta, S. Banerjee, G. R. Mallavarapu, S. Sharma, S. P. S. Khanuja, A. K. Shasany, and S. Kumar, Hort. Sci. **37**, 632 (2002). https://doi.org/10.21273/HORTSCI.37.4.632
31. S. Sasikumar, S. Raveendar, A. Premkumar, S. Ignacimuthu, and P. Agastian, Ind. J. Biotech. **8**, 223 (2009).
32. N. Goel, N. Singh, and R. Saini, Nat. Sci. **7**, 129 (2009).
33. K. Yadav and N. Singh, Fasci. Biol. **18**, 151 (2011).