In Vitro Flower Induction from Shoots Regenerated from Cultured Axillary Buds of Endangered Medicinal Herb Swertia chirayita H. Karst.

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In vitro flowering and effective micropropagation protocol were studied in Swertia chirayita, an important medicinal plant using axillary bud explants. The Murashige and Skoog’s medium (MS) supplemented with benzyl amino purine (BAP) 1.0 mg L−1 and adenine sulfate 70.0 mg L−1 was found optimum for production of multiple shoots. In the present study, incubation of flowering cultures on BAP supplemented medium (during shoot multiplication) was found necessary for flowering (6 weeks). However, concentrations of auxins-like IBA (0–2.0 mg/L) were ineffective to form reproductive buds. Subculture duration, photoperiod, and carbon source type do have influence on the in vitro flowering. The mature purple flowers were observed when the cultures were maintained in the same medium. This is the very first report that describes in vitro flowering system to overcome problems associated with flower growth and development as well as lay foundation for fruit and seed production in vitro in Swertia chirayita.

1. Introduction

India is ranked the 6th among 12 mega diversity countries of the world [1] and Uttarakhand is one of the states in India which is known for its great diversity. Swertia chirayita is an important medicinal plant found in Uttarakhand. Swertia chirayita is considered the most important plant for its bitterness, antihelminthic [2], hypoglycemic, hepatoprotective [3], and antiviral [4] properties. The novel techniques of plant tissue culture provide a viable alternative for managing these valuable resources in a sustainable manner. There are few reports about the tissue culture of Swertia chirayita. Documented literature reveals that there is a limited literature reported by few workers on in vitro propagation of Swertia chirayita, where they have used the nodal explants, in vitro grown seedlings, nodal meristems, and immature seed culture [5–9]. Balaraju et al. (2009) published reports on in vitro propagation of S. chirata using shoot tip explants derived from in vitro grown seedlings [10]. Chaudhuri et al. (2008) and Wang et al. (2009) reported direct shoot regeneration from in vitro leaves [11, 12]. But there is no report about the in vitro flowering of Swertia chirayita till date. The flowering process is one of the critical events in the life of a plant. This process involves the switch from vegetative stage to reproductive stage of growth and is believed to be regulated by both internal and external factors. A flowering system in vitro is considered to be a convenient tool to study specific aspects of flowering, floral initiation, floral organ development, and floral senescence [13]. The application of cytokinins, sucrose concentrations, photoperiod, and subculture time to promote flowering in vitro is well documented in many plant species [14, 15]. This is the very first report on in vitro flowering of this valuable medicinal plant and may open up new gates in the field of its conservation and continuous supply of plant material throughout the year by knowing its flowering behavior in vitro. This study is part of a larger programme
designed to investigate the *in vitro* conservation protocol of *Swertia chirayita* and describes *in vitro* flowering system to overcome problems associated with flower growth and development as well as fruit and seed production *in vitro* and hence may open up new gates in the conservation and sustainable exploitation of this very important plant.

2. Materials and Methods

2.1. Plant Material. The nodal segments from juvenile plants of *Swertia chirayita* grown *ex situ* were collected from Hitech Nursery, Deovan, Chakrata (7,699 ft., lat. 30°43.642', long. 77°51.941'), India, during the month of July and prepared herbarium was submitted to Botanical Survey of India, Northern Regional Centre, Dehradun (BSD), for identification of species level and plants were identified as *Swertia chirayita* (Roxb. ex Fleming) (VS 02) Family: Gentianaceae (Acc. number 113342). Surface sterilization was done as per the protocol given by Sharma et al. (2013) [16].

2.2. Culture Conditions. The basal media comprised of the mineral salts and organic nutrients of the MS medium (Murashige and Skoog, 1962) [17] containing 2.5% sucrose, solidified with 0.2% clarigel (HiMedia), and supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) and 70 mg/L adenine sulfate was used for culture establishment [16, 18]. The Subcuturing was performed at an interval of 3 to 4 weeks. Each treatment was replicated 12 times and all experiments were repeated at least thrice.

To examine the effect of photoperiod, 3 light/dark cycles, that is, 12/12, 16/8, and 8/16, were used in monitoring flowering *in vitro*. To examine the subculture time, explants were subcultured to fresh MS medium supplemented with 1.0 mg/L 6-benzylaminopurine (BAP) and 70 mg/L adenine sulfate on an interval of 4, 6, and 8 weeks. Five different sources of carbohydrates, that is, glucose sucrose, maltose, fructose, and lactose for a same concentration (2.5%), were examined for best flowering response. After bud formation, the cultures were shifted to continuous light of low intensity for induction of fully opened flowers. Subsequently, they were maintained under 16/8 h light/dark cycle for fruit development.

3. Results

The *in vitro* flowering was observed in the present study of *Swertia chirayita* and had not been reported earlier. *In vitro* flowering offers a unique system in the study of molecular basis and hormonal regulation of flowering. Flower initiated in MS media supplemented with BAP (1.0 mg/L) and adenine sulfate (70 mg/L) (Figure 1(a)) after 4–6 weeks of cultures. The production of flowering shoots continued for many subcultures spanning a period of more than two years. Flowers produced from tissue cultures systems presented normal morphological aspects. They were monocious and differentiated from lateral branches as field-grown plants (Figure 1(c)). Besides, anthesis was observed in floral buds development.

Maximum numbers of flowers (buds) (12 per culture) were obtained when shoots were cultured on MS medium containing 1.0 mg/L BAP + 70 mg/L adenine sulfate (Figure 1(b)) and incubated at 16/8 h light/dark period after 6 weeks. In the present study, incubation of flowering cultures on BAP supplemented medium (during shoot multiplication) was necessary for flowering (6 weeks). However, concentrations of auxins-like IBA (0–2.0 mg/L) were ineffective to form reproductive buds (data not shown). The production of flowers was promoted in approximately the same proportion. Flowering was induced *in vitro* in excised shoot cultures of *Swertia chirayita* devoid of any preformed bud. Photoperiod was found to be important for *in vitro* flowering. Maximum *in vitro* flowers were obtained at 16 hrs ± 2 light periods; it was observed that plants incubated under 12 hrs or shorter photoperiods (8 hrs) were negatively affected for floral bud development. Optimum temperature for efficient *in vitro* flowering was 24°C ± 2°C with a relative humidity of 60–70%. The nature of carbon source (mono or disaccharides) in the medium has an important influence on the formation of reproductive buds. The carbohydrates slightly differed in their ability to support the formation of reproductive buds. In general, sucrose was best closely followed by glucose; maltose and fructose were also effective for formation of flowering shoots whereas lactose was totally ineffective (Table 1).

4. Discussion

Flowering is considered to be a complex process regulated by both internal and external factors and its induction under *in vitro* culture is extensively rare. Physiological studies have sought for many years about what is florigen and have shown that flowering time control is influenced by environmental factors and endogenous cues. Plants can integrate these signals, such as day length, vernalization, ambient temperature, irradiance, water/mineral availability, and presence/absence of neighbors, to relate flowering time. Flowering *in vitro* has been promoted by cytokinins at optimum concentrations.

Results obtained from our previous study [16] revealed that, after 4 weeks of initial culture, nodal explants cultured on MS medium with BAP (1.0 mg/L) and 0.007% (70 mg/L) adenine sulfate developed maximum number of multiple shoots and cytokinin especially that BAP with adenine sulfate was found to be the key component for multiple shoot establishment. In the present investigation, effectiveness of BAP in inducing bud break was observed and has been reported in many other plant species [19–22]. Cytokinins is a common requirement for *in vitro* flowering [23]. A number of studies report the use of cytokinins for *in vitro* flowering in species like *Murraya paniculata* [24], *Fortunella hindsii* [25], *Gentiana triflora* [26], *Pharbitis nil* [27] and *Ammi majus* [28]. There are reports that indicate the beneficial effects of cytokinin especially BAP on the induction of *in vitro* flowering for medicinal plants like *Withania somnifera*, *Rauvolfia tetraphylla*, and *Anethum graveolens* [29–31] which are in accordance with our investigation. BAP is found to be playing an important role not only as a growth regulator but also as a factor regulating floral organ formation of regenerated plantlets [32]. It has been reported that phytohormones
affected flowering by mediating growth changes within the apical meristem and that cytokinins, in particular, played a key role in the initiation of mitosis and the regulation of cell division and organ formation. Auxins have frequently been reported to inhibit the formation of flowering buds in vitro in both long-day plants and short-day plants; low concentrations, however, may promote flowering even when higher ones are inhibitory [33]. Chrungoo and Farooq (1984) reported that, in saffron plants, NAA had an inhibitory effect on sprouting, vegetative growth, and flowering [34] and this has been in accordance with the present study where incorporation of IBA was not having promontory or inductive effect on flowering initiation. Carbohydrate source is also found to be an important factor and, in the present study, a lower concentration of 2.5% was found optimum for *Swertia chirayita* for flower initiation and maturation. This has been evident from study on *Arabidopsis thaliana* which reported that presence of sucrose in aerial parts of the plant promotes flowering [35]. Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by moving between shoot and root.

Light is the most important environmental factor that induces changes in plant physiology and morphology, regulating flowering season cycles [36–38]. Day length and light quality play a crucial role in flower induction both in vivo and in vitro possibly due to altered photosynthetic turnover on flowering and are believed to be essentially perceived by expanded leaves; then, “florigen” (sucrose and isopentenyladenine) will be produced and moved directly or indirectly to shoot apical meristem (SAM) to guide flowering determination [39]. In some plants, vernalization alone is sufficient for flowering evocation, but others require
subsequent exposure to inductive photoperiods (usually long days), and in them the changes at the apex wrought by vernalization and the photoperiodic stimulus are presumably different, possibly complementary [33]. Swertia chirayita is a high altitudinal plant enjoying gloomy and cold situation in nature, but importance of photoperiod instead of the vernalization for in vitro flowering of this plant has been demonstrated in the present study and maximum flowering frequency was observed with 16 h photoperiod. Subculture duration was also found to be an important factor for in vitro flowering in Swertia chirayita and importance of subculture duration has also been demonstrated by Wang et al. (2002) in other plant species [14].

5. Conclusion

In conclusion, our work has laid a preliminary foundation for a further research of in vitro flowering of Swertia chirayita. In tissue culture, in vitro flowering serves as an important tool for many reasons. One of the most important ones is being able to shorten the life cycles of plants; other aims include studying flower induction and initiation and floral development. Controlling the environment and media components enables the manipulation of different variables that affect these processes. So, this technique is of practical importance and can also serve for mass production of specific organs with unique compounds for pharmaceutical, nutritional, and other uses.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] T. V. Ramachandra and A. Suja, “Sahyadri: western ghats biodiversity information system,” in Biodiversity in Indian Scenario, N. Ramakrishnan, Ed., chapter 1, pp. 1–22, Daya Publishing House, New Delhi, India, 2006.
[2] S. Medda, S. Mukhopadhyay, and M. K. Basu, “Evaluation of the in-vivo activity and toxicity of amarogentin, an antileishmanial agent, in both liposomal and niosomal forms,” Journal of Antimicrobial Chemotherapy, vol. 44, no. 6, pp. 791–794, 1999.
[3] R. P. Rastogi and B. N. Mehrotra, Compendium of Indian Medicinal Plants, vol. 5, CDRI, Lucknow and National Institute of Science Communication, New Delhi, India, 1998.
[4] M. S. Zheng and Z. Y. Lu, “Antiviral effect of mangiferin and isomangiferin on herpes simplex virus,” Chinese Medical Journal, vol. 103, no. 2, pp. 160–165, 1990.
[5] A. Ahuja, S. Koul, B. L. Kaul et al., “Media compositions for faster propagation of Swertia chirata,” Patent WO 03/045132 A1, 2003.
[6] P. Joshi and V. Dhawan, “Axillary multiplication of Swertia chirayita (Roxb. Ex Fleming) H. Karst., a critically endangered medicinal herb of temperate Himalayas,” In Vitro Cellular & Developmental Biology, vol. 43, no. 6, pp. 631–638, 2007.
[7] S. Koul, K. A. Suri, P. Dutt, M. Sambyal, A. Ahuja, and M. K. Kaul, “Protocol for in vitro regeneration and marker glycoside assessment in Swertia chirata Buch.-Ham. ex Wall.—an endangered medicinal herb,” In Vitro Cellular & Developmental Biology, vol. 43, no. 5, pp. 467–472, 2007.
[8] R. K. Chaudhuri, A. Pal, and T. B. Jha, “Production of genetically uniform plants from nodal explants of Swertia chirata Buch.-Ham. ex Wall.—an endangered medicinal herb,” In Vitro Cellular & Developmental Biology, vol. 43, no. 5, pp. 467–472, 2007.
[9] R. K. Chaudhuri, A. Pal, and T. B. Jha, “Regeneration and characterization of Swertia chirata Buch.-Ham. ex Wall. plants from immature seed cultures,” Scientia Horticulturae, vol. 120, no. 1, pp. 107–114, 2009.
[10] K. Balaraju, P. Agastian, and S. Ignacimuthu, “Micropropagation of Swertia chirata Buch.-Hams. ex Wall.: a critically endangered medicinal herb,” Acta Physiologica Plantarum, vol. 31, no. 3, pp. 487–494, 2009.
[11] R. K. Chaudhuri, A. Pal, and T. B. Jha, “Conservation of Swertia chirata through direct shoot multiplication from leaf explants,” Plant Biotechnology Reports, vol. 2, no. 3, pp. 213–218, 2008.
[12] L. Wang, L. An, Y. Hu, L. Wei, and Y. Li, “Influence of phytohormones and medium on the shoot regeneration from leaf of Swertia chirata Buch.-Ham. ex Wall. in vitro,” African Journal of Biotechnology, vol. 8, no. 11, pp. 2513–2517, 2009.
[13] C. J. Goh, “Studies on flowering in orchids—a review and future directions,” in Proceedings of the Nagoya International Orchid Show (NIOC ‘92), pp. 44–49, Nagoya, Japan, 1992.
[14] G. Y. Wang, M. F. Yuan, and Y. Hong, “In vitro flower induction in roses,” In Vitro Cellular & Developmental Biology, vol. 38, no. 5, pp. 513–518, 2002.
[15] N. H. Vu, P. H. Anh, and D. T. Nhut, “The role of sucrose and different cytokinins in the in vitro morphogenesis of rose (hybrid tea) cv. 'First Prize’,” Plant Cell, Tissue and Organ Culture, vol. 87, no. 3, pp. 315–320, 2006.
[16] V. Sharma, B. Kamal, N. Srivastava, A. K. Dobriyal, and V. Jadon, “Effects of additives in shoot multiplication and genetic validation in Swertia chirayita revealed through RAPD Analysis,” Plant Tissue Culture and Biotechnology, vol. 23, no. 1, pp. II–19, 2013.
[17] T. Murashige and F. Skoog, “A revised medium for rapid growth and bioassays with tobacco tissue cultures,” Physiologia Plantarum, vol. 15, no. 3, pp. 473–497, 1962.
[18] V. Sharma, In vitro rapid mass multiplication and molecular validation of Swertia chirayita [Ph.D. thesis], HNB Garhwal University, Uttrakhand, India, 2012.
[19] N. Goel, N. Singh, and R. Saini, “Efficient in vitro multiplication of Syrian rue (Peganum harmala L.) using 6-benzylaminopurine pre-conditioned seedling explants,” Nature and Science, vol. 7, pp. 129–134, 2009.
[20] D. Lal and N. Singh, “Mass multiplication of Celastrus paniculatus Willd.—an important medicinal plant under in vitro conditions using nodal segments,” Journal of American Science, vol. 6, no. 7, pp. 55–61, 2010.
[21] D. Lal, N. Singh, and K. Yadav, “In vitro studies on Celastrus paniculatus,” Journal of Tropical Medicinal Plants, vol. 11, no. 2, pp. 169–174, 2010.
[22] K. Yadav and N. Singh, “In vitro propagation and biochemical analysis of field established wood apple (Aegle marmelos L.),” Analele Universității Din Oradea, vol. 18, no. 1, pp. 23–28, 2011.

[23] R. Scorza, “In vitro flowering,” Horticulture Review, vol. 4, pp. 106–127, 1982.

[24] H. B. Jumin and M. Ahmad, “High-frequency in vitro flowering of Murraya paniculata (L.) Jack,” Plant Cell Reports, vol. 18, no. 9, pp. 764–768, 1999.

[25] H. B. Jumin and N. Nito, “In vitro flowering of Fortunella hindsii (Champ.),” Plant Cell Reports, vol. 15, no. 7, pp. 484–488, 1996.

[26] Z. Zhang and D. W. M. Leung, “A comparison of in vitro with in vivo flowering in Gentian,” Plant Cell, Tissue and Organ Culture, vol. 63, no. 3, pp. 223–226, 2000.

[27] E. Galoch, J. Czaplewska, E. Burkacka-Laukaitys, and J. Kopcewicz, “Induction and stimulation of in vitro flowering of Pharbitis nil by cytokinin and gibberellin,” Plant Growth Regulation, vol. 37, no. 3, pp. 199–205, 2002.

[28] D. Pande, M. Purohit, and P. S. Srivastava, “Variation in xanthotoxin content in Ammi majus L. cultures during in vitro flowering and fruiting,” Plant Science, vol. 162, no. 4, pp. 583–587, 2002.

[29] S. Anitha and B. D. R. Kumari, “In vitro flowering in Rauvolfia tetraphylla L.,” Pakistan Journal of Biological Sciences, vol. 9, no. 3, pp. 422–424, 2006.

[30] S. Jana and G. S. Shekhawat, “Plant growth regulators, adenine sulfate and carbohydrates regulate organogenesis and in vitro flowering of Anethum graveolens,” Acta Physiologiae Plantarum, vol. 33, no. 2, pp. 305–311, 2011.

[31] K. V. Saritha and C. V. Naidu, “In vitro flowering of Withania somnifera Dunal.—an important antitumor medicinal plant,” Plant Science, vol. 172, no. 4, pp. 847–851, 2007.

[32] A. B. Mandal, A. Maiti, and R. Elanchezhian, “In vitro flowering in maize (Zea mays L.),” Asia-Pacific Journal of Molecular Biology and Biotechnology, vol. 8, no. 1, pp. 81–83, 2000.

[33] L. T. Evans, “Flower induction and the florigen concept,” Annual Review of Plant Physiology, vol. 22, pp. 365–394, 1971.

[34] N. K. Chrungoo and S. Farooq, “Influence of gibberellic acid and naphthaleneacetic acid on the yield of saffron and on growth in saffron crocus (Crocus sativus L.),” Journal of Plant Physiology, vol. 27, pp. 201–205, 1984.

[35] M. Roldán, C. Gómez-Mena, L. Ruiz-García, J. Salinas, and J. M. Martínez-Zapater, “Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of Arabidopsis in the dark,” Plant Journal, vol. 20, no. 5, pp. 581–590, 1999.

[36] C. P. Victório, R. M. Kuster, and C. L. S. Lage, “Light quality and production of photosynthetic pigments in in vitro plants of Phyllanthus tenellus Roxb.,” Brazilian Journal of Biosciences, vol. 5, pp. 213–215, 2007 (Portuguese).

[37] C. P. Victório, E. S. Tavares, and C. L. S. Lage, “Plant anatomy of Phyllanthus tenellus Roxb. cultured in vitro under different light qualities,” Brazilian Journal of Biosciences, vol. 5, pp. 216–218, 2007 (Portuguese).

[38] G. B. Kerbauy, Fisiologia Vegetal, Guanabara Koogan, Rio de Janeiro, Brazil, 2nd edition, 2008.

[39] G. Bernier and C. Péreilleux, “A physiological overview of the genetics of flowering time control,” Plant Biotechnology Journal, vol. 3, no. 1, pp. 3–16, 2005.