ISSN: 0976-1675

www.rjas.org

Research Journal of Agricultural Sciences

© Centre for Advanced Research in Agricultural Sciences

Research Paper

DI: 6109-0705-212

Efficient *In-vitro* Propagation of *Saussurea costus*

Sumeet Kour*, Seema Singh, Jasfeeda Qadir, Gowhar A Shapoo and Zahoor Ahmad Kaloo

Department of Botany,

University of Kashmir, Hazratbal - 190 006, Srinagar, Jammu and Kashmir

Received: 07 May 2020; Revised accepted: 12 July 2020

A B S T R A C T

Saussurea costus (Falc.) Lipsch., is an imperative medicinal plant species growing in Kashmir Himalaya. *S. costus* is used in the treatment of countless infirmities. The underground portion of this plant species encloses various sesquiterpene lacones such as costunolide, dehydrocostus lactone and many others which can be developed as bioactive molecules. It is enlisted in Appendix Ist of Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). During the present study, different explants viz. leaf, petiole, pedicel, etc. of *S. costus,* were subjected to *in-vitro* studies so as to develop proficient protocols for its regeneration. In addition to this, seed germination was also achieved by giving different treatments. Leaf explants produced maximum amount of callus on Murashige and Skoog's (MS) medium supplemented with 6-Benzylamino purine (BAP) (2.5 mg/l) in 90% cultures within 14 days. MS medium containing BAP (2.5 mg/l) was the most effective medium for shoot regeneration. Petiole and pedicel also showed callus formation and shoot regeneration but number of days taken was more and percent culture response was less. The *in vitro* regenerated shoots from leaf explants also develop roots on MS basal medium within a period of 15 days.

Key words: *Saussurea costus*, Callus, Explants, *In-vitro* propagation, Seed germination, Threatened

Saussurea costus commonly known as Kuth (Kritikar and Basu 2001) is a robust perennial herb1-2.5m tall. Leaves of *S. costus* are unevenly toothed. Basal leaves are very large possessing a long-winged petiole, upper leaves are smaller, subsessile, shortly petioled. Rhizomes are firm measuring 40-45cms in length and dark brown in colour. Flower heads are bluish purple to almost black coloured, rounded and often assembled together in the axils of leaves (Pandey *et al.* 2007) (Fig 1-A,B). Sesquiterpene lactones have been described to be the major elements of *Saussurea costus*. Costunolide, dehydrocostus lactone and cyanopicrin isolated from this plant species have been recognized to have potential to be established as bioactive molecules (Yang *et al*. 1997). It is one of the most important medicinal plant species well-known for anti-microbial, anti-ulcer, anti-inflammatory, anti-cancer and anti-hepatotoxic properties (Madhuri *et al*. 2012). It is effective against common cold, cough, fever, asthma, diarrhea, skin diseases, heart diseases and nervous disorders (Zahara *et al.* 2014). *S. costus* is also used as a promising traditional herbal medicine for oral cancer therapy. The methanolic extract impedes cell growth and induces apoptosis in KB human oral cancer cells (sung *et al.* 2013). De hydrocostus lactone and costunolide obtained from the underground portion of *S. costus* revealed strong larvicidal activity against mosquito *Aedes albopictis* (Liu *et al*. 2012).

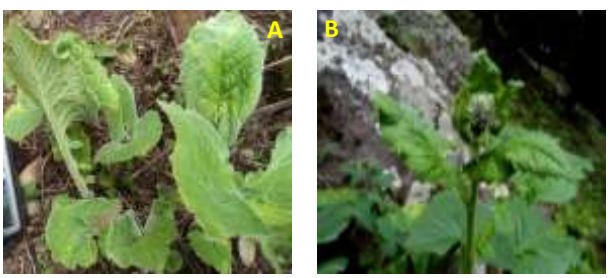

Fig 1(A, B) Morphology of *Sassurea costus* (Pic: G. A. Shapoo)

In India this plant is endemic to sub alpine regions of Jammu and Kashmir, Himachal Pradesh and Uttaranchal,

***Corresponding author:** Sumeet Kour, Department of Botany, University of Kashmir, Hazratbal - 190 006, Srinagar, Jammu and Kashmir

e-mail: sumeetkour07@gmail.com

from an altitude of 2600-4000 m asl (Shah 2006). This plant propagates mainly by seeds and also by rhizomes (Kour *et al.* 2016). Owing to its far-ranging use, it is uprooted from wild resulting it to fall in the IUCN category of Critically Endangered species. Moreover, high mortality rate of seedlings occurs due to chilling conditions in early stages added a constraint in propagation through seeds. Thus, *in-vitro* propagation is an efficient method for conservation of *S. costus.* Unabated as the plant extraction continues to be, far are not days when this economically important species will be extinct. It is indeed a need of the hour for this species which calls for the rescue of whatever is left that and as such the present study for its conservation was carried out.

MATERIALS AND METHODS

Surveys were conducted in different parts of Kashmir Himalaya. Plant species was collected from Forest block of Mahadev peak of Dhara, Srinagar and was subsequently transplanted at the Kashmir University Botanical Garden (KUBG). Herbarium specimen was processed and deposited at Kashmir University Herbarium (KASH) Voucher Specimen Number 2317 [Ref. No. F1 (Specimen. voucher. CBT) KU/2017].

Various explants such as leaf, petiole, pedicel were obtained from transplanted plants at KUBG. They were first thoroughly washed under running tap water in order to remove dirt and dust followed by washing with labolene (1% v/v) and surfactant tween-20 (1%v/v). The detergent was removed by washing the explants with double distilled water. Then they were treated under Laminar Air Flow Hood with chemical sterilant 2% or 4% sodium hypochlorite for 8-10 min. This was followed by washing with autoclaved double distilled water and finally inoculation was carried out on sterilized nutrient medium. Murashige and Skoog's (Murashiage and Skoog 1962) medium, gelled with 8% agar, supplemented with different concentrations of auxins and cytokinins both individually and in combination were used. Auxins like 2, 4- D; IAA; NAA; IBA and cytokinins like BAP and Kn were used in concentration range of 0.1-5 mg/l. The pH of the media was adjusted to 5.8 before autoclaving at 121°C and 15 lbs pressure. The cultures were incubated at 22±4°C.

RESULTS AND DISCUSSION

In-vitro response from leaf explants

Callus production: The leaf explants produced callus on MS medium supplemented with BAP at a concentration of 2.5mg/l, BAP 4mg/l, NAA1.5 mg/l, NAA 2.5mg/l, BAP (2.5mg/l) + NAA (0.5mg/l) and BAP (2.5mg/l) + IBA (0.5mg/l). MS medium containing BAP 2.5 mg/l was found to be more effective in producing hard and green coloured callus in 90% cultures within 14 days (Fig 2, Table 1).

a) BAP (2.5mg/l) b) BAP (4mg/l) c) NAA (1.5mg/l) d) NAA (2.5mg/l) e) BAP (2.5mg/l) + NAA (0.5mg/l) f) BAP (2.5mg/l) + IBA (0.5mg/l)

Fig 2 Callus production from leaf explant on MS medium containing

Table 1 Effect of different treatments on callus production from leaf explant

Treatments	No. of days for callus regeneration	Texture and colour of callus	Percent culture response
MS Basal	-	-	-
MS + BAP (2.5mg/l)	14	Hard and yellowish green	90
MS + BAP(4mg/l)	21	Hard and light green	80
MS + NAA(1.5mg/l)	62	Hard and light green	40
MS + NAA(2.5mg/l)	58	Hard and green	40
MS + BAP(2.5mg/l) + N AA(0.5mg/l)	29	Hard and red	70
MS + BAP(2.5mg/l) + I BA (0.5mg/l)	46	Hard and green	20

N= 30 Replicates per treatments

Table 2 Effect of different treatments on shoot regeneration from leaf explant

Treatments	Mean No. of shoots	Mean length of shoots	No. of days	Percent culture response
MS Basal	-	-	-	-
MS + BAP (2.5mg/l)	6.2±0.33	5.4±0.83	20	100
MS + Kn (1mg/l)	2.8±0.43	4.1±0.33	33	30
MS + BAP (2.5mg/l) + NAA (0.5mg/l)	4.03±0.49	2.5±0.33	20	70

N= 30 Replicates per treatments

Shoot regeneration

Leaf callus when sub cultured on MS medium supplemented with BAP (2.5mg/l) produced shoots with a mean length of 5.4±0.83cm in 100% cultures within 20 days. MS medium fortified with a combination of BAP (2.5mg/l) and NAA (0.5mg/l) also produced shoots with a mean length of 2.5±0.33cm in 70% cultures within 20 days (Fig 3, Table 2).

a) BAP (2.5mg/l) b) Kn (1mg/l) c) BAP (2.5mg/l) +NAA (0.5mg/l)

Fig 3 Shoot regeneration from leaf callus on MS medium containing

a) BAP (2.5mg/l) b) BAP (2.5mg/l) + NAA (0.5mg/l) c) 2,4-D (1mg/l) d) BAP (4mg/l)

Fig 4 Callus production from petiole explant on MS medium containing

In-vitro response from petiole explant

Callus production

Callus was induced from petiole explants when MS medium was supplemented with BAP (2.5mg/l) and BAP (2.5mg/l) + NAA (0.5mg/l). The callus was hard and creamish in colour and was obtained in 90% and 80% cultures within 15 and 27 days respectively of inoculation (Fig 4, Table 3).

Table 3 Effect of different treatments on callus production from petiole explant

Treatments	No. of days	Texture and colour	Percent culture response
MS Basal	-	-	-
MS + BAP (2.5 mg/l)	15	Hard and light green	90
MS + BAP (2.5 mg/l) + NAA (0.5 mg/l)	27	Hard and creamish	80
MS + 2,4D (1 mg/l)	25	Hard and green	70
MS + BAP (4 mg/l)	30	Hard and creamish	40

N= 30 Replicates per treatments

Shoot regeneration

Petiole callus when sub cultured on MS medium supplemented with BAP (2.5mg/l) regenerated shoots with a mean shoot length of 2.5±0.25cm with maximum number of shoots in 80% cultures within 29 days. However, shoot length was found to be more i.e. 2.9 cms on MS medium supplemented with BAP (2.5mg/l) + NAA (0.5mg/l) (Fig 5, Table 4).

Table 4 Effect of different treatments on shoot regeneration from petiole explant

Treatments	Mean no. of shoots	Mean length of shoots	No. of days	Percent culture response
MS Basal	-	-	-	-
MS + BAP (2.5 mg/l)	3.5 ± 0.56	2.5 ± 0.25	29	80
MS + BAP (2.5 mg/l) + NAA (0.5 mg/l)	3.0 ± 0.43	2.9 ± 0.38	40	40

N= 30 Replicates per treatments

a) BAP (2.5mg/l)

b) BAP (2.5mg/l) +NAA (0.5mg/l)

Fig 5 Shoot regeneration from petiole callus on MS medium containing

a) BAP (2.5mg/l)

b) BAP (2.5mg/l) + NAA (0.5mg/l)

c) BAP (3mg/l) + IAA (2mg/l)

Fig 6 Callus production from pedicel explant on MS medium containing

In-vitro response from pedicel explants

Callus production

MS medium fortified with BAP (2.5mg/l) and BAP (3mg/l) + IAA (2mg/l) proved effective in producing hard callus in 80% and 60% cultures. The callus produced was greenish in colour in case of BAP (2.5mg/l) and whitish in MS medium supplemented with BAP (3mg/l) + IAA (2mg/l) (Fig 6, Table 5).

Table 5 Effect of different treatments on callus production from pedicel

Treatments	No. of days	Texture and color	Percent culture response
MS Basal	-	-	-
MS + BAP (2.5 mg/l)	25	Hard and pale yellow	80
MS + BAP (3 mg/l) + IAA (2 mg/l)	40	Hard and whitish	60
MS + BAP (2.5 mg/l) + NAA (0.5 mg/l)	30	Hard and creamish	60

N= 30 Replicates per treatments

Table 6 Effect of different treatments on shoot regeneration from pedicel explant

Treatments	Mean no. of shoots	Mean length of shoots	No. of days	Percent culture response
MS Basal		-	-	-
MS + BAP (2.5 mg/l) + NAA (0.5 mg/l)	4.4±0.9	1.5±0.3	40	40
MS + Kn (1 mg/l)	-	-	-	-
MS + BAP (2.5 mg/l) + NAA (0.5 mg/l)	-	-	-	-

N= 30 Replicates per treatments

Shoot regeneration

Pedicel callus when sub cultured on MS medium supplemented with BAP (2.5mg/l) + NAA (0.5mg/l) regenerated shoots with a mean shoot length of 1.5±0.3cmin 40% cultures within 40 days (Fig 7, Table 6). From the above observations it is evident that leaves proved be the elite explants in terms of mean of shoots and number of days taken for shoot regeneration (Table 7).

Table 7 Elite explant in terms of callus formation and shoot regeneration

Explant	No. of days for callusing	Percent culture response	No. of days for shoot regeneration	Mean no. of shoots	Percent culture response
Leaf	14	90	20	6.2±0.33	100
Petiole	15	90	29	3.5±0.56	80
Pedicel	25	80	40	4.4±0.9	40

Fig 7 Shoot regeneration from pedicel callus on MS medium fortified with BAP (2.5mg/l) +NAA (0.5mg/l

Seed germination

Seeds were inoculated on MS basal medium and on cotton containing autoclaved double distilled water. The seeds showed best response on cotton wetted with autoclaved double distilled water. Seeds were also treated with different concentrations of GA_3. The seeds showed synchronous germination and there was great increase on the germination percentage and the number of days taken for germination was less within a week (Fig 9, Table 8).

Table 8 Effect of different treatments for seed germination

Treatments	Mean no. of days for seed germination	Percent response
GA_3(150ppm)	22	80
GA_3(250ppm)	20	70
GA_3(300ppm)	20	70
GA_3(500ppm)	15	90
Vermiculite	20	100
MS Basal	No response	No response

N= 10 replicates per treatment

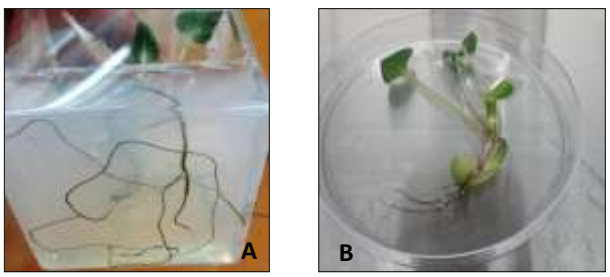

Fig 8 (A, B) Rooting of *in-vitro* regenerated shoots on MS basal medium

Fig 9 a) Seed germination on sterilized cotton wetted with autoclaved double distilled water, b) Seed germination in Vermiculite under greenhouse conditions

Various explants viz. leaf, petiole and pedicel responded differently in terms of callus production and shoot regeneration in response to various PGR's added to the MS medium individually and in combination at different concentrations. Leaf explants produced maximum callus when inoculated on MS medium supplemented with BAP (2.5mg/l) in 90% cultures within an average of 14 days. However, callus was also obtained when MS medium was fortified with BAP (4.0mg/l), NAA (2.5mg/l) and a combination of BAP 2.5mg/l + NAA 0.5mg/l and BAP 2.5mg/l + IBA 0.5mg/l. The amount of callus and percent culture response was less. The results are in accord with the results obtained from leaf explants of *Onobrychis sativa* (Mohajer *et al.* 2012). However maximum callus production was achieved on MS medium supplemented with BAP (2.5mg/l) and NAA (0.5 mg/l). It has been found that petiole explants produced maximum callus in 90% cultures when MS medium was supplemented with BAP (2.5 mg/l) within time duration of 15 days. Callus differentiation in petiole explants was also initiated on MS medium containing BAP (2.5 mg/l) + NAA (0.5 mg/l); BAP (4mg/l); and 2-4 D(1mg/l), but the amount of callus produced and percent culture response was less in these cases. Callus was also obtained from petiole explants of *Amorphophallus albus* on MS medium fortified with BAP (0.9mg/l) + NAA (0.9mg/l) (Jainbin *et al.* 2008). During the present study callus was also obtained from pedicel explants by adding various growth hormones (BAP, Kn, IAA, NAA, 2, 4-D) in varying combinations to MS and MS medium was supplemented with BAP (2.5mg/l). MS medium containing BAP (2.5mg/l) + IAA(2mg/l) was also effective in inducing differentiation of callus but the time taken was comparatively more and amount of callus produced along with percent culture response was less. The results are in accord with those of Karakas and Turker who obtained callus on pedicel explants of *Bellis perennis* L. in MS media containing 0.5 mg/l TDZ and 0.5 mg /l IAA (Karakas and Turker 2013). Shoot regeneration was achieved on callus produced from leaf and petiole and pedicel explants. A maximum mean number of shoots 6.2±0.33 with mean shoot length 5.4±0.83 were produced in 100% cultures within 20 days. Shoots also regenerated when leaf callus was sub-cultured on MS medium containing BAP (2.5mg/l) + NAA (0.5mg/l) and Kn (1mg/l). Number of regenerated shoots (2.8±0.43 and 4.03±0.49 and with 2.5±0.33cm and 4.1±0.33cm mean shoot length) and percent culture response (30% and 70%). Petiole callus initiated on MS medium containing BAP (2.5mg/l) and regenerated shoots in 29days but mean number of shoots 3.5±0.56 with 2.5±0.25cm mean shoot length and percent culture response (80%) were less as compared to

leaf regenerated callus. Shoots also regenerated when petiole callus was sub-cultured on MS medium containing BAP (2.5mg/l) + NAA (0.5mg/l). Number of regenerated shoots was 3.0±0.43 with 2.9±0.38 cm mean shoot length and40% percent culture response. Pedicel regenerated 4.4±0.9 mean number of shoots with 1.5±0.3cm mean shoot length from callus initiated on MS medium supplemented with BAP (2.5mg/l) + NAA (0.5mg/l). Winand *et al.* (1986) reported that BAP in combination with NAA is suitable for multiple shoot formation. Similarly, shoot regeneration was achieved in *Dittrichia viscose* (L.) W. Greuter (sin. *Inula viscose* (L.) Aiton); *Inula racemosa* Hook. F and *Inula verbascifolia* (Willd.) on MS medium supplemented with BAP (Romano 1997, Jabeen *et al.* 2007, Perica *et al.* (2008).

Saussurea costus, a critically endangered medicinal plant species growing in Kashmir Himalaya can be rescued from extinction by subjecting to *in-vitro* conservation stratergies. Growth regulators viz. auxins (IAA, IBA, NAA and 2, 4-D) and cytokinins (6-Benzylamino purine and Kn) were found to be responsible for inducing cellular totipotency in mature and differentiated tissues of leaf, petiole and pedicel which were used as explants. Most responsive explant for callus production and subsequent shoot regeneration was found to be leaf explant. For leaf explants, MS medium supplemented with BAP (2.5 mg/l) was found to be the most effective medium for callus initiation and MS medium supplemented with BAP (2.5 mg/l) was effective medium for shoot regeneration. For petiole explants, MS medium supplemented with BAP (2.5 mg/l) and BAP (2.5 mg/l) + NAA (0.5 mg/l) was effective for callus initiation. MS medium fortified with BAP (2.5 mg/l) was effective medium for shoot regeneration. For pedicel explants, most effective medium for callusing and shoot regeneration was BAP (2.5 mg/l) and BAP (2.5 mg/l) + NAA (0.5 mg/l) respectively. Leaves proved be the best explants in terms of mean of shoots and number of days taken for shoot regeneration. The *in-vitro* regenerated shoots from leaf explants develop roots on MS basal medium within a period of 15 days. It can be concluded that for *ex-situ* conservation of this prized plant species these *in-vitro* propagation techniques will prove helpful in rescuing this plant from extinction.

LITERATURE CITED

Jabeen N, Shawl A S and Dar G H. 2007. Micropropagation of *Inula racemosa* Hook.f. - A valuable medicinal plant. *International Journal of Botany* **3**: 296-301.

Jianbin H U, Xiaoxi G, Jun L I U, Conghua X I E and Jianwu L I. 2008. Plant regeneration from petiole callus of *Amorphophallus albus* and analysis of somaclonal variation of regenerated plants by RAPD and ISSR markers. *Botanical Studies* **49**: 189-197.

Karakas F P and Turker A U. 2013. An efficient *in vitro* regeneration system for *Bellis perennis* L. & comparison of phenolic contents of field-grown and *in-vitro* grown leaves by LC-MS/MS. *Industrial Crops and Products* **48**: 162-170.

Kour S, Singh S, Kaloo Z A and Shapoo G A. 2016. Effective *ex-situ* conservation strategies of *Saussurea costus* (Falc.) Lipsch an endangered medicinal plant of Kashmir Himalaya. *Saussurea* **6**(5): 342-349.

Kritikar K R and Basu B D. 2001. *Indian Medicinal Plants*. 6 Second Edition, Oriental Enterprises. pp 1961-1965.

Liu Z L, He Q, Chu S S, Wang C F, Du S S and Deng Z W. 2012. Essential oil composition and larvicidal activity of *Saussurea lappa* roots against the mosquito *Aedesalbo pictus* (Diptera: Culicidae). *Parasitol Research* **110**: 2125-2130.

Madhuri K, Elango K and Ponnusankar S. 2012. *Saussurea lappa* (Kuth root): review of its traditional uses, phytochemistry and pharmacology. *Oriental Pharmacy and Experimental Medicine* **12**: 1-9.

Mohajer S, Taha R M, Khorsana A and Yacob J S. 2012. Induction of different types of callus and somatic embryogenesis in various explants of Sainfoin (*Onobrychis sativa*). *Australian Journal of Crop Science* **6**: 1305-1313.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiology Plant* **15**: 473-497.

Pandey M M, Rastogi S and Rawat A K S. 2007. *Saussurea costus*: Botanical, chemical and pharmacological review of an ayurvedic medicinal plant. *Journal of Ethnopharmacology* **110**: 379-390.

Perica M C, Vrsek I and Mitic B. 2008. *In-vitro* propagation of *Inula verbascifolia* (Willd.) Hausskn. sub species *verbascifolia. Plant Biosystems* **142**: 1-4.

Romano A. 1997. Micropropagation of *Dittrichia viscosa* (L.) W. Greuter (sin. *Inula Viscosa* (L.) Aiton). ISHS *Acta Horticulturae* **502**: II *WOCMAP Congress.*

Shah R. 2006. *Natures Medicinal Plants of Uttaranchal*: (*Herbs, Grasses and Ferns*). Vol. 1/2. Gyanodaya Prakashan, Nanital, Uttarakhand, India.

Sung M M, Sang J Y, Joong K K, Heung J K, Mi S C, Bo R P, Su G K, Byung O K, Sook Y L, Hong A, Hong S C, Do K K and Chun S K. 2013. Anticancer activity of *Saussurea lappa* extract by apoptotic pathway in KB human oral cancer cells. *Pharmaceutical Biology* **51**(11): 1372-1377.

Winand C H, Ramaut J L and Gasper T H. 1986. Multiplication vegetative par culture *in-vitro* d, *Arnica Montana* L. *Journal De Pharmacie De Belgique* **41**: 147-150.

Yang H, Xie J and Sun H. 1997. Study on chemical constituents of *Saussurea lappa*. *Acta Botanica Yunnanica* **19**: 92-96.

Zahara K, Tabassum S, Sabir S, Arshad M, Qureshi R, Amjad M S and Chaudhari S K. 2014. A review of therapeutic potential of *Saussurea lappa*-An endangered plant from Himalaya. *Asian Pacific Journal of Tropical Medicine* **7**(5): 60-69.