ACTIONS FOR *EX-SITU* CONSERVATION OF *Saussurea lappa* (DECNE.) CLARKE

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Abstract. *Saussurea lappa* (Decne.) Clarke (Asteraceae) is a well-known traditional medicinal herb in India and is enlisted in Appendix – I of CITES (Endangered Plant List). An efficient regeneration protocol through indirect organogenesis from leaf explants of *S. lappa* has been developed. Mature and cotyledonary leaf explants were inoculated on Murashige and Skoog (MS) culture medium containing 3% (w/v) sucrose and 0.8% (w/v) agar supplemented with various concentrations of auxins and cytokinins individually or in different combinations. Maximum callus induction (100%) with best visual growth was obtained on MS medium supplemented with 2,4-D (2.0 mg/L) and BAP (2.0 mg/L). The appearance of the callus varied depending upon the combinations and concentrations of plant growth regulators (PGRs) used. The highest shoot regeneration percentage (66.66%) with maximum number of shoots (4.9 shoots/explant) were obtained on MS medium containing BAP (3.0 mg/L) and NAA (0.10 mg/L). MS half strength media with NAA (0.5 mg/L) resulted in highest rooting percentage (85%) followed by IAA (0.5 mg/L). After acclimatization, plantlets grew normally under greenhouse and field conditions. This protocol could be used for rapid mass production, germplasm conservation and provides a basis for genetic improvement of *S. lappa.*

Keywords: *Saussurea lappa*; endangered; *in vitro*; indirect regeneration

INTRODUCTION

Saussurea lappa (Decne.) Clarke (family Asteraceae) commonly known as Kuth or Costus is an endemic perennial medicinal plant distributed in western Himalayas and neighbouring valleys at altitudes of 2500-3000 m [22]. It is a critically endangered medicinal plant species enlisted in Appendix – I of CITES (Endangered plant list) [14]. The roots of this plant contain an essential oil known as costus oil. The important constituents of the costus oil include saussurine, resinoids, inulin, essential oil and other alkaloids [31].

The species has found applications in pharmaceutical industry to treat more than forty-three diseases [37]. Diverse medicinal properties of *S. lappa* for example anti-inflammatory, suppress hepatitis B, anticancerous, antibacterial, antihepatotoxic, anti-arthritic, antiviral, antifungal, antiproliferative and antioxidant have attracted entrepreneurs to set eyes on this plant [7]. Its root extract is used as tonic useful in skin disease, epilepsy, paralysis, vermicide, diuretic, rheumatism, bronchitis, nervous disorders, brain stimulant, irregular menstruation, heart diseases, carminative, aphrodisiac, antihelminthic, treating deaf and ophthalmic condition [6, 22]. Because of its diverse medicinal properties, the species was overexploited by local population and pharmaceutical companies for the preparation of various valuable medicines. Large-scale uprooting from wild is the major factor threatening its existence. The conventional propagation of this plant is through root cuttings which is a commercial valuable part of the plant. The percentage of seed germination is very poor [11].

Due to excessive and destructive exploitation, it is getting fast depleted. The modern biotechnological techniques have come as a boon creating new dimensions in the field of agriculture for getting modern product with high yield and at faster rate [36]. The present investigation porpose an efficient protocol for indirect organogenesis of *S. lappa* by manipulating growth regulators on leaf explants. Also, the effect of subculture frequencies on *S. lappa* callus development is described.

MATERIALS AND METHODS

Plant material and sterilization. Healthy seeds of *S. lappa* were procured from Lahul valley of Himachal Pradesh (India). Healthy plants of *S. lappa* plants were collected from Forest Department, Jammu & Kashmir (India) and maintained in pots in the green house of Department of Botany, Kurukshetra University, Haryana (India). Surface sterilized seeds were inoculated on half strength MS medium [18] to raise seedlings for production of cotyledonary leaf [7]. Cotyledonary leafs excised from aseptically raised seedlings and mature leaf segments were used as explants. Explants were surface sterilized by washing with Teepol solution (5%) followed by washing under running tap water for fifteen minutes to remove the adhering dust particles. Thereafter, the explants were disinfected using 0.1 % (w/v) of mercuric chloride for 3 minutes followed by a brief rinse with 70% ethanol. These disinfected explants were then thoroughly washed with sterilized double distilled water to remove the traces of mercuric chloride.

Culture media and *in vitro* culture induction. The surfaces sterilized explants were trimmed into small pieces (0.8 X 1.0 cm) and inoculated on MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, India) supplemented with auxins (2,4-D, NAA) and cytokinin (BAP) in various concentrations of for callus induction and mass multiplication. The pH of the medium was adjusted to 5.8 prior and autoclaved at 1.5 kg cm^{-2} at 121°C for 20 min. The cultures were incubated under 16 hours

photoperiod with a photon flux density (PFD) of 40 µmol $m^{-2}s^{-1}$ at 25 ± 2°C and 70% humidity. After 4 weeks of inoculation, the efficiency of plant growth regulators (PGRs) was determined by recording the time required for callus induction, percent callus induction, colour and texture of callus. After 40 days of callus induction, both cotyledonary leaf and mature leaf explants callus were again subcultured (i.e. the 1st, 2nd & 3rd cycle) on their respective medium for callus multiplication and finally a mass of calli was harvested.

For shoot regeneration, the best *in vitro* regenerated callus from 3rd successive sub culture were excised aseptically and transferred to various concentrations (0.5- 3.0 mg/L) of BAP alone and in combination with IAA, NAA and 2,4-D.

Root development and ex-vitro acclimatization. In this study, we have used the *in vitro* rooting protocol established by us in our earlier research [7]. For root induction, the regenerated shoots (2.5-3.0 cm) were excised and cultured on either full or half strength MS media supplemented with (0.5- 2.0 mg/L) of IAA or NAA. The well rooted plantlets were gently washed under running tap water to remove the adhering agar with minimum injury. These plants were acclimatized and hardened in autoclaved sand: soil ratio (3:1). The plantlets were covered with glass jars to ensure humidity. They were supplied with half strength MS salt solution on alternate days. In third week, glass jars were removed for 3-4 hrs daily to expose the plants to the field conditions. After 4 weeks, these plants were transferred to bigger pots and were maintained under greenhouse for acclimatization.

Data analysis. All the experiments were repeated thrice with a minimum of twenty replicates per treatment. The statistical calculations were all carried out using SPSS (version11.5) statistical software. The difference between means was analyzed by one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test at $P \leq 0.05$.

RESULTS

Callus Induction. The MS medium without PGRs did not show callus induction. The effects of different explants and the interactions between different plant growth regulators on callogenesis are presented in Tables 1 and 2. A wide range of variations in percent callus induction, growth and nature of callus were observed depending on concentrations and combinations of PGRs used.

It was noticed that callus proliferation usually started from the cut surface of the explant used and finally covered the whole explant. Significant differences in the percent callus induction and friability were observed among the different explants used (Table 1; Fig. 1a-b). Mature leaf explants showed great ability to induce callus, followed by cotyledonary leaf explants. In case of mature leaf explants, MS media supplemented with 2.0 mg/L of 2,4-D + 2.0 mg/L of BAP recorded the highest (86.66%) per cent callus induction in comparison among all the tested combinations (Table 1). Glassy white soft friable appearance of callus was observed in mature leaf explant on MS media supplemented with 2.0 mg/L of 2,4-D alone and 2.0 mg/L of 2,4-D + 2.0 mg/L of BAP, while creamish brown friable appearance of callus was observed on MS media supplemented with 2.0 mg/L of BAP (Fig. 1a). Among the various treatments tested with cotyledonary leaf explant, maximum percent callus response was observed on MS media supplemented with 2.0 mg/L of 2,4-D + 0.5 mg/L of BAP (Table 1). The different appearance of the callus was observed on all the treatments of cotyledonary leaf explant. The cotyledonary leaf callus on MS media supplemented with NAA (2.0 mg/L) recorded creamish white friable soft, BAP (0.5 mg/L) recorded whitish green compact, NAA (2.0 mg/L) + BAP (0.5 mg/L) recorded whitish green friable, 2,4-D (2.0 mg/L) recorded creamish white friable soft, 2,4-D (2.0 mg/L) + BAP (0.5 mg/L) recorded glassy white soft, while 2,4-D (2.0 mg/L) + BAP (0.5 mg/L) + NAA (2.0 mg/L) recorded glassy brown soft appearance of callus. Differentiation was not observed in any of the tested combinations of Table 1.

After 40 days of the 3rd subculture cycle, the best visual growth of callus with highest (96.66%) percent callus proliferation was recorded on MS media supplemented with 2.0 mg/L of 2,4-D + 2.0 mg/L of BAP from mature leaf callus (Table 2; Fig. 1c-d). This medium also produced shoot buds along with dark green friable callus. MS medium with 2.0 mg/L of BAP also produced creamish green brown friable appearance of callus with the appearance of shoot buds. In case of cotyledonary leaf callus, the per cent culture response between 67.77% and 71.11% was observed from medium with 2,4-D (2.0 mg/L) alone and with combination with BAP + NAA, which was comparatively lower in comparison to callus formation on the same medium from mature leaf callus. Therefore, was not tested for further shoot regeneration.

Shoot regeneration. The *in vitro* regenerated from the 3rd successive subculture of mature leaf segments on MS medium supplemented with 2.0 mg/L of 2,4-D + 2.0 mg/L of BAP were excised transferred to MS medium supplemented with various concentrations of BAP alone and with various combinations with NAA, 2,4-D and IAA for shoot induction (Table 3). MS medium devoid of any plant growth regulator did not show any response. MS medium supplemented with various concentrations (0.5 - 2.0 mg/L) of BAP resulted in the production of shoots with regeneration of green and compact appearance of callus (Fig. 1e). Among the various concentrations of BAP tested alone, MS medium supplemented with 2.0 mg/L of BAP recorded higher percent response with the production of 4 shoots per culture (Table 3). An increased visual growth of callus was observed with an increase in the

concentration of BAP in medium. However, among the various treatments used, the highest percent culture response (66.66%) with very good visual growth of callus was recorded on MS medium supplemented with 3.0 mg/L of BAP + 0.10 mg/L of NAA with the production of 4.9 number of shoots per culture followed by BAP (2.0 mg/L) + NAA (0.25 mg/L) (Fig. 1f). The combinations BAP (2.0 mg/L) with IAA (0.5 mg/L) and NAA failed to produce shoots.

Root development. The MS medium devoid of any plant growth regulator failed to develop any roots in excised shoots (Table 4). The half strength MS medium supplemented with various concentrations of IAA and NAA resulted in best results over full strength. Half strength MS medium containing IAA (0.5 mg/L) recorded an average number of 3.2 roots with 76% rooting. An increase in the concentration of PGRs along with strength of MS medium resulted into the production of more callus comparatively (Table 4).

Table 1. Effect of different plant growth regulators on different types of explants after 40 days of culture incubation

Explant + conc. of growth regulator in MS medium (mg/L)	Percent culture responding	Appearance of callus	Visual growth of callus*	Differentiation
Mature leaf explant + 2,4-D (2.0)	80.00^{d}	Glassy White, Soft, Friable	+ + + + +	-
Mature leaf explant + BAP (2.0)	75.55^{e}	Creamish brown, Friable	+ + + +	-
Mature leaf explant + 2,4-D (2.0) + BAP (2.0)	86.66^{ab}	Glassy White, Soft, Friable	+ + + + +	-
Cotyledonary leaf + NAA (2.0)	69.99^{e}	Creamish white Friable Soft	+ + + +	-
Cotyledonary leaf + BAP (0.5)	69.99^{e}	Whitish Green, Compact	+ + + +	-
Cotyledonary leaf + BAP (0.5) + NAA (2.0)	80.00^{c}	Whitish Green, Friable	+ + + + +	-
Cotyledonary leaf + 2,4-D (2.0)	79.99^{d}	Creamish White, Friable, Soft	+ + + +	-
Cotyledonary leaf + 2,4-D (2.0) + BAP (0.5)	84.44^{bc}	Glassy White, Soft	+ + + + +	-
Cotyledonary leaf + 2,4-D (2.0) + BAP (0.5) + NAA (2.0)	83.33^{bcd}	Glassy Brown, Soft	+ + + + +	-

*- No Callus, + Poor Callus (less than 50% coverage), ++ Less Callus (50% to 75% coverage), +++ Moderate Callus (more than 75% coverage), ++++ Good Callus (100% coverage), +++++ Very Good Callus (100% coverage + overlap)

Table 2. Effect of different plant growth regulators on 3rd sub culturing of callus after 40 days

Explant + conc. of growth regulator in MS medium (mg/L)	Per cent culture responding	Appearance of callus	Visual growth of callus*	Differentiation
Mature leaf explant + 2,4-D (2.0)	90.00^{b}	Glassy White, Soft	+ + + +	-
Mature leaf explant + BAP (2.0)	88.88^{c}	Creamish Green Brown, Friable	+ + +	Shoot buds
Mature leaf explant + 2,4-D (2.0) + BAP (2.0)	96.66^{a}	Dark Green, Friable	+ + + + +	Shoot buds
Cotyledonary leaf + NAA (2.0)	69.99^{ef}	Glassy Brown, Soft	+ + +	-
Cotyledonary leaf + BAP (0.5)	68.88^{fg}	Creamish Brown, Friable	+ +	-
Cotyledonary leaf + BAP (0.5) + NAA (2.0)	73.33^{d}	Glassy Brown, Soft	+ + +	-
Cotyledonary leaf + 2,4-D (2.0)	67.77^{fgh}	Glassy Brown, Soft	+ + +	-
Cotyledonary leaf + 2,4-D (2.0) + BAP (0.5)	72.22^{de}	Glassy Brown, Soft	+ + +	-
Cotyledonary leaf + 2,4-D (2.0) + BAP (0.5) + NAA (2.0)	71.11^{de}	Glassy Brown, Soft	+ +	-

*- No Callus, + Poor Callus (less than 50% coverage), ++ Less Callus (50% to 75% coverage), +++ Moderate Callus (more than 75% coverage), ++++ Good Callus (100% coverage), +++++ Very Good Callus (100% coverage + overlap)

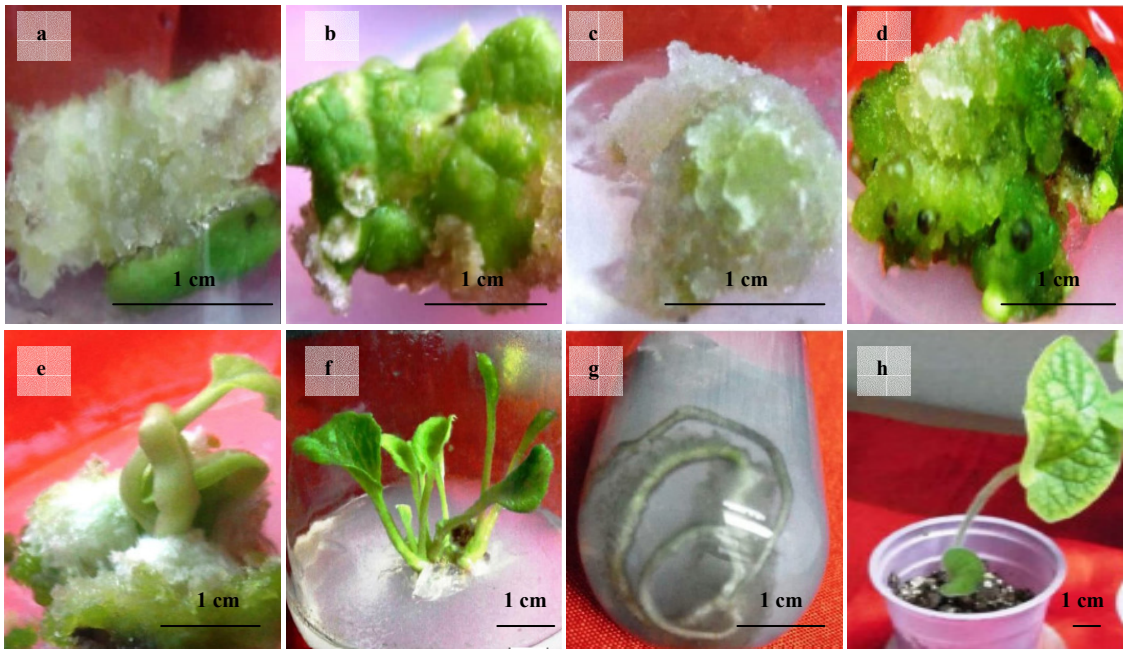

Figure 1. *In vitro* propagation of *S. lappa*: **(a)** Callus initiation from cotyledonary leaf explants on MS medium with 2,4-D (2.0 mg/L); **(b)** Callus initiation from mature leaf explants on MS medium with BAP (2.0 mg/L); **(c)** Callus proliferation from cotyledonary leaf explants after first subculture on MS medium + 2,4-D (2.0 mg/L) + BAP (0.5 mg/L); **(d)** Callus proliferation from mature leaf explants after third subculture on MS medium + BAP (2.0 mg/L) + 2,4-D (2.0 mg/L); **(e)** Callus induction along with shoot formation on MS medium with BAP (0.5 mg/L); **(f)** Indirect shoot regeneration from subcultured matured leaf callus on MS medium with BAP (3.0 mg/L) + NAA (0.10 mg/L); **(g)** Root initiation on full strength MS medium with IAA (2.0 mg/L); **(h)** Acclimatized plantlet.

Table 3. Effect of different plant growth regulators alone and in combination on shoot regeneration from callus derived from 3rd sub culture of mature leaf explant in medium containing 2,4-D (2.0 mg/L) + BAP (2.0 mg/L)

Medium and conc. of growth regulator in MS medium (mg/L)	Per cent culture response/ bud break	No. of days required for the bud break	Appearance of callus	Visual growth of callus*	No. of shoots per culture
MS Medium	-	-	-	-	--
MS+BAP (0.5)	40.00^{f}	75^{d}	Green, Compact	++	1^{d}
MS+BAP (2.0)	56.66^{d}	54^{b}	Green, Compact	+++++	4^{c}
MS +BAP (2.0) + NAA (0.5)	59.99^{c}	-	Whitish Greenish, Friable	+++++	-
MS +BAP (2.0) + IAA (0.5)	11.11^{g}	-	Whitish Greenish, Friable	+++++	-
MS +BAP (3.0) + 2,4-D (0.5)	84.44^{a}	-	Whitish Greenish, Friable	+++++	-
MS + BAP (2.0) + NAA (0.25)	47.77^{e}	61^{c}	Greenish, Compact	++++	4.6^{b}
MS + BAP (3.0) + NAA (0.10)	66.66^{b}	51^{a}	Greenish, Compact	++++	4.9^{a}

*- No Callus, + Poor Callus (less than 50% coverage), ++ Less Callus (50% to 75% coverage), +++ Moderate Callus (more than 75% coverage), ++++ Good Callus (100% coverage), +++++ Very Good Callus (100% coverage + overlap)

Table 4. Effect of half strength MS medium and full strength MS medium with or without various concentrations of NAA and IAA in root regeneration from excised shoots after 40 days of culture

Medium	Plant growth Regulator	Rooting (%)	Number of roots	Root morphology
MS half strength	-	-	-	-
MS full strength	-	-	-	-
MS half strength	0.5 mg/L IAA	76^{b}	3.2^{b}	Long ,Thin
MS full strength	2.0 mg/L IAA	70^{c}	1.9^{d}	Long ,Thick
MS half strength	0.5 mg/L NAA	85^{a}	6.4^{a}	Long, thin and very less callus formation [7]
MS full strength	2.0 mg/L NAA	20^{d}	2.0^{cd}	No rooting and Profuse callus formation

- (no response)

Full strength MS medium supplemented IAA (2.0 mg/L) resulted in 70 percent rooting with an average production of 1.9 long and thick roots. (Fig. 1g). Among auxins, both NAA and IAA proved to be the good root inducer, but IAA also induced roots formation without callus production. However, the highest number of roots was observed on culture medium with NAA (0.5 mg/L).

After 4 weeks, these plantlets were transferred to under natural field conditions of photoperiod and temperature. More than 60% of *in vitro* raised plantlets were characterized by well-developed leaves with roots and showed no morphological abnormalities (Fig. 1h).

DISCUSSION

Plant tissue culture offers a fast alternative to conventional propagation technique and it is used as a complementary strategy for the conservation and utilization of genetic resources [7, 12, 15]. Indirect regeneration involves the formation of callus. Callus is an undifferentiated mass of cells, obtained by culturing explants on nutrient medium [19, 20]. The MS medium devoid of any PGRs did not report callus induction. It may be due to the insufficient level of endogenous growth hormones in explants to induce callusing and requires an exogenous supply of PGRs to trigger cell division [8]. Under *in vitro* conditions, the application of cytokinins causes a decrease in cell wall lignification, facilitating callus initiation and growth [13]. It was noticed that callus proliferation usually started from the cut surface of the explant used and finally covered the whole explant. It may be due to accumulation of auxins at the point of injury, which stimulated cell proliferation in the presence of growth regulators [1]. Dhital *et al*. [3] also reported that leaf explants produced earlier callusogenesis with higher frequency of callus induction in potato cultivars. Contrary, Shirin *et al.* [27] emphasized internodal segments, as a best explant source for *in vitro* callus induction. The variation in the apperance of callus with different PGRs have also been noticed by Ehsandar *et al.* [4] and Kumlay & Ercisli [13] on *Solanum tuberosum.* It could be due to differences in endogenous growth regulators in the explants [16].

Among the cytokinins, BAP is the most frequently used hormone, which interacts actively with 2,4-D or NAA for callus initiation and its maintenance [21]. The optimal concentration of the PGRs depend on plant genotype and type of explants used [17]. Faisal & Anis [5] obtained higher frequency of callus formation on MS medium containing 2,4-D in *Tylophora indica*. 2,4-D is very ideal auxin to initiate callus and somatic embryogenesis in different medicinal plant species, whereas IAA, IBA, NAA could also be used as stimulants in a lesser extent [9, 25].

Dark green and compact calli have good regeneration ability for shoot proliferation [10]. The auxin and cytokinin ratio acts as an important factor in determining organogenesis in micropropagation experiments [32]. Different endogenous level of cytokinins and auxins along with exogenous plant hormones that exist in cultured medium combine together to induce organogenesis. BAP was more efficient for shoot induction due to its ability to induce and produce natural hormones such as zeatin within the tissue through natural hormone system [26]. The vital role of BAP for *in vitro* shoot induction from different explants of medicinal plant species is well documented [2]. Lower concentration of NAA in combination with BAP also facilitates better morphogenesis and enhanced the rate of shoot buds differentiation in *Cassia* sp. [23, 24].

The proliferation of callus depends on the media composition and optimization of subculture period for maintaining its growth in order to obtain higher callus

biomass with regenerative potency [17]. It is recommended to subculture a callus at every 4–6 weeks [18].

Many researchers have reported the effectiveness of half strength MS medium for root induction [30]. Effective *in vitro* rooting on regenerated shoots grown on MS medium supplemented with NAA or IAA has been well documented in *Aegle marmelos* [34], *Stevia rebaudiana* [28] and *Puya berteroniana* [30]. Reports on acclimatization and hardening were reported for *Glycyrrhiza glabra* [35], *Stevia rebudiana* [29], *Spilanthes acmella* [33] and *Simmondsia chinensis* [12].

The success of any micropropagation protocol depends finally on the establishment of regenerated plantlets under *ex-vitro* field conditions with higher survival rate. Plants raised under *in vitro* conditions are maintained under controlled environmental factors such as: constant temperature, low light intensity, high humidity, supplementary sugar supply and growth regulators. Sudden changes in environment condition cause low survival or reduced growth rate [36].

In conclusion, this experimental study has resulted in an expeditious indirect regeneration protocol from mature leaf explants may be highly useful for raising quality planting material for mass multiplication and conservation of *S. lappa*. It could also facilitate phytochemical production, genetic transformation and pharmacological studies of this potential medicinal plant.

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