

***In vitro* Regeneration of *Saussurea heteromalla* (D. Don.) Hand-Mazz Using Auxins and Current Status in Galiyat Abbottabad**

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Current Status of *Saussurea heteromalla* was investigated in Galiyat areas of District Abbottabad, viz., Jahaffar, Seri, Beeran Gali, Banj, Haryala, Daryala Gali, Sarbhanna, Barriyan, Akhreela and Broangiala. *Saussurea heteromalla* was found common in Seri, Sarbhanna and Barriyan; endangered in Haryala, Jahaffar, Banj, Daryala Gali and Beeran Gali and absent in Akhreela and Broangiala. *In vitro* regeneration of *Saussurea heteromalla* (D. Don.) Hand-Mazz on MS media was conducted and shoots were developed on full strength MS medium supplemented with 1 mgL⁻¹ GA₃. The developed shoots were transferred for root induction to half strength MS medium fortified with various concentrations of Indole butyric acid (IBA) and α -Naphthalene acetic acid (NAA) i.e. T1 (control), T2 (2 mgL⁻¹ IBA), T3 (3 mgL⁻¹ IBA), T4 (4 mgL⁻¹ IBA), T5 (1 mgL⁻¹ NAA), T6 (2 mgL⁻¹ NAA) and T7 (3 mgL⁻¹ NAA). Maximum mean shoot length (6.3 cm), mean number of leaves (7), mean number of nodes (5.25); highest root emergence percentage (71%), means root length (1.5 cm), mean number of roots (3) and highest survival rate (100%) was recorded in treatment T4. However, treatments T5 and T6 also seem to be effective for *in vitro* rooting of *Saussurea heteromalla*. Treatments T2, T3 and T7 showed minimum root growth. It was concluded that IBA at higher concentration is more effective for *in vitro* rooting and better shoot growth of *Saussurea heteromalla* whereas NAA also initiate rooting but at lower concentration.

Keywords: *Saussurea heteromalla*; Medicinal plants; *In vitro* regeneration; Current Status

Introduction

Saussurea heteromalla (D. Don.) Hand-Mazz is an endangered medicinal plant belongs to family Asteraceae (Compositae) locally known as Kali-Ziri. It is a tall perennial herb native to Western Himalayas, at altitudes of 550-4000m and found in open places. In Pakistan it is distributed in Baluchistan, Kurram, Punjab, Chitral, Swat, Hazara, Salt Range, Rawalpindi, Lahore, Murree and Pakistani Kashmir (Butola & Samant, 2010). The leaves, roots, seeds and whole plant is reported to have medicinal value and have been used in the treatment of various ailments of humans and livestock such as nervous breakdown, liver problems, hysteria, cardiac troubles, carminative, febrifuge, antidote against horse bite, vitiligo, wounds, fever, colic and skin diseases (Quattrocchi, 2012; Butola & Samant, 2010). Plant is reported to possess arctiin, arctigenin and chlorojanerin. Arctiin and arctigenin are reported to possess anti-inflammatory activity while chlorojanerin is known to have anti-ulcer and anti-viral properties (Saklani et al., 2011).

There are only few literature reported about Status of *Saussurea heteromalla* in Pakistan such as Qureshi et al., (2008) reported that *Saussurea heteromalla* was common in Abbottabad District; Butola & Samant, (2010) reported its Conservation Status was common overall in Pakistan; Khan et al., (2014) found its Status in Poonch Valley Azad Kashmir was endangered; Hussain et al., (2016) found it to be occasional in Rawalakot Azad Jammu and Kashmir (Pakistan) and Khalid & Shah (2016) reported its Conservation Status was rare in Mohmand Agency, (FATA).

Habitat destruction, overgrazing and overexploitation by local herbalists due to its wide range of medicinal value have threatened its abundance even occurrence/existence. It is therefore necessary to take immediate step for rapid mass multiplication of this medicinally important plant species through tissue culture techniques, in order to save as well as to maintain sustainable amount of plant derived drugs. Nowadays, Plant tissue culture techniques have been increasingly applied for rapid mass multiplication and conservation of rare, endangered and other economically important plants. The plants propagated through tissue culture techniques have been used for reintroduction to recover the populations of endangered plants in their wild habitats, thus it save plants from extinction (Sen & Sharma, 1991). *In vitro* regeneration of *Saussurea heteromalla* has not been done so far. Therefore the prime objective of present study was to develop a suitable lab protocol for successful *in vitro* rooting and growth of *Saussurea heteromalla* on MS medium supplemented with different concentrations of auxins. Beside this present research work was also conducted to determine the Current Status of *Saussurea heteromalla* in Galiyat areas of District Abbottabad by using IUCN Conservation Status Criteria via questionnaire.

Materials and Methods

The present study was carried out in Potato Tissue Culture Laboratory at Hazara Agriculture Research Station (HARS), Abbottabad in 2019.

Field trips and data enumeration

In order to determine the Current Status of *Saussurea heteromalla* in Galiyat areas of District Abbottabad, 10 areas were visited viz Jahaffar, Seri, Beeran Gali, Banj, Haryala, Daryala Gali, Sarbhanna, Barriyan, Akhreela and Broangiala. Different information about the Current status of plant was recorded via questionnaire from local inhabitants. Besides local inhabitant, information about the Current status of *Saussurea heteromalla* was also recorded by our own field visits and general observation technique. Fresh plants of *Saussurea heteromalla* were identified through identification key 'Flora of Pakistan' (Nazimuddin and Qaiser, 1983).

Preparation and sterilization of culture medium

Murashige & Skoog's (1962) basal medium was prepared by using 1 mgL⁻¹ Ca-pentothenate, 100 mgL⁻¹ myoinositol with 30g/L sucrose, 6 g/L agar and 1 mgL⁻¹ GA3. pH of the medium was adjusted to 5.8 by adding few drops of either 1N NaOH or 1N HCL solution prior to autoclaving. These test tubes were sterilized in an autoclave at 121°C and 15psi pressure for 15-20 minutes.

Surface sterilization and inoculation of explants

For *in vitro* study nodal segments explants (0.5 – 1.0 cm) were excised and thoroughly washed with tap water for 2-3 times. After that they were surface sterilized with 0.1% (w/v) Mercuric chloride (HgCl₂) solution for 1 minute followed by washing thrice with sterilized distilled water. Then nodal segments were treated with 0.1% (w/v) Antibiotic (Penicillin) solution for 1 minute followed by three times washing with sterilized distilled water. Finally explants were treated with 0.3% (w/v) Carbendazim solution for 2 minutes and then rinsed thrice with sterilized distilled water to remove final traces of sterilant prior to inoculation. In order to expose fresh tissues nodal segments containing axillary buds were trimmed at both ends and then inoculated aseptically on artificial nutrient medium in laminar flow cabinet. After inoculation the cultured test tubes were maintained at 20 ± 2°C in growth chamber for 16 hours light and 8 hours dark periods.

Rooting medium

For *in vitro* rooting MS Half Strength medium was prepared and divided into 7 portions. Each portion represent a treatment containing a different concentration of Indole butyric acid (IBA) and α-Naphthalene acetic acid (NAA); and labeled as T1 (0), T2 (2 mgL⁻¹ IBA), T3 (3 mgL⁻¹ IBA), T4 (4 mgL⁻¹ IBA), T5 (1 mgL⁻¹ NAA), T6 (2 mgL⁻¹ NAA) and T7 (3 mgL⁻¹ NAA). MS medium without auxins served as controlled (T1).

Data collection

Data were recorded after 1 week interval from each test tube. Data regarding root induction was observed for 3 weeks. The data parameters were Shoot emergence, Shoot length, Number of nodes, Number of leaves; Root emergence, Number of roots, Root Length; Survival and Death Rate.

Statistical analysis

All the experiments were carried out in a Complete Randomized Design (CRD) and the data were analyzed by using computer software Statistix 8.1. Means were compared by using Least Significance Difference (LSD) test at 5% probability level.

Results and Discussion

Current status of *Saussurea heteromalla*

Present research work demonstrated that *Saussurea heteromalla* was common in 3 localities i.e., Seri, Sarbhanna and Barriyan, the reasons for its abundance may be because of suitable soil, climatic and topographic conditions. In Haryala, Jahaffar, Banj, Daryala Gali and Beeran Gali it is recorded as being endangered (Table 1), this might be because of habitat destruction, overgrazing and to some extent overexploitation. In Akhreela and Broangiala, *Saussurea heteromalla* is absent; as both these localities are found at high altitude and also these are rocky areas. According to IUCN Red List Categories and Criteria, (2001) a species is said to be an endangered when its population has declined between 50% and 70% and cause of the decline may either be unknown or known. This definition fit into 5 localities of Galiyat, hence overall Status of *Saussurea heteromalla* in Galiyat is endangered.

Table 1. Current Status of *Saussurea heteromalla* in Lower Galiyat Areas.

S. No	Localities	Current Status (%)		
		Common	Endangered	Absent
1	Jahaffar	40 abc	60 a	0 b
2	Seri	80 a	40 a	0 b
3	Haryala	20 bc	80 a	0 b
4	Daryala Gali	20 bc	60 a	20 a
5	Banj	40 abc	60 a	0 b
6	Sarbhanna	80 a	20 a	0 b
7	Barriyan	60 ab	40 a	0 b
8	Beeran Gali	40 abc	60 a	0 b
9	Akhreela	0 c	20 a	80 a
10	Broangiala	20 bc	20 a	60 a

Means in a column followed by the same letters are not significantly different at P ≤ 0.05.

In vitro regeneration

For shoot regeneration, nodal segments were inoculated on MS basal medium supplemented with 1 mgL⁻¹ GA3. After 2 days of culturing shoot emergence were observed in all cultured segments (Figure 1). Data recorded after 1st week of culturing showed that mean shoot length was 2.31 cm with 2.89 mean numbers of leaves and 2.18 mean numbers of nodes (Figure 2). A significant increase was observed in mean shoot length (5.1 cm), mean numbers of leaves (5.25) and mean numbers of nodes (4.27) (Figure 3) after 2nd week of culturing. GA3 promote shoot elongation by stimulating cell division and elongation (Taiz & Zeiger, 2002).

Results showed that GA3 plays a vital role not only in shoot elongation but also significantly increased the number of nodes and leaves (Figure 3) which corroborates with the observation made by Rostami and Shahsava, (2012). George et al., (2008) reported that pre-treatment of plant material with GA3 enhances subsequent root formation on root-inducing medium.

Table 2. Rooting response after 1st, 2nd and 3rd week of Sub-culturing on rooting medium.

Treatments	Root Emergence %			Root Length (cm)			No of Roots		
	1 st week	2 nd week	3 rd week	1 st week	2 nd week	3 rd week	1 st week	2 nd week	3 rd week
T ₁	0 b	0 b	0 b	0 a	0 a	0 b	0 a	0 b	0 b
T ₂	14 ab	29 ab	29 ab	0.09 a	0.12 a	0.45 b	0.14 a	0.29 b	0.67 b
T ₃	14 ab	29 ab	43 ab	0.1 a	0.21 a	0.73 b	0.17 a	0.43 b	0.83 b
T ₄	57 a	57 a	71a	0.57 a	0.75 a	1.5 a	0.57 a	1.5 a	3 a
T ₅	29 ab	29 ab	50 ab	0.4 a	0.67 a	1.2 ab	0.33 a	0.57 b	1.2 b
T ₆	29 ab	29 ab	57 ab	0.21 a	0.41 a	0.97 b	0.43 a	0.71 b	1.43 ab
T ₇	14 ab	29 ab	43 ab	0.12 a	0.35 a	0.79 b	0.29 a	0.43 b	0.89 b

Means in a column followed by the same letters are not significantly different at P ≤ 0.05.

Table 3. Survival and Death Percentage of regenerated plantlets of *Saussurea heteromalla* after 3rd week of sub-culturing on rooting medium.

Treatments	Survival Rate (%)	Death Rate (%)
T ₁	0 c	100 a
T ₂	57 b	43 b
T ₃	86 ab	14 bc
T ₄	100 a	0 c
T ₅	100 a	0 c
T ₆	100 a	0 c
T ₇	86 ab	14 bc

Means in a column followed by the same letters are not significantly different at P ≤ 0.05.

Table 4. Comparison of Growth rate of *Saussurea heteromalla* after 5th week of culturing.

Treatments	Shoot Length (cm)	No of Leaves	No of Nodes
T ₁	0 c	0 c	0 c
T ₂	4.89 b	4.67 b	4.47 b
T ₃	5 ab	5.12 ab	4.77 ab
T ₄	6.3 a	7 a	5.25 a
T ₅	6 a	6.43 ab	5.14 a
T ₆	5.7 a	6.14 ab	5 a
T ₇	5.13 ab	5.29 ab	4.83 ab

Means in a column followed by the same letters are not significantly different at P ≤ 0.05.

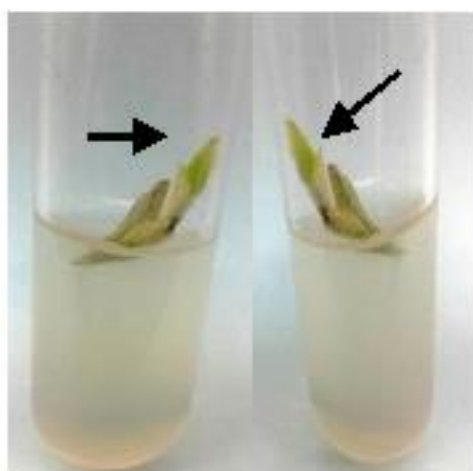


Figure 1. Shoot emergence in the cultured nodal segment of *Saussurea heteromalla* after 2 days of culturing.



Figure 2. Shoot development of *Saussurea heteromalla* after 1st week of culturing.



Figure 3. Shoot development of *Saussurea heteromalla* after 2nd week of culturing.

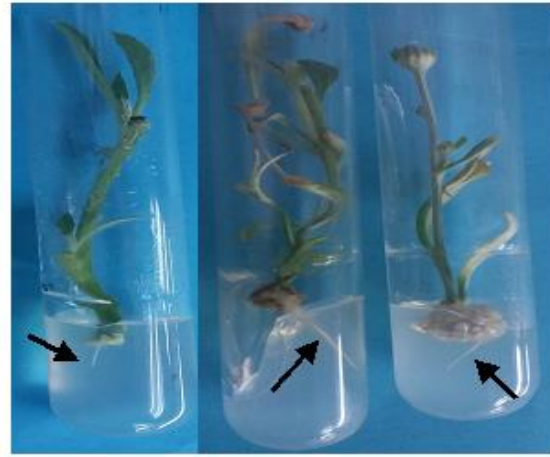


Figure 4. Root emergence in the regenerated shoot of *Saussurea heteromalla*.

Root emergence percentage

After 3rd week of sub-culturing on various rooting media root induction was observed in all treatments except control (T1) which did not develop any root emergence due to lack of auxins (Table 2) (Figure 4) and according to George, (1993) auxin plays a major role in root formation and development. Highest root emergence percentage was recorded in treatment T4 (4 mgL⁻¹ of IBA) which corroborates with the early findings of Grigoriadou et al., (2011) on *Achillea occulta*. Exogenously added IBA under in vitro conditions may induce changes in peroxidase, IAA oxidase activity and phenolic contents allowing the establishment of the favorable endogenous hormone balance for root initiation in excised shoots (Qaddoury and Amssa, 2004). In treatments T5 (1 mgL⁻¹ of NAA) and T6 (2 mgL⁻¹ of NAA), 50% and 57% root emergence was recorded. This reduction in root emergence showed that IBA is better than NAA as it trigger early cell division and root primordia formation than NAA. The superiority of IBA over NAA in inducing rooting has been reported in several studies on *Chrysanthemum morifolium* (Nalini, 2012), *Spilanthes calva* (Razaq et al., 2013) and *Saussurea costus* (Sharma et al., 2018). Lowest root emergence percentage was recorded in treatment T2 (2 mgL⁻¹ of IBA), this is because of basal callusing which interferes with root induction. As in vitro rooting is associated with basal callusing and intensity of which depends on concentration of the auxins (Arora & Bhojwani, 1989). In present study, high concentration of IBA induces maximum rooting and reduction in concentration of IBA reduces rooting percentage whereas low concentration of NAA induces maximum rooting and high concentration suppressed the root emergence.

Root length

Statistical analysis after 3rd week of sub-culturing on rooting medium revealed that highest mean root length was recorded in treatment T4 (4 mgL⁻¹ of IBA) followed by treatments T5 (1 mgL⁻¹ of NAA) and T6 (2 mgL⁻¹ of NAA) (Table 2) (Figure 5). The reason for these differences in mean root length among auxins (IBA and NAA) is due to difference in physiological phenomenon which controls their uptake, transport, metabolization and subsequent gene activation (Ludwig-Muller, 2000). Cosgrove, (1999) discussed that cell elongation involves sequential changes in activity of enzymes and the enzymes required for cell enlargement processes are triggered by the auxin. IBA promotes root length by triggering the synthesis of enzymes involved in cell enlargement (Wada et al., 1998) and is possible reason for maximum mean root length in treatment T4. Treatment T7 (3 mgL⁻¹ of NAA) mean root length per shoot was 0.79 cm. This inferior effect of NAA on root length is due to its high concentration as Taiz & Zeiger, (2002) reported that root growth is strongly inhibited at high concentration of auxin because at high level it induce the production of ethylene, an inhibitor of root growth.

Number of roots

The effect of PGRs were observed in each treatments and statistical analysis after 3rd week of sub-culturing on rooting medium revealed that maximum mean number of roots was recorded in treatment T4 (4 mgL⁻¹ of IBA) (Figure 6) (Table 2). This may be because of exogenously added IBA is converted into free IAA by β -oxidation and also the release of free IBA from IBA conjugates through hydrolysis (Epstein & Ludwig-Muller, 1993). Both free IAA and IBA serve as a source of auxin during later stages of root development and may be the possible reason for maximum number of roots in treatment T4. Exogenous IBA enhanced not only endogenous level of IAA but also increase indole-3-acetyl-aspartic acid (IAA_{sp}) that is required for normal growth of root stem cell as the root formation proceeded (Garcia et al., 1994). Treatments T6 (2 mgL⁻¹ of NAA) and T5 (1 mgL⁻¹ of NAA) showed 1.43 and 1.2 mean number of roots per shoot respectively. This inferior effect of NAA on the number of roots as compared to IBA is due to its more persistence nature which enables it to remain around the root tissues and block the formation of root stem cells (De Klerk et al., 1997). Lowest mean number of roots per regenerated shoot was observed in treatment T2 (2 mgL⁻¹ of IBA). This might be due to deficiency of free auxin required for the development of root meristemoids.

Survival and death percentage

On 3rd week of shifting of developed shoots on rooting medium, data regarding survival and death rate was recorded (Table 3). Highest survival rate was observed in treatments T4, T5 and T6, as these treatments possess well developed root and shoot system which is responsible for proper translocation and metabolism of nutrients from medium. Lowest survival rate was recorded in treatment T2, this might be because of poor rooting system which badly effect the growth of regenerated shoots. Treatment T1 (control) did not show any root emergence because of lack of auxin, so its survival rate was 0% and death rate was 100% (Figure

7) as discussed by De Klerk, (2002) that the success of tissue culture depends on in vitro rooting of plantlets, without rooting plantlets will unable to withstand ex vitro condition.

Hardening and acclimatization

At the time of transplantation, data regarding Shoot growth of 5th week old plantlets was recorded which showed a significant difference ($P \leq 0.05$) among treatments (Table 4) (Figure 8). Highest shoot growth was recorded in treatment T4. This maximum shoot growth is because of well develop rooting system which help in absorption of nutrients from medium. The lowest shoot growth was recorded in treatment T2. This might be due to poor rooting system which retard shoot growth. In treatments T5 and T6 in which NAA was used, although have good shoot growth but show leaf necrosis which may be due to harmful secondary metabolites formed by NAA as reported by Ali et al., (2009) and Li et al., (2013). For hardening and acclimatization completely regenerated plantlets of various treatments were taken out of the culture tube (Figure 9) and washed gently with distill water to remove all the traces of medium adhere to the basal portion of the plantlets. After that, plantlets were transplanted in sterilized peat moss in green house (Figure 10).

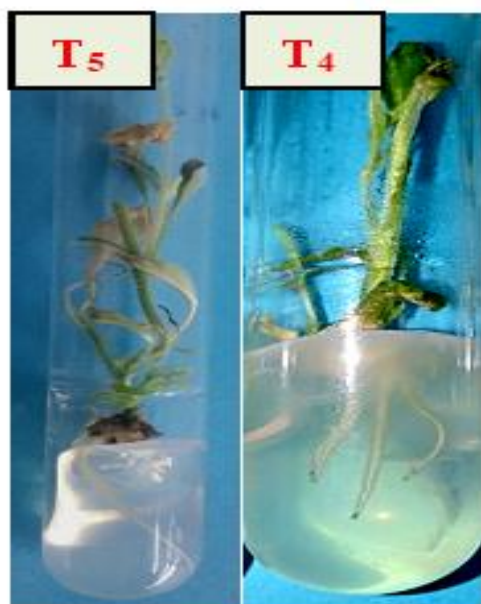


Figure 5. Treatments T4 and T5 showing root length after 3rd week of sub-culturing on rooting medium.

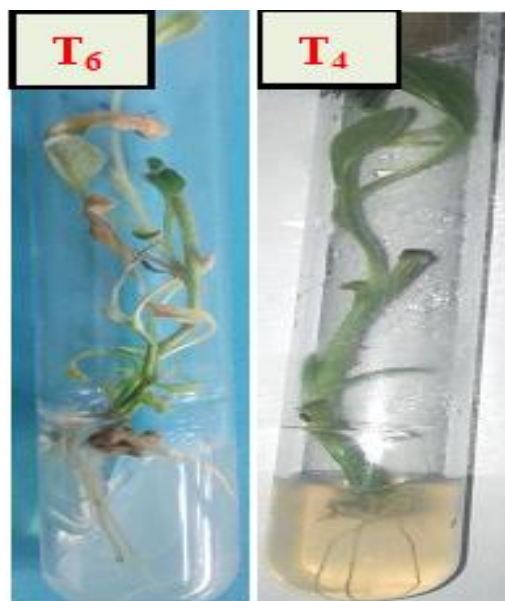


Figure 6. Treatments T4 and T6 showing number of roots 3rd week of sub-culturing on rooting medium.

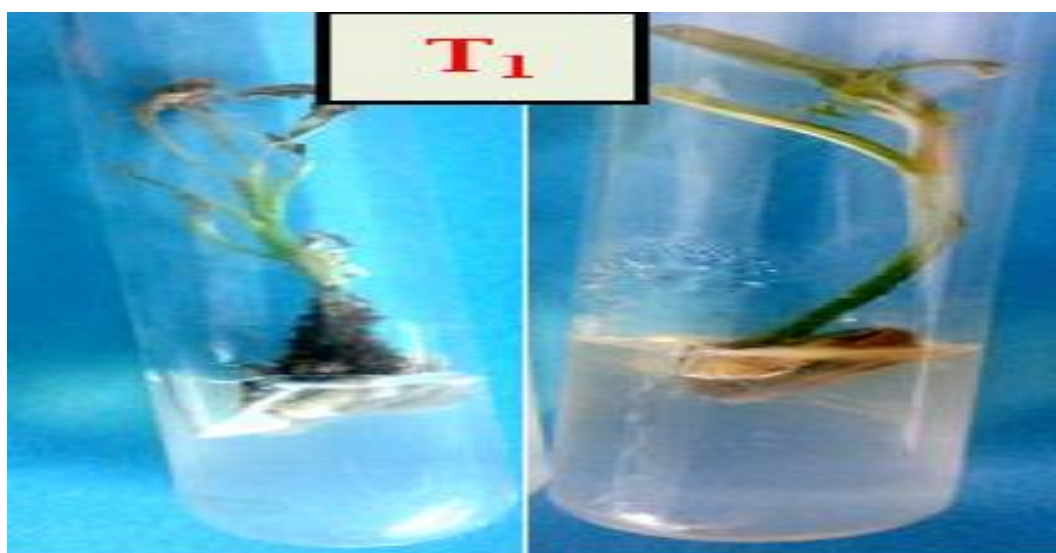


Figure 7. Treatment T1 showing plant death after shifting to rooting medium.



Figure 8. Treatments T2, T3, T4, T5, T6 and T7 showing growth rate after 5th week of culturing.



Figure 9. Regenerated plantlet with well-developed roots taken out of the culture tube.



Figure 10. Hardening in green house.

Conclusion and Recommendations

Present research project concluded that overall status of *Saussurea heteromalla* in Galiyat areas of District Abbottabad is endangered and IBA at higher concentration is more effective for *in vitro* rooting and better shoot growth of *Saussurea heteromalla* whereas NAA also initiate rooting but at lower concentration. As present research project demonstrated *Saussurea heteromalla* to be endangered in Galiyat, therefore it has been recommended to stop over exploitation for medicinal purposes, to limit the interference of anthropogenic activities and livestock grazing; by doing so we can prevent it from extinction. This research work is the first attempt towards *in vitro* regeneration of *Saussurea heteromalla*, further research has been recommended for standardization of its successful *in vitro* regeneration by using other plant growth regulators (cytokinins).


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