

Integration of ex-situ multiplication approaches with in-situ conservation of *Gentiana kurroo* through reintroduction and recovery programs: a critically endangered and endemic species of the North-West Himalaya

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Research Article

Keywords: Critically Endangered, *Gentiana kurroo*, conservation, endemic, species recovery, species distribution modelling

Posted Date: April 17th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4253275/v1>

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Additional Declarations: Competing interest reported. The authors declare no conflicts of interest.

Abstract

Gentiana kurroo Royle (Gentianaceae), a highly medicinal, critically endangered and endemic species of the Western Himalayas with restricted distribution in India and Pakistan. The perspective focuses on the *ex-situ* mass multiplication and *in-situ* conservation of the species through reintroduction under the species recovery initiative. The integration of effective conservation strategies viz macro and micro and intervention of modern technological tools for in-situ restoration of species were invoked. The macro propagation was carried out through seed germination and vertically segregating the profusely branched tuft of plants into small tufts along with the rhizome. The species was micro propagated through axillary bud explants in MS medium fortified with 4.4 μ M BAP and 0.53 μ M NAA was found to be the optimum for shoot induction having 98.23% of shoot formation with 11.32 mean shoot number per culture and shoot length of 4.6 cm. Shoots rooted in half strength MS medium supplemented with IBA (4.9 μ M) and 0.3% activated charcoal. Profuse rooting with average 24.58 roots per shoot was reported after 3-weeks of incubation. The plants were acclimatized in the glass house and were finally transferred to the open environment with 83% survival percentage. Under the species reintroduction and recovery programs, the propagated saplings were introduced into three different wild habitats located in Chakrata and Mussoorie forest divisions of Uttarakhand and 44% survival was reported after six months of field transfer. The research demonstrates the successful coherence of ex-situ propagation methods with in-situ conservation through reintroduction initiatives. The efforts might be helpful in *in-situ* preservation of the germplasm and simultaneously to cater the escalating medicinal requirements of *Gentiana kurroo*.

INTRODUCTION

Gentiana kurroo Royle, a critically endangered, perennial medicinal herb of Western Himalaya, is known by various names in different language and regions. These include 'Karu' in Hindi, 'Traayamaana' in Sanskrit, 'Himalayan gentian' or 'Indian gentian' in English and 'Nilkanth' in Jammu & Kashmir. The species is a native of western and northwestern Himalaya with its limited presence in India and Pakistan. In India, it has been reported from a very few disjunct populations in Uttarakhand, Himachal Pradesh and Jammu & Kashmir (Garg 1987; Tomar et al. 2011; Raina et al. 2003; Ved et al. 2015). The species is mainly being exploited for its medicinal roots, which is the source of bitter glycosides (gentiopicrin and gentianin) and alkaloids (gentiomin). The roots are marketed under the trade name "Indian Gentian" (Sharma et al. 2014). The plant is used in the treatment of various ailments such as cough, stomach disorders, jaundice, fever etc. Medically it served as an antiperiodic, expectorant, antibilious, astringent, stomachic, antihelminthic, blood purifier and carminative (Kirtikar and Basu 1935). In addition, it is also used as veterinary medicine (Kaul 1997).

Earlier the species was reckoned as common in the Western Himalaya, but in the recent few decades the populations of the species are dwindling in its natural habitat and consequently became extremely rare (Clarke 1983; Sastri 1962). The unabated exploitation of species from wild has brought the species near to extinction vortex (Garg 1987). Hence, considering the magnitude of compounding threats on the natural population of *G. kurroo*, the Ministry of Commerce, Government of India has included it in the

negative list of exports vide Notification no. 2 (RE-98) 1997–2002 dated 13th April 1998 (Raina et al. 2003). Furthermore, the IUCN has listed the species as 'Critically Endangered' (IUCN 2020).

G. kurroo is a microclimatic species and grows only on exposed, dry and calcareous rocky slopes. The specialized habitat requirement of the species significantly increases the magnitude of threats and decreases its regeneration potential. Further, habitat destruction of *G. kurroo* has compounded the threat on its population, as observed during the field surveys. The rocky habitats of the species are being mined illegally for the raw materials of the cement industries. Situation further becomes grim when it was observed during the field surveys that the species has been completely wiped out from some previously reported localities in Uttarakhand, including the type locality Bhadraraj in Mussoorie Forest Division.

The dwindling status of *G. kurroo* in the wild and its critically endangered status urged the conservation biologist to ponder about the existence of species and urgently devise some alternative methods for successful propagation and perpetuation of the species.

Attempts were made to propagate the species through tissue culture using different explants, such as apical meristem (Kaushal et al. 2015), callus regeneration from leaves, petioles and roots (Sharma et al. 2014), shoot tip and nodal segments (Sharma et al., 1993). Besides this, studies on somatic embryogenesis (Kaushal and Sidana 2018), cryopreservation of shoot tips (Sharma et al. 2020) and genetic fidelity assessment of ten years old micropropagated plantlets (Kaur et al. 2009) were also conducted on *G. kurroo*. The species was also macro-proliferated by using longitudinal section of mother rhizome and above ground part (Tomar et al. 2011). Srivastava et al. (2022) highlighted the ornamental potential of the species for the successful conservation and preservation of the germplasm. But previous studies were focused on the *ex-situ* conservation only and the issue of *in-situ* conservation of species remains unaddressed. Currently, no efforts are underway for replenishment of species in the wild and sustainable harvesting of the species (Behera and Raina 2011).

Besides *ex-situ* conservation, *in-situ* conservation approaches are in dire need for successful perpetuation of species in the wild and to maintain a small viable population. Species reintroduction & recovery programs might bridge the gap between *ex-situ* and *in-situ* conservation approaches and could deal effectively in conserving threatened plants in their natural habitats. Similar efforts are already underway in various developed countries such as United States, Canada, Great Britain, Australia etc. and desired results have been obtained in many countries. In the United States alone 1158 species have special recovery programs (USFW 1992). Over the past few years populations of 47 species have been stabilized through various recovery processes and are now de-listed from the recovery program (<https://ecos.fws.gov/ecp0/reports/delisting-report>).

Similar efforts were also made in India, particularly in the Western Ghats, to reintroduce four critically endangered and endemic species into wild viz. *S. kathalekanensis* (Vasudeva et al. 2003), *Hubbardia heptaneuron* Bor, a monospecific and endemic genus on the verge of extinction, *Ceropegia fantastica* (Yadav et al. 2009) and *Buchanania barberi* Gamble, a critically endangered tree endemic to the south Western Ghats of Kerala (Dhyani et al. 2022).

The present perspective focuses on the *ex-situ* mass multiplication (i.e. macro & micro propagation) of *G. kurroo* with the aim of reintroduction of species into suitable wild habitat under the species recovery program and to maintain the minimum viable population under *in-situ* conservation approach.

MATERIAL & METHODS

Macro proliferation

To multiply the species through macro proliferation the seeds of *G. kurroo* were collected from wild in the November end and were stored at 4°C. The healthy seeds were segregated and were shown in compost enriched soil in at the end of May month. The seedlings developed were shifted to polybags containing soil and compost mixture in 4:1 (v/v). The proliferated healthy plants were further divided vertically into small tuft and maintained in the polyhouse.

Micro propagation

The macro-proliferated live plants maintained in the Experimental Botanical Garden, Botanical Survey of India, Northern Regional Centre, Dehradun, India were used as a source of explants. Axillary bud explants were initially washed under running tap water for 30 minutes followed by 1% Tween-20 (Himedia Laboratories, India) to remove dirt particles, traces of soil for 30 minutes to properly remove tween-20 solution from the explants. Thereafter, explants were disinfected with different surface sterilizing agents i.e. ethanol (70%), sodium hypochlorite (6%) and mercuric chloride (0.1%) for 5 min each and after that explants were rinsed thrice by autoclaved distilled water. The surface disinfected explants were inoculated into basal MS medium supplemented with different PGRs alone or in combinations to screen out the morphogenic response.

Shoot initiation and Shoot multiplication:

Axillary bud explants of *Gentiana kuroo* were inoculated into MS media supplemented with different concentration of BAP (2.22–8.90 µM), TDZ (2.27–6.8 µM) and Kinetin (3.25–9.3 µM) alone. Subsequently, the optimal concentration of BAP (4.4 µM), TDZ (4.54 µM) and kinetin (6.9 µM) was further tested in combination with different concentrations of naphthalene acetic acid (NAA) (0.53–2.65 µM). Shoot proliferation was performed in the medium having similar composition and supplemented with activated charcoal (1.5 mg/l). All the cultures were sub-cultured at a regular interval of 2–3 weeks. Shoot multiplication rate was calculated on the basis of percentage of explants with a positive response, number of total shoots per explants and shoot height after 6-weeks of incubation.

Rooting

The *in vitro* developed shoots (3–6 cm in length) were shifted to root induction into basal and modified MS medium (quarter and half-strength) augmented with different concentration of IBA (2.46, 4.9, 7.36, 9.8 µM), IAA (2.85, 5.7, 8.5 & 11.42 µM), NAA (2.65, 5.3, 7.9 & 10.6 µM) and 0.3% activated charcoal.

Cultures were incubated under the same conditions as above and rooting percentage, number of roots and root length were recorded after 8-weeks of incubation.

All the cultures (i.e. seed, PLBs, shoot & roots) were maintained in the culture room at $24 \pm 2^\circ\text{C}$, under a 16/8h light and dark cycle with a light intensity of $47.29 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by white fluorescent LED tubes (40 W; Wipro, India). All the plant growth regulators (PGRs) used was procured from Himedia Laboratories, Mumbai, India and glass wares used were from Borosil, India.

Hardening and Transplantation

Plantlets were taken out from the culture flasks after 8 weeks of incubation and washed gently under running tap water to detach the traces of the medium from the roots. Plantlets were shifted to plastic cups (Size: 8×7 cm) containing sand and were maintained in the glass house at $25 \pm 2^\circ \text{C}$ for 4-weeks. In order to acclimatize plants to field conditions, plantlets were transferred to soil pots (Size: 20×18 cm) containing soil and compost (2:1 v/v) for acclimatization to the open environment.

All the experiments were conducted in triplicates and each set of experiment was carried out with 20 explants. Analysis of variance and mean separation was carried out using Duncan's multiple range test (DMRT) utilizing SPSS software.

Reintroduction of species under species recovery programme

The *ex-situ* multiplied saplings of the species were introduced into those habitats where very few individuals of the species are growing (Khadamba, Chakrata Forest Division) and were growing earlier but due to some bio-anthropogenic factors they were completely wiped out from the habitat (Bhadraraj, Mussoorie Forest Division, the type locality of the species). Some new suitable habitats were also selected for the introduction of species (Morthat and Deb Van, Chakrata Forest Division, Dehradun), as depicted in the species distribution models through maxent modeling. The ultimate aim of the efforts is to increase the area of occupancy of the species and consequently effective conservation and preservation of germplasm.

RESULT

Macro proliferation of *G. kurroo*

G. kurroo was macro-proliferated by the germination of seeds in the nursery. The seeds started germination after a month of sowing and 67% germination was observed after two months of sowing (Fig. 1a). Thereafter, the individual seedlings were shifted to polybags for the further growth and proliferation. The species was macro-proliferated by vertically segregating a fully grown healthy tuft (8–12 shoots) of *G. kurroo* (Fig. 1b) into a small tuft. Each tuft was shifted to polybags for the growth and development and were maintained in the polyhouse at lower temperature (Fig. 1c).

Shoot induction and proliferation

The axillary bud explants inoculated into the basal MS medium (control) did not produce any morphogenic response. Supplementing basal MS medium with different concentration of cytokinins alone (BAP, TDZ, and Kinetin) a significant enhancement in shoot development was observed (Table-1). Optimum response was observed in MS medium enriched with 4.4 μM (BAP), 4.5 μM (TDZ) and 6.9 μM (kinetin) separately with 68.15, 51.27 and 46.29% shoot development, respectively (Fig. 2a & b). Further, supplementing the optimal concentration of BAP, TDZ and Kinetin with different concentration of NAA (0.53–1.9 μM), a synergistic response was observed and a significant increase was observed in the shoot development. The MS medium enriched with 4.4 μM (BAP) and 0.53 μM (NAA) was considered to be the optimal medium for shoot induction and 98.23% of shoot formation with per culture 8.32 mean shoot number and 4.6 cm shoot length (Fig. 2c & d).

Root Induction and Hardening

The developed shoots were shifted for root induction into basal and modified MS medium (half and quarter strength). Incipient root development was observed in $\frac{1}{2}$ MS medium, while MS and $\frac{1}{4}$ MS medium did not produce any morphogenic response. Augmenting $\frac{1}{2}$ MS medium with different concentration of IBA (2.46, 4.9, 7.36, 9.8 μM), IAA (2.85, 5.7, 8.5 & 11.42 μM), NAA (2.65, 5.3, 7.9 & 10.6 μM) and 0.3% activated charcoal exhibit significant increase in the root development (Table-2). Half-strength MS medium enriched with IBA (4.9 μM) was observed to be the optimum medium and 92.11% rooting response was observed with 24.58 roots per shoot with root length of 5.8 cm (Fig. 2e).

To optimize the acclimatization, initially plantlets with well-developed roots were shifted to plastic cups containing sand and were kept inside the glass house to acclimatize to the open environment for 4-weeks. To maintain the humidity, plants were initially covered with polythene for one-week with reducing time interval. Thereafter, plantlets were shifted to pots containing compost enriched soil and were maintained in the polyhouse for one month. Thereafter, pots were shifted to net house to properly acclimatize the plants and were maintained for one month (Fig. 2g). Fully acclimatized plants were finally transferred to the open environment with 83% success (Fig. 2h).

Reintroduction of species under species recovery programme

The multiplied plantlets of *G. kurroo* were introduced into suitable wild habitats, as depicted in the species distribution modeling of *G. kurroo* in the North-West Himalaya (Fig. 3), under the species recovery programme. Three different localities of the district Dehradun, Uttarakhand viz. Bhadraraj, Mussoorie Forest Division (Type locality of the species), Morthat, Deoban, and Khadamba in Chakrata Forest Division were selected for the reintroduction of species and approximately 5000 saplings were introduced (Figs. 4, 5 & 6). Post reintroduction, a follow-up visit of the three localities were conducted after six months of reintroduction and 44% survival was reported.

Discussion

G. kurroo is a highly medicinal and ornamental species of the North-West Himalaya. Owing to its high medicinal value, the species has been unsustainably being exploited from wild and consequently the species is facing severe risk of extinction. More than 80% decrease has been observed in the populations of species. Due to its dwindling number and critically endangered status, species needs immediate action for the mass multiplication and effective conservation in the wild.

The increasing over-exploitation pressure on the wild populations of species and limitations of conventional propagation practices, needed to be overcome by recruiting some effective multiplication (macro and micro) techniques for the mass multiplication of species and to make the plantlets available for reintroduction in the wild. Simultaneously, to effectively address the challenge of escalating pharmaceutical demands of the species (Sharma et al. 2014). Application of effective propagation technology for the *G. kurroo* offers not only a means for the mass multiplication of existing germplasm, but also reduces the compounding pressure on the natural populations of the species and consequently in the conservation of this critically endangered species. Increasing the population size of species in the wild is one of the effective remedial steps in the field of restoration of species in the wild (Ravikanth et al. 2018) and simultaneously the approach 'conservation through cultivation' needs to be applied for the effective management of species in the wild and to meet the increasing market demands.

In the present study species was multiplied through macro and micro propagation methods. Macropropagation was initiated through seed germination (67%) which is in accordance to Raina et al. (2003). The species was macro-proliferated by vertically segregating a profusely branched plant into a small tuft and previous reports also validate the current finding (Tomar et al. 2011; Behera and Raina 2012). Chopra et al. (1958) observed this method more effective in the propagation of high altitude Himalayan species.

Miropropagation through direct organogenesis was induced in axillary bud explants for the mass multiplication of the species and combination of BAP and NAA was found to be most effective in inducing bud break and shoot initiation in axillary bud explants of *G. kurroo* which are in accordance with Kaur et al. (2009), Sharma et al. (2014) and Panwar and Joshi (2020). The in vitro developed shoots showed optimum morphogenic response in ½ MS media enriched with IBA. Half-strength MS media enriched with IBA also found effective for root induction in *G. kurroo* by Sharma et al. (1993) and Kaushal et al. (2015). The supremacy of IBA over other auxins in root induction were also observed in *Nepenthes khasiana* (Joshi et al. 2021), *Tricholepis roylei* (Panwar and Joshi, 2020). The plantlets were successfully acclimatized to the open environment.

The macro & micro propagated plantlets of *G. kurroo* were successfully introduced into three different suitable wild habitats viz. Bhadraraj (Mussoorie Forest Division), Morthat and Khadamba (Chakrata Forest Division) of the district Dehradun, Uttarakhand with overall 44% success rate. Similar findings, under species specific recovery programme, were also obtained in *Semecarpus kathalekanensis*

(Vasudeva et al. 2003), *Hubbardia heptaneuron* (Yadav et al. 2009), *Ceropegia fantastica* (Chandore et al. 2010) and *Buchanania barberi* (Dhyani et al. 2022).

In conclusion, *Gentiana kurroo* is a critically endangered species of the North-West Himalaya and *ex-situ* multiplication and reintroduction of saplings under species recovery program might be helpful in restoration of its population in the wild. Application of Species Distribution Modeling analysis in conservation strategies of *G. kurroo* will further complement the efforts by predicting suitable habitats in the North-West Himalaya and launching reintroduction programs for the *in-situ* conservation, restoration and recovery of the species. Long-term monitoring and periodic assessment programs also need to be developed for the effective conservation and management of the critically endangered *G. kurroo*. The need for immediate action on mass multiplication and conservation of species in the wild is emphasized, with a focus on effective propagation techniques to address the challenges of over-exploitation and pharmaceutical demands. The study presents successful macro and micro propagation methods for the mass multiplication of species which could be a boon in conservation of this critically endangered species.

Declarations

Acknowledgement

Authors are thankful to National Mission on Himalayan Studies (NMHS) for providing grant (GBPNI/NMHS-2017-18/LG-03/570, dated: 26/02/2018) for conducting the study. Authors are also thankful to the Head of office, Botanical Survey of India, Northern Regional Centre, Dehradun for providing the necessary facilities. Authors also extend heartfelt thanks to Mr. Satish, Mr. Yunus, and Uttarakhand Forest Department for their help during the field reintroduction programs.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

GSP and AB conceived and designed the experiments, conducted experiments and analysed the data. GSP, PK and AS conducted field work. GSP, PK and AAM wrote the manuscript and revised it.

DATA AVAILABILITY STATEMENT

The data that support the findings of the study are available from the corresponding author upon reasonable request. Occurrence data used to build habitat suitability models are available by the corresponding author and some of the coordinates were downloaded from the GBIF. All environmental data that support the findings of this study are available in the public Domain and were downloaded from <https://WorldClim.org> and Maxent software at <https://github.com/mrmaxent/Maxent>.

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Tables

Table 1: Effect of PGRs on shoot proliferation from axillary bud explants of *Gentiana kurroo* Royle inoculated in MS medium.

Plant growth Regulators (μM)	Explants with Shoots (%)	No. of shoots per explants	Shoot length (cm)
MS ₀	-	-	-
BAP			
2.2	18.09	1.37 $\pm$ 0.9	1.9 $\pm$ 0.2
3.1	36.87	2.86 $\pm$ 0.5	3.1 $\pm$ 0.4
4.4	68.15	4.8 $\pm$ 0.72	3.4 $\pm$ 0.47
6.6	59.04	3.26 $\pm$ 0.68	3.2 $\pm$ 0.4
TDZ			
2.27	17.58	3.27 $\pm$ 0.8	1.8 $\pm$ 0.4
3.18	35.47	4.00 $\pm$ 0.8	2.8 $\pm$ 0.2
4.54	51.27	3.67 $\pm$ 0.7	3.3 $\pm$ 0.2
6.8	46.38	4.48 $\pm$ 0.6	3.1 $\pm$ 0.2
Kinetin			
3.25	13.48	1.98 $\pm$ 0.8	1.7 $\pm$ 0.6
4.6	34.23	2.89 $\pm$ 0.6	2.7 $\pm$ 0.3
6.92	46.29	3.55 $\pm$ 0.3	3.3 $\pm$ 0.2
9.3	41.85	2.07 $\pm$ 0.36	3.0 $\pm$ 0.15
BAP + NAA			
4.4 +0.53	98.23	8.32 $\pm$ 1.0	4.6 $\pm$ 0.6
4.4 +1.06	91.89	5.8 $\pm$ 0.9	4.4 $\pm$ 0.9
4.4 +1.59	80.26	4.9 $\pm$ 0.6	4.3 $\pm$ 0.8
TDZ+NAA			
4.5+0.53	74.82	3.89 $\pm$ 1.0	4.1 $\pm$ 0.6
4.5+1.06	89.01	6.1 $\pm$ 1.2	4.3 $\pm$ 0.8
4.5+1.59	79.72	4.6 $\pm$ 1.03	4.2 $\pm$ 0.6
Kinetin + NAA			
6.9+0.53	68.86	5.44 $\pm$ 0.7	4.2 $\pm$ 0.5
6.9+1.06	80.82	4.18 $\pm$ 1.0	4.0 $\pm$ 0.6

6.9±1.59	77.66	4.05 ± 0.8	3.9 ± 0.6
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#Data are presented as the mean ±SD. Means followed by different letter within columns indicate significant differences at $p \leq 0.05$

Table 2. Effect of auxins on root induction in *in-vitro* regenerated shoots of *G. kurroo* in half-strength MS medium.

IBA	Auxins (μM)		Rooting (%)	No. of roots per shoot	Root Length (cm)
	NAA	IAA			
MS ₀	0	0	-	-	-
½ MS	0	0	10.14 ^f	1.9 ± 0.39 ^f	0.5 ± 0.2 ^e
¼ MS	0	0	9.4	1.1 ± 0.36 ^f	0.3 ± 0.1 ^e
2.46	0		66.13 ^d	10.87 ± 0.42 ^c	3.2 ± 0.9 ^c
4.9	0	0	92.11 ^a	24.58 ± 0.87 ^a	5.8 ± 0.29 ^a
7.36	0	0	81.32 ^b	16.9 ± 0.41 ^b	4.2 ± 0.3 ^b
9.8	0	0	75.38 ^c	14.54 ± 1.0 ^b	4.1 ± 0.49 ^b
0	2.65	0	46.30 ^e	8.26 ± 1.2 ^d	2.0 ± 0.4 ^d
0	5.3	0	66.18 ^b	14.10 ± 0.92 ^b	4.3 ± 0.82 ^b
0	7.9	0	58.21 ^b	10.56 ± 0.42 ^c	3.4 ± 0.9 ^c
0	10.6	0	52.61 ^c	8.14 ± 0.69 ^d	3.2 ± 0.9 ^c
0	0	2.85	44.31 ^e	6.23 ± 0.29	2.6 ± 0.28 ^{cd}
0	0	5.7	69.15 ^b	12.21 ± 0.5 ^c	3.9 ± 0.35 ^{bc}
0	0	8.5	57.59 ^b	9.1 ± 1.0 ^d	3.1 ± 0.4 ^{cd}
0	0	11.42	50.47 ^c	7.3 ± 0.9 ^e	2.7 ± 0.39 ^{cd}

#Data are presented as the mean ±SD. Means followed by different letter within columns indicate significant differences at $p \leq 0.05$

Figures



Figure 1

Macro-propagation of *Gentiana kurroo*: **a.** seedlings developed from seed germination after two months of sowing, **b.** profusely branched plantlets used for the macro-proliferation by segregating rhizome vertically, **c.** multiplied plantlets maintained in polyhouse.

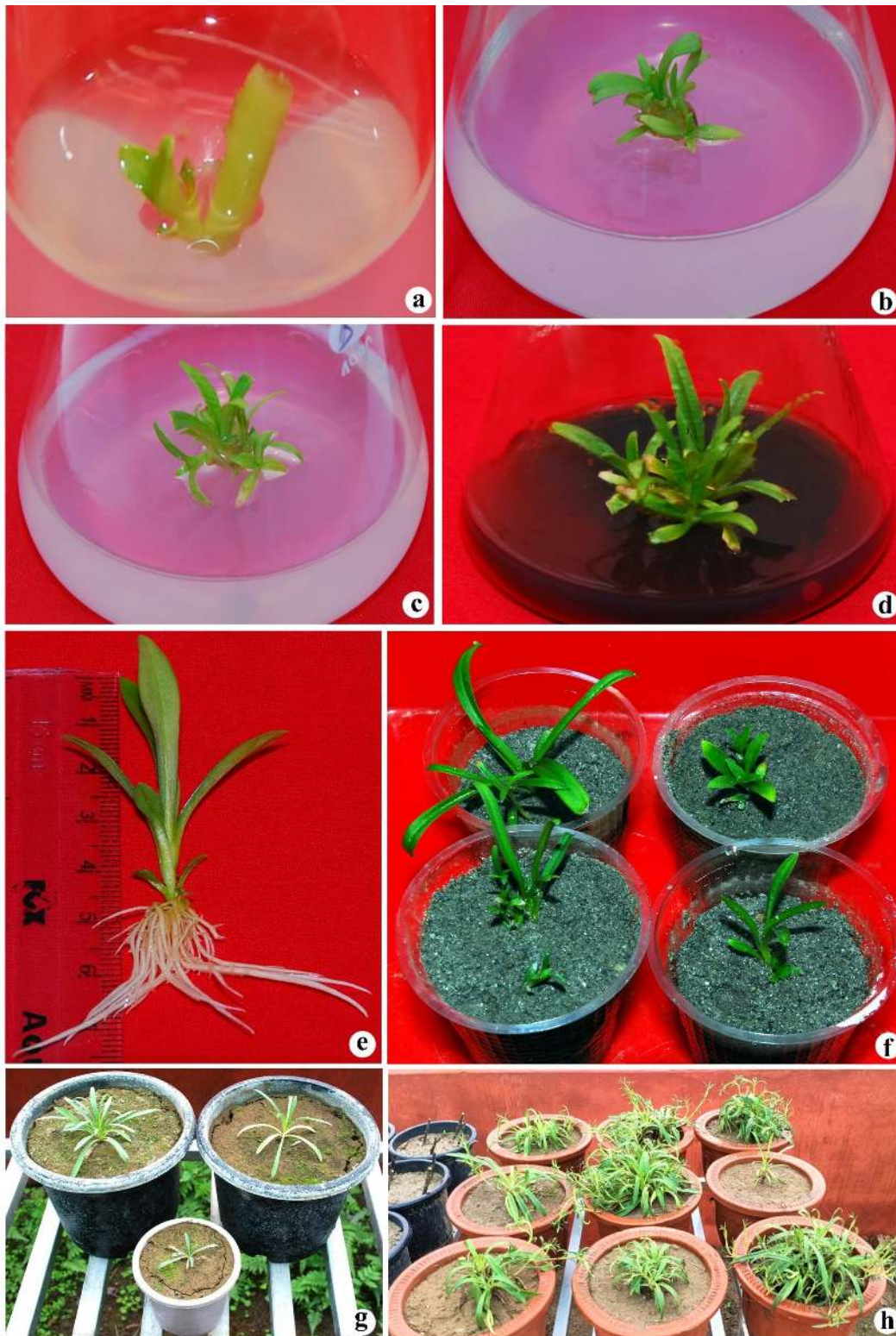


Figure 2

In vitro propagation of *Gentiana kurroo*: **a.** bud break in axillary bud explants inoculated in MS medium, **b** & **c.** proliferation of shoots in MS medium enriched with BAP (4.4 μM) and NAA (0.53 μM), **d.** root induction in *in-vitro* developed shoots inoculated in $\frac{1}{2}$ MS medium enriched with IBA (4.9 μM) and AC, **e.** Plantlet with fully developed shoots and roots, **f.** Plantlets shifted for hardening into plastic cups

containing sand. **g.** Plants transferred to the open environment for the acclimatization, **h.** fully acclimatized plants growing in the open environment.

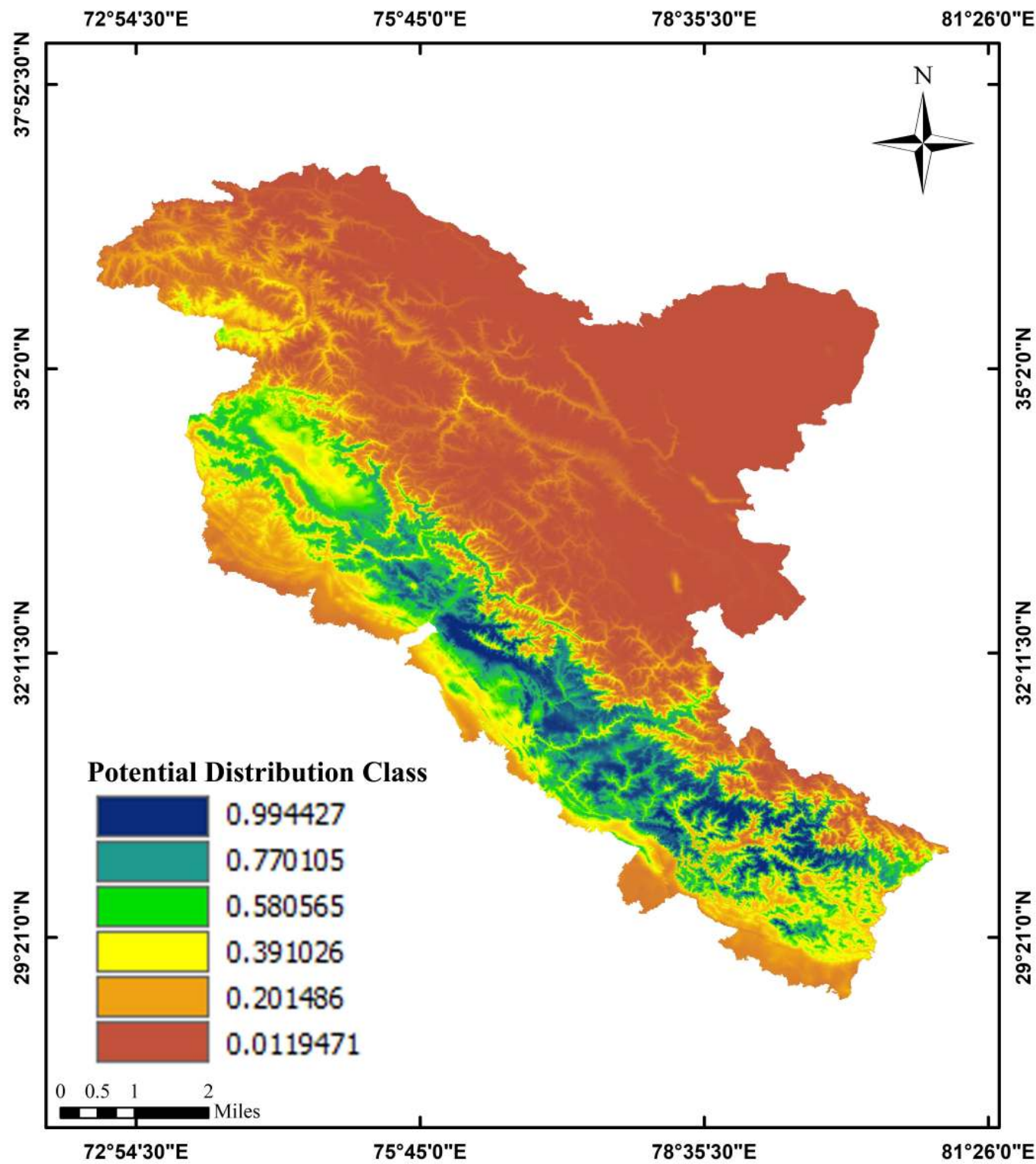


Figure 3

Gentiana kurroo habitat suitability analysis in the North-West Himalaya using maxent modeling.

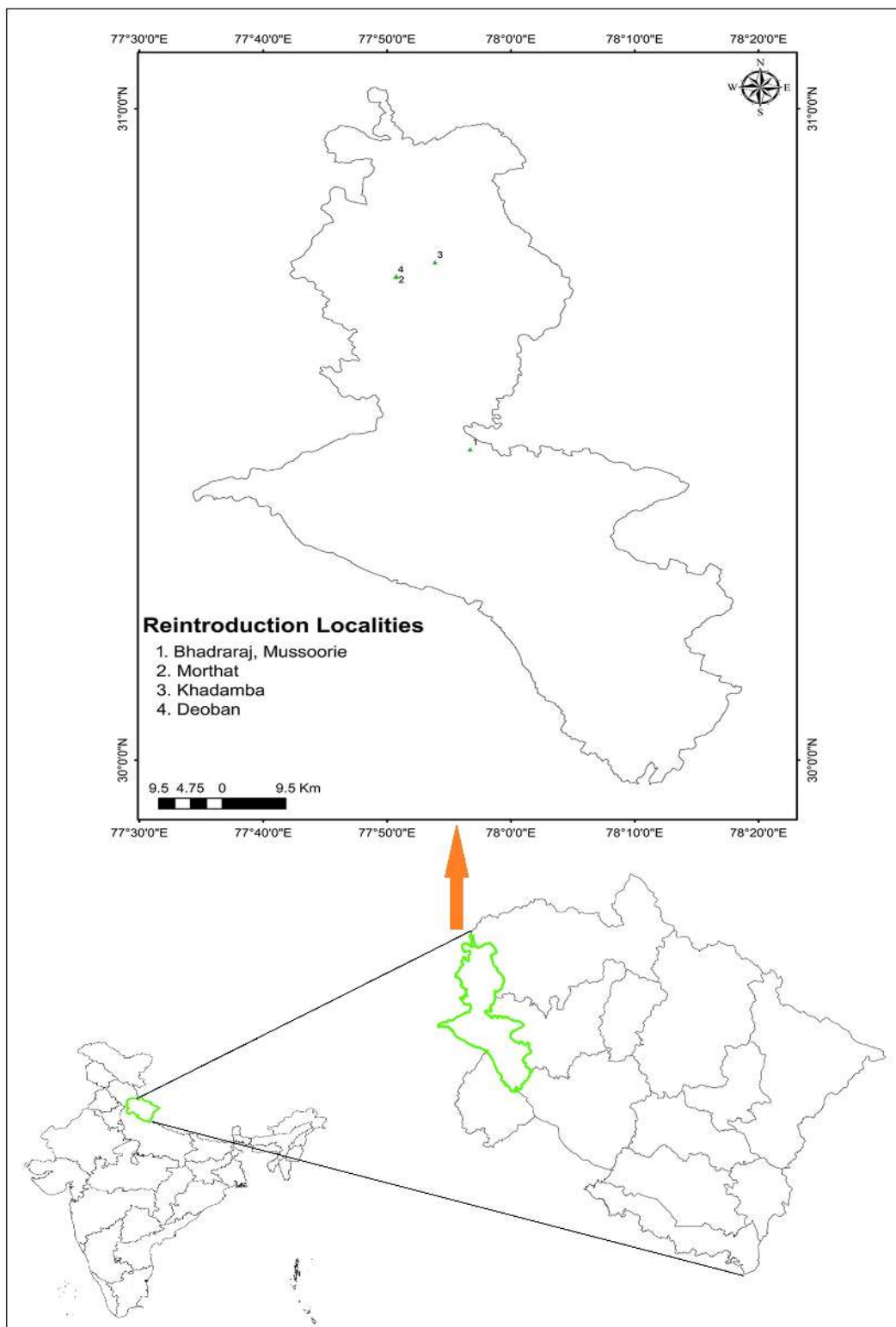


Figure 4

Reintroduction sites of *Gentiana kurroo* in Chakrata and Mussoorie forest divisions of Dehradun, Uttarakhand, India.



Figure 5

Reintroduction of *Gentiana kurroo* in Bhadraraj, Mussoorie Forest Division, Uttarakhand the type locality of the species, under the species recovery programme.



Figure 6

Reintroduction of *Gentiana kurroo* in the wild locality at Khadamba, Chakrata, Dehradun Uttarakhand under the species recovery programme.