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Clonal Propagation of *Rhynchostylis retusa* (Lin.) Blume through *in vitro* Culture and their Establishment in the Nursery

Pinaki Sinha* and Miskat Ara Akhter Jahan

Biological Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Kudrat-I-Khuda Road, Dhaka, Bangladesh

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Abstract

For high frequency regeneration of *Rhyncnostylis retusa* (Lin.) Blume apical nodal segments were used. Half strength MS + 2% sucrose + 1.5 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone + 10% (v/v) coconut water (CW) + 0.5 g/l activated charcoal (AC) was the best nutrient medium, on which 89% cultures induced 8 microshoots per culture. Subculture of microshoots for further 8 weeks on the same nutrient medium enhanced the number of microshoots up to 95. For further proliferation of microshoots, their development into shoots as well as formation of secondary microshoots from the base of the old ones, the best medium was half strength of MS + 2% sucrose + 2 g/l peptone + 10% (v/v) CW + 0.5 g/l AC + 150 mg/l L-glutamine. Plantlets with roots were obtained in half strength of MS + 2% sucrose + 2 g/l peptone + 10% (v/v) CW + 0.5 g/l AC + 5.0 g/l banana powder, on which cent per cent shoots rooted within eight weeks. The pH of all the categories of cultures were maintained at 5.6 before adding 2.2 g/l gelrite and autoclaving, and the cultures were incubated at 2000 - 3000 lux for 16/8 hrs light/dark at 24 ± 2ºC. Regeneration of plantlets continued due to repeated subculture of microshoots and regenerants were acclimatized and established in the nursery.

Introduction

Rhynchostylis retusa (Lin.) Blume is a large epiphytic plant having a robust stem of about 25 cm long. Leaves are strap-shaped and about 25 cm long and the pendulous inflorescences are about 60 cm long which densely flowered. Flowering sits in summer to autumn, specially in May-June. It grows in Bangladesh, India and Ceylon up to Philippines. In India it is found at the base of

*Author for correspondence: E-mail: pinakiju@yahoo.com

Himalayas from Garhwal to Bhutan and Khasia Hills up to 1000 ft elevation (Mukherjee 2002). In Bangladesh, the plant grows naturally as epiphyte on the stem of big trees in the southern region, specially in Patuakhali and Barisal districts. Reports on *in vitro* clonal propagation of any *Rhynchostylis* species are limited. Vijrabhaya and Vijrabhaya (1970) cultured excised apices of *R. giganteas* on a composite agar medium supplemented with NAA and coconut milk. Vij et al. (1984) were able to micropropagate *R. retusa* by culturing young leaf segments, from plants growing both *in vitro* and *in vivo*, on Mitra et al. (1976) medium (Mitra et al. 1976) supplemented variously with IAA, NAA, 2,4-D, Kn, GA and organic supplements, peptones, urea, casein hydrolysate and yeast extract. Plantlets, upon excision from mother plant, grew well. Bui et al. (1999) reported the effect of the auxin/cytokinin ratio as well as its interaction with TDZ on bud regeneration of *R. retusa* and on the influence of sucrose on bud development and rooting. The combination of BA and NAA led to a higher regeneration frequency than either alone, and TDZ in combination with BA or, to a lesser degree with BA and NAA, increased the number of shoots compared to TDZ alone. Various protocols developed for orchid microcloning show considerable variations with respect to media composition and environmental conditions (Arditti and Ernst 1993). Because there is no extensive literature on *in vitro* clonal propagation of *R. retusa,* the present study attempts to address this deficiency.

Materials and Methods

Mature plants were collected from a *Rhynchostylis retusa* colony naturally growing on the trees in the villages of Patuakhali district, Bangladesh, and used as source materials. Apical nodal segments (NS) were used as explants. Surface sterilization was made following Sinha et al. (2010), and the surface sterilized explants were prepared for inoculation as follows. For direct induction of protocorm like bodies (PLBs)/microshoots, explants were cut into 10 mm long nodal segments with one node in each segment. Explants were cultured initially in KC (Knudson 1946), VW (Vacin and Went 1949), half strength of MS and MS media, each supplemented with different concentrations and combinations of sucrose, auxins, cytokinins, peptone, coconut water, and activated charcoal. The cultures were gelled with 2.2 g/l gelrite (Duchefa, the Netherlands). The pH of the medium was adjusted to 5.6 before autoclaving at a pressure of 1.06 kg cm^{-2} at 121ºC for 20 min. Medium was poured in 25 × 150 mm culture tubes containing 20 ml of medium or 100 ml conical flasks containing 40 ml of medium.

The medium that responded best was used in subsequent experiments. For proliferation of microshoots and their development into shoots, each clump of

microshoots induced on explants was subcultured for 8 weeks on the same nutrient medium on which microshoots were induced. The microshoots clump was then dissected vertically into 8 pieces and subcultured on specific medium with specific additives along with different concentrations of casein hydrolysate (50 - 200 mg/l) and L-glutamine (50 - 200 mg/l). Media were gelled with 2.2 g/l gelrite. In all experiments the explants were cultured in 250 ml conical flasks or jam bottles (86 x 120 mm) containing 50 ml medium. The pH of the media was adjusted to 5.6. All cultures were incubated at 24 ± 1°C, and under cool white fluorescent light of 2000-3000 lux for 16 hr per day.

In the microshoot proliferation medium, old microshoots developed into leafy structures from the base of which new microshoots emerged profusely. For the whole plant formation, these leafy structures or shoots were subcultured on the specific medium with additives and different concentrations of banana powder (Duchefa, the Ntherlands) (BP) (5 - 10 g/l) and potato homogenate (PH) (50 - 100 g/l), gelled with 2.2 g/l gelrite for plantlet formation. Ten shoots were cultured in each culture vessel and each treatment was replicated in 15 culture vessels.

For acclimatization the rooted plantlets (50 - 70 mm in height) were taken out from the medium and washed with tap water to remove the gel that adhered to the roots. They were then implanted in a plastic basket containing three types of substrates: substrate A [coconut husk (20 mm^3)], substrate B [coconut husk (20 mm^3) and charcoal (10 mm^3) (2 : 1)] and substrate C [tree bark (10 mm^3) and charcoal (10 mm^3) (2 : 1)]. To maintain high humidity the plantlets were treated with three different periods of misting: 4-hour (treatment 1), 6-hour (treatment 2) and 8-hour (treatment 3) intervals for 30 days. After acclimation, the plantlets were transplanted to 15 cm clay pots perforated at the bottom and side and containing coconut husk (20 mm^3) and charcoal (10 mm^3) (2 : 1) and maintained under shade net at 30/25°C (day/night). Plants were watered every day and fertilized with 30N-10P-10K solution (3 g/l of water) at 10-day intervals for 12 months. After 12 months the plants were fertilized with 20N-20P-20 K solution (3 g/l of water) at 5-, 10-, and 15-day intervals.

All experiments were repeated three times. Each treatment had 15 replicates. The morphogenetic response of explants for microshoot induction was evaluated in eight weeks of culture. For microshoots proliferation and plantlet formation, results were evaluated in eight weeks of culture. Morphogenetic response was expressed as percentage of explants with microshoots in relation to the number of surviving explants. For acclimaon 50 or 25 plantlets were taken for each treatment. Data were statistically analyzed and in some parameters means were compared using DMRT (Duncan 1955).

Results and Discussion

Results showed that half strength of MS nutrient medium was superior in all respects to other media, in which microshoots were induced directly from the explants instead of PLBs. Since the half strength of MS was found to be superior, the following experiments were carried out in that medium

Nodal explants were cultured individually on half strength of MS containing 2% sucrose, 10% CW and 2 g/l peptone. Effects of BA, Kn and TDZ individually and in combination with NAA and IAA in different concentrations on microshoot induction were recorded. BA, Kn or TDZ alone in the medium was not overall successful for induction of either PLBs or microshoots from the nodal segment. All combinations of BA-NAA, BA-IAA, Kn-NAA, Kn-IAA, TDZ- NAA and TDZ-IAA responded in inducing microshoots but BA-NAA showed the most potential for the induction of microshoots from nodal explants (Table 1, Fig. 1A). The medium with 1.5 mg/l BA + 0.5 mg/l NAA was best for micro-shoots induction in maximum number (89%) of cultures. The highest number of microshoots per culture was 8.0 induced within 39.5 days. Among the combinations of TDZ-NAA 1.5 mg/l TDZ + 0.5 mg/l NAA was the best, in which 4.7 microshoots (58.5%) were induced per culture within 48 days. Effects of other combinations of auxin-cytokinin were inferior in respect of microshoots development (Data are not shown).

Reports on *in vitro* clonal propagation of any *Rhynchostylis* sp. is limited (Vajrabhaya and Vajrabhaya 1970, Vij et al. 1984, Sood and Vij 1986, Bui et al. 1999, Kumar et al. 2003). In previous experiments on *Rhynchostylis retusa* young leaf segments of mature plants (Vij et al. 1984, Kumar et al. 2003) and root segments (Sood and Vij 1986, Kumar et al. 2003) were used as explants. Vajrabhaya and Vajrabhaya (1970) cultured apices of *Rhynchostylis gigantea* on a composite agar medium with NAA and CW and obtained callus, which upon subsequent subculture on fresh medium differentiated into plantlets. For micropropagation of *Rhynchostylis retusa*, Vij et al. (1984) used young leaf segments which were cultured on Mitra et al. (1976) medium. Root segments of the same species was also used as explants and cultured on Mitra et al. (1976) medium (Sood and Vij 1986). TCLs of stem nodes and internodes of *Rhynchostylis gigantea* were cultured on MS (Bui et al. 1999). In the present study apical nodal segments were used. Sood and Vij (1986) obtained PLBs from root explant culture of *Rhynchostylis retusa.* In the present study, instead of PLBs microshoots induced directly from the explants cultured on half strength of MS supplemented with 2% sucrose + 1.5 mg/l BA + 0.5 mg/l NAA + 2 g/l peptone + 10% CW + 0.5 g/l AC. Many orchid species require auxins and/or cytokinins for neoformation of PLBs and shoot development. The ratio of auxin and cytokinin for these

organogenesis depends upon the species studied (Arditti and Ernst 1993, Bui et al. 1999). NAA is frequently used in combination with cytokinin such as BA in many monopodial orchid species (Goh and Wong 1990, Valmayor et al. 1986). In *Vanda*, the highest PLB production was obtained from shoot tip explants using NAA and BA (Valmayor et al. 1986). *In vitro* regeneration of *Rhynchostylis gigantea* via protocorms in the presence of an auxin, NAA (0.1 mg/l) was first

Table 1. Effects of BA and NAA on microshoots induction from nodal segment of *R. retusa*, cultured on half strength of MS with 2% sucrose, 10% CW and 2 g/l peptone. Data were recorded in 8 weeks after inoculation. Results are mean ± SE of 15 cultures, repeated three times.

Treatments (mg/l)		% of cultures induced microshoots	*No. of microshoots per explant
BA	NAA		
0.0	0.0	-	-
0.5	0.2	25.6 ± 3.1d	1.8 ± 0.3 d
1.0	0.2	31.2 ± 3.5d	3.1 ± 0.6 d
1.5	0.2	62.3 ± 5.3 c	4.5 ± 1.3 c
2.0	0.2	65.8 ± 5.1 c	4.8 ± 1.5 c
2.5	0.2	67.5 ± 5.7c	5.2 ± 1.7 c
0.5	0.5	28.4 ± 3.4 d	2.3 ± 0.4 d
1.0	0.5	31.5 ± 3.1d	3.2 ± 0.6 d
1.5	0.5	89.5 ± 6.4 a	8.0 ± 1.8 a
2.0	0.5	75.6 ± 5.8 b	6.2 ± 1.3 b
2.5	0.5	68.7 ± 5.2c	5.4 ± 1.1 c

*Mean values followed by the same letter are not significant at $p \geq 0.05$ by DMRT.

reported by Vajrabhaya and Vajrabhaya (1970). Besides auxins, the other regulators that affect the process of regeneration in monopodial epiphytic orchid species are Kn, NAA or IAA; they acted synergistically on the formation of PLBs (Arditti and Ernst 1993). In another study on *Rhynchostylis gigantea* (Bui et al. 1999) it was shown for the first time the direct regeneration of bud primordia, in which a combination of BA and NAA together led to a higher regeneration frequency than BA or TDZ alone. The optimal combination for maximal bud regeneration was 3 μM BA and 3 μM TDZ, giving rise to 11.7 buds per explant. In the present study on *Rhynchostylis retusa* the optimum concentrations of BA and NAA was 1.5 and 0.5 mg/l, respectively, on which 8.0 microshoots were induced from nodal segment explants, TDZ and TDZ-NAA combinations were not as effective as BA-NAA.

Here, it was also observed that once the microshoots induced with the hormonal effects of BA-NAA combination, they proliferated with repeated

subculture in the same nutrient medium. In this study high frequency induction of microshoots was successfully obtained by adding peptone, CW and activated charcoal to the medium. The induced microshoots were subcultured in PGR free medium containing organic supplements for shoot multiplication.

The initially induced microshoot clumps were subcultured in the same nutrient medium as mentioned above. Through the eight-week subculture, the micro shoots were increased in number up to an average number of 95 (Fig. 1B). Each microshoot clump (mentioned in materials and method) was subcultured on gelrite-gelled half strength of MS supplemented with 2% sucrose + 2 g/l peptone + 10% (v/v) CW + 0.5 g/l AC (without PGR) along with different concentrations (50 - 200 mg/l) of L-glutamine or CH for 8 weeks. Results showed that eight-week subcultures on medium having 150 mg/l L-glutamine the shoots (including leaves) elongated properly. The mean height of the shoots (including leaves) was 68.8 mm. Moreover, a large number (an average of 10 microshoots from each shoot) of new microshoots were induced from the base of the old shoots (Fig. 1C). The long shoots were isolated and subcultured again on the same nutrient medium, where new microshoots were again induced from the basal portion of the shoots. The shoots cultured on the medium having CH were elongated and exhibited root induction but no new microshoots were induced. The effect of CH concentration used on shoot height was not significantly different among them but significantly different from effects of L-glutamine. As a result, the medium determined to be most effective, for microshoots proliferation and their development into shoots in half strength of MS + 2% sucrose + 10% CW + 2 g/l peptone + 0.5 g/l activated charcoal + 150 mg/l L-glutamine.

The rooted shoots were procured and subjected to acclimation. Besides this, the organic supplements, banana powder (BP) (2.5 - 10 g/l) and potato homogenate (PH) (25 - 100 g/l) were tested to observe their efficacy on root induction. BP and PH were added individually to the medium (half strength of MS with 2% sucrose + 2 g/l peptone + 10% (v/v) CW + 0.5 g/l AC). The addition of 5 g/l banana powder in the medium enhanced root induction and growth of both shoots and roots. An average of 5 stout roots induced in 100% cultures within eight weeks. The mean length of root was 35 mm. With the formation of roots, the plantlets grew vigorously. The addition of 50 g/l PH to the medium also increased the number of roots, but the value was less than that developed in a medium supplemented with BP. So, through this experiment the most efficient medium discovered for plantlets formation of *Rhynchostylis retusa* was half strength of MS medium + 2% sucrose + 10% CW + 2 g/l peptone + 0.5 g/l AC + 5 g/l BP, in which 100% shoots rooted (Fig. 1D) in 8 weeks of culture.

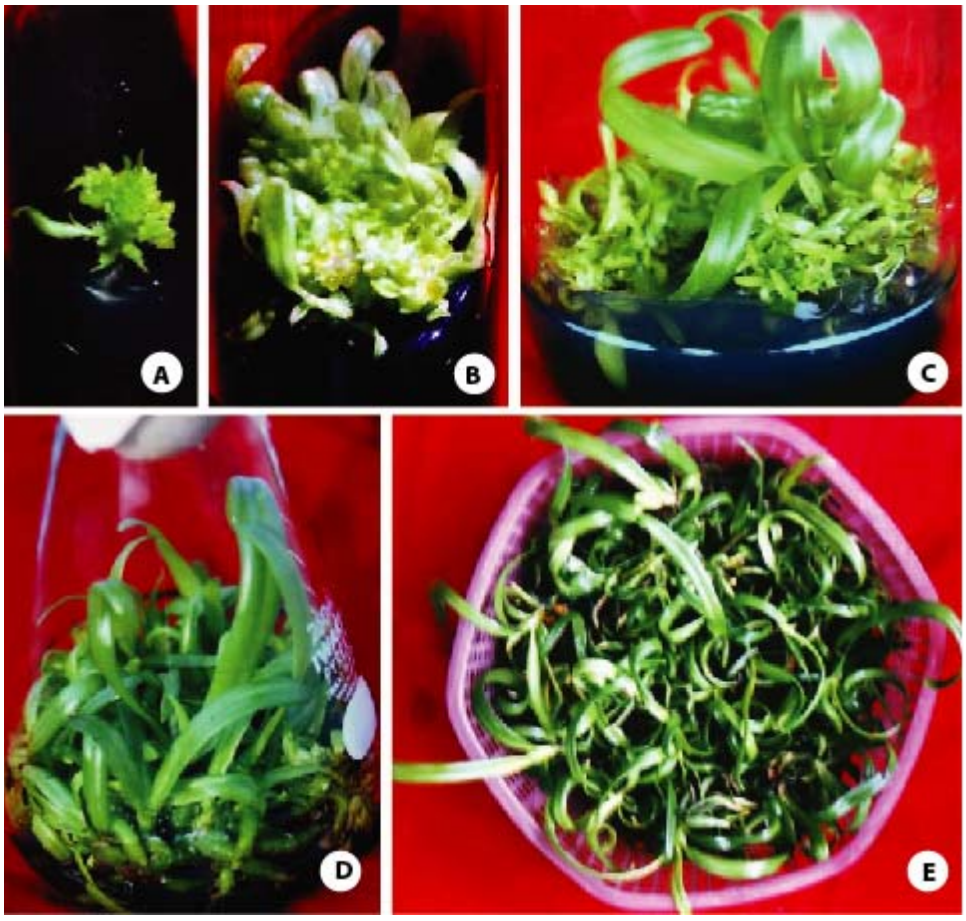

Fig. 1A-E: Microshoots induction and regeneration of *Rhynchostylis retusa* from nodal explants. A. Microshoots induction from the explants cultured for 8 weeks. B. Multiplication of microshoots cultured in the same medium for further 8 weeks. C. Development of microshoots into shoots and induction of new microshoots from the base of the old ones. D. Regenerated plantlets with roots. E. Acclimatd plantlets.

Within the first 36 weeks after initiation of culture 1500 plantlets were obtained from a single explant of leaf base segment. Repeating the subculture of microshoots on the proliferating medium and subsequently on the rooting medium, the regeneration of plantlets continued and 13500 plantlets could be produced during the next every 24 weeks.

The survival of regenerated plants during acclimatization did not depend significantly on the substrate used in the culture basket (Table 4). Air humidity had the most significant effect on the survival of the plantlets. Humidity maintained by misting at 6-hour intervals was found to be the optimum in which 94.5% plantlets survived with healthy status. If the misting interval was shorter

(4-hour), the survival rate was only 63.5% due to excessive moisture. When humidity was maintained by misting at 8-hour intervals, transplanted plantlets gradually declined due to withering and only 50.0% plantlets survived. The acclimated plantlets (Fig. 1E) were transplanted to 15 cm clay pots perforated at the bottom and side and kept in the nursery under a shade net. Plants were watered every day and fertilized with 30N-10P-10K solution (3 g/l of water) at 10-day intervals. During rearing for the first 12 months no mortality was observed; instead, growth was exhibited through the emerging out new leaves.

Table 2. Effects of L-glutamine and casein hydrolysate (CH) individually on microshoot proliferation and their development into shoots cultured on half strength of **MS + 2% sucrose + 10% CW + 2 g/l peptone + 0.5 g/l AC. Data were recorded in 8 weeks of culture. Results are mean ± SE of 15 cultures, repeated three times.**

L-glutamine/ CH	Nodal segment derived microshoots	
(mg/l)	ψShoot height (mm)	Remarks
L-glutamine		
50	33.7 ± 1.8 d	*
100	48.8 ± 2.3 c	*
150	68.8 ± 2.6 a	*
200	51.5 ± 2.8 c	*
CH		
50	58.1 ± 2.7 b	**
100	58.5 ± 2.1 b	**
150	58.2 ± 1.8 b	**
200	57.9 ± 1.6 b	**

ψMean values ± SE followed by the same letter in a column are not significant at $p \leq 0.05$ by DMRT. *Shoot development with shoot growth and root induction as well as new microshoots formation from the base of the old ones. **Only shoot development and root induction, no new microshoot formation.

In the present study high frequency induction of microshoots was successfully obtained by adding peptone, CW and activated charcoal to the medium. After inducing microshoots, during subcultures PGR was no longer used; instead, only the organic supplements, as mentioned above, were used for high frequency proliferation. In the present study L-glutamine and CH were tried individually in the medium for shoot growth and proliferation. L-glutamine was found to be superior to CH. Effects of L-glutamine were also reported on PLBs proliferation in different orchid cultures (Sinha et al. 2003, 2007a,b). Amino acids were added as supplemental sources of nitrogen to the growth medium for the culture of plant cells and tissues in numerous studies

Table 3. Effects of different concentrations of banana pulp (BP) and potato homogenate (PH) on root induction in regenerated shoots derived from nodal segment explants of *Rhynchostylis retusa* cultured on half strength of **MS medium with 2% sucrose + 10% CW + 2 g/l peptone + 0.5 g/l AC. Data were recorded 8 weeks after inoculation. Results are mean of 15 cultures, repeated thrice.**

BP/PH (g/l)	Shoots derived from nodal segments	
	*No. of roots	*Length of individual root (mm)
0	2.5 ± 0.6d	11.7 ± 0.5d
BP 2.5	3.1 ± 0.6c	17.0 ± 1.8c
BP 5.0	5.0 ± 1.4a	33.5 ± 3.8a
BP 7.5	4.1 ± 0.9b	29.0 ± 3.5b
BP 10.0	3.7 ± 0.5b	17.5 ± 1.5
PH 25	3.6 ± 0.8bc	24.8 ± 4.5b
PH 50	4.0 ± 0.6b	27.4 ± 6.5b
PH 75	3.6 ± 0.8bc	29.7 ± 7.5b
PH 100	3.0 ± 0.4c	18.8 ± 0.5c

*Mean values ± SE followed by the same letter in a column are not significant at $p \leq 0.05$ by DMRT.

Table 4. Acclimation of *in vitro* regenerated plants of *Rhynchostylis retusa* treated with different types of misting in different substrates. Data were taken after four weeks of culture.

Variants of Acclimation		*% of acclimated plants (mean ± SE)
Substrates	Humidity	
A	Treatment 1	63.2 ± 6.5 b
	Treatment 2	92.5 ± 5.2 a
	Treatment 3	42.4 ± 3.8 c
B	Treatment 1	62.5 ± 4.3 b
	Treatment 2	94.7 ± 4.5a
	Treatment 3	43.2 ± 3.5c
C	Treatment 1	63.8 ± 4.2 b
	Treatment 2	95.6 ± 4.6 a
	Treatment 3	53.5 ± 3.6 c

*Twenty plants were taken for each treatment and the experiments were repeated three times. Mean values followed by the same letter in a column are not significant at $p \leq 0.05$ by DMRT.

(Gamborg 1970). In the present study the high frequency proliferation of micro-shoots was possible due to the synergistic effect of the organic compounds

present in CW and L-glutamine. CW and L-glutamine in the culture medium positively affected the multiplication rates of somatic embryos just as they did for other plant species (Kako 1973, Pierik 1987). Likewise PLBs positively affected the rates of other orchid species (Alam et al. 2006, Sinha et al. 2003, 2007a,b). Encouraging results were also obtained in *Cattleya* cultured in half strength of MS supplemented with CW and activated charcoal (Torres and Jiménez 2004).

For rooting of regenerated shoots of *Rhynchostylis gigantea* chlofenuron (CPPU) was used (Bui et al. 1999). In the present study of *Rhynchostylis retusa* addition of banana powder in the medium was highly effective for root induction in 100% of the cultures. Kusumoto (1979) also obtained positive results by adding banana pulp to the medium of *Cattleya* culture. In the present experiment identical shoots were separated from the culture and subcultured for their development into plantlets in obtaining a large number of identical plantlets.

In the present study callus induction was not needed rather microshoots were induced directly from the explants cultured in medium containing limited doses of BA and NAA. The protocol for high frequency regeneration of *Rhynchostylis retusa* as established through this study could be utilized in commercial cultivation for sustainable use and conservation as well.

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