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Micropropagation protocol for Mongolian rare shrub *Lycium truncatum* Y.C. Wang

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ABSTRACT

In this study, micropropagation protocol for the Mongolian rare shrub *Lycium truncatum* L. has been developed through axenical seed germination. There are successful micropropagation procedures for some *Lycium* species; nonetheless, *L. truncatum* requires both *in-vitro and ex-vitro* optimization.The 4-week-old sterile seedlings with spontaneous root formation, cultivated in full-strength hormone-free MS media were utilized as initial explants for *in vitro* culture. The highest shoot proliferation (4.6 shoots/explant) was achieved on Driver and Kuniyuki Walnut medium (DKW) supplemented with 2 mg/L kinetin (KIN) and 0.1 mg/L 1-naphtaleneacetic acid (NAA) combination. The proliferating shoots on the half-strength MS media supplemented with 0.1mg/L NAA displayed robust root formation. The rooted plantlets were acclimated in sterile soil in a pot and cultivated at room temperature for 2-3 months with a high survival rate of more than 90% before transferring to the greenhouse.

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KEYWORDS: *Lycium truncatum*, Nodal segments, DKW medium, Micropropagation, Acclimatization

INTRODUCTION

The genus *Lycium* is a woody shrub that comprises approximately 97 species and is widely distributed in arid to semiarid regions in Eurasia, Africa, North America, and South America (Gong *et al.*, 2022). *Lycium* species have suitable physiological characteristics for drought and salt resistance making them suitable to combat desertification and rehabilitation (Dimitrova *et al.*, 2017). Out of several reported species of *Lycium*, *L. truncatum* Y.C. Wang, *L. ruthenicum* Murray, and *L. Potaninii* Pojark are grown in Mongolia. *L. truncatum* Y.C. Wang, belongs to the Solanaceae family and is distributed in a very limited habitat range in Mongolia, including the Depression of Great Lakes, East Gobi, and Transaltai Gobi, Alashan Gobi desert region. In Mongolia due to the consecutive drought, desertification, pasture degradation by livestock, and mining activity, the *L. truncatum* is more likely considered a rare woody shrub registered in the Mongolian Red Book and Mongolian Checklist of native vascular plants (Baasanmunkh *et al.*, 2022).

L. truncatum similar to the other *Lycium* species is rich in nutrients such as vitamin C, β-carotene, organic acids and tannins. The fruits of *Lycium* are known to have ophthalmic and diuretic effects. *L. barbarum* var. aurantiocarpum, *L. chinense* var potaninii, and *L. ruthenium*, are used for medicinal purposes and are considered superfoods. The root bark of *L. truncatum* is used as a medicinal ingredient in China (Yao *et al.*, 2018). Several studies have described the regeneration of the *Lycium* genus through adventitious shoot organogenesis and somatic embryogenesis. Moreover, the agrobacterium-mediated transformation has also been successful in obtaining transgenic plantlets on *L. barbarum* (Kairong*et al.*, 1999; Hu *et al.*, 2001, 2002, 2006). *L. barbarum* has received the most attention due to its high commercial value for its secondary metabolites. There are optimized protocols for *in vitro* propagation of *L. barbarum* through seedling or axillary shoot proliferation. *L. barbarum* was propagated *in vitro* on Murashige and Skoog (MS) medium supplemented with BAP, 6- furfurylaminopurine (KIN), or thidiazuron (TDZ) by axillary bud proliferation (de Oliveira Prudente *et al.*, 2019). In another study, the highest shoot formation of *L. barbarum* was achieved on MS medium

supplemented with 2.22 µM BAP and wheat starch instead of agar (Fira *et al.*, 2016). *L. barbarum in vitro* propagation protocol was also developed through axillary shoot proliferation where DKW medium supplemented with 0.5 mg/L BAP and 3% sucrose had the best results. The plantlets were dipped in 100 mg/L indole-3-butyric acid (IBA) solution to stimulate the growth of *ex-vitro* rooting (Silvestri *et al.*, 2018). The pre-existing meristems in nodal segments of *L. barbarum* allowed them to be an ideal explant as it helped to maintain the genetic uniformity of the species (Frabetti *et al.*, 2009).

The axillary bud and leaves of *L. ruthenicum* were studied to be a more suitable explant for the micropropagation of the species (Gao *et al.*, 2021). In a study, the optimal medium for axillary bud sprouting was MS supplemented with 1.0 mg/L BAP and 0.2 mg/L NAA. The optimal medium for proliferation was MS supplemented with 0.1 mg/L BAP and 0.2 mg/L NAA. The optimal medium for plantlet rooting was MS + 0.2 mg/L IBA, achieving a rooting rate of 98%. The suitable substrate was a mixture of nutritional soil, river sand, and vermiculite in a 2:1:1 ratio (Conghui *et al.*, 2019).

Currently, merely the biochemical characterization of fruits and genotype analysis of seed morphology of the *L. truncatum* have been studied (Zhurba *et al.*, 2021a, b). Despite the numerous efficient *in vitro* micropropagation studies on the *Lycium* genus, there is no direct technique for *L. truncatum*. Hence the present study was aimed to produce an efficient *in vitro* micropropagation and regeneration protocol of *L. truncatum* that can eventually help to preserve its genetic pool.

MATERIALS AND METHODS

Plant Material and Sterilization

Mature *Lycium truncatum* seeds were obtained for this study in August 2020 from the Umnugobi province, Mongolia (42°48'50.93"N, 107°0'11.48" E). To accelerate germination, seeds were sown in 200 µM of gibberellic acid (GA_3) solution for one hour followed by surface sterilizing in 70% (v/v) ethanol for 30 seconds, then 2.5% sodium hypochlorite (NaOCl) solution containing 2-3 drops of Tween-20 for 15 min, subsequently rinsed with sterile distilled water 5 times. The sterile seeds were germinated on half-strength MS medium (Murashige & Skoog, 1962) without plant growth regulators, supplemented with 3% (w/v) sucrose, and the pH of the medium was adjusted to 5.75-5.85 with 1 M KOH or HCl before adding 0.8% (w/v) of plant agar (PhytoTech Labs, USA).

Stock Explant Preparation

Initial seedlings (after 4 weeks of germination) were used to obtain an adequate number of explants for subsequent experiments. Micro-cut aseptic nodal segments with two to three nodes were cultivated on various MS mediums (full MS, ½ MS, ¼ MS) to determine appropriate culture conditions for *in vitro* establishments. In addition, the micro-cuttings were prepared with leaves or without leaves (with petiole fragments) and inserted vertically into the media. The regenerated explants (%), nodes per shoot (N), shoot length (cm), and spontaneously formed rooting rate were recorded after 4 weeks of culture.

Growth Regulators and Culture Conditions

All *in vitro* cultures were incubated in the growth room at 25±2 °C under a 16 h photoperiod with 60 µmol m^{-2} s^{-1} light intensity provided by cool-white fluorescent lamps and relative humidity of 48±2% for 4 weeks. The vessels used for seed germination, stock culture, and multiplication were 200 mL jars dispensed with 40 mL of medium. Five explants were subcultured per jar with 6 times replication, moreover, all the explant data were recorded after 4 weeks.

Shoot Multiplication

To determine a suitable shoot multiplication media, multiple plant-growing mediums (MS (Murashige & Skoog, 1962), DKW (Driver & KuniyukiWalnut, 1984), and Woody Plant Medium (WPM) (Lloyd & McCown, 1980) supplemented with various cytokinin with two different concentrations (0.1 mg/L; 1 mg/L) were tested.Initially, the cytokinin, BAP, KIN, N6-(2-isopentenyl) adenine (2-iP), zeatin (ZEA), or TDZ was added to the medium solely. After evaluating the effects of cytokinin, KIN was selected as the most effective cytokinin and combined with auxin, NAA, to improve the overall efficiency of plantlet regeneration. Accordingly, the combinations of 1 mg/L or 2 mg/L KIN with 0.1 mg/L NAA were evaluated.

Rooting and Acclimatization

For the explants proliferated through the shoot multiplication experiment, two to three nodal segments excised from shoots at the end of a 4-week proliferation period were used for root formation. To improve root morphology strength, a half-strength MS medium was supplemented with auxin, indole-3-acetic acid (IAA), IBA, or NAA, solely at two different concentrations (0.1 or 1 mg/L). Rooting media were prepared in 50 mL test tubes containing 20 mL of medium. *In vitro*, rooted plantlets were evaluated after 4 weeks of culture and transplanted one by one into a sterilized mixture of black soil, peat moss, and vermiculite and watered with 100 mL of ¼ MS solution in an aseptic condition. Furthermore, each pot with a plantlet was covered with polyethylene film to maintain high humidity and placed in an *ex-vitro* culture room at 26±2 °C. Ten days after transplanting, small holes have been made in the polyethylene covering to reduce humidity gradually. The acclimatization and survival rates were reported one month after the plants were grown in a greenhouse.

Statistical Analysis

Statistical data analysis was performed using monofactorial ANOVA and Tukey HSD test in Excel to compare each treatment (with a significance level set at $p<0.05$).

In our experiment 5 explants were inoculated per vessel with 6 times of replications. The average values obtained for each

vessel were used in statistical data analysis. The survival plantlets in each treatment were classified as regenerated explants, and the regeneration rates were expressed as as percentages. Any failed regenerated explants were marked as zero. Except for the shoot multiplication experiment with various cytokinin treatments, the number of nodes per shoot, shoot length, and shoot proliferation per explant were all recorded. However, in the shoot multiplication experiment, only regenerated explants and shoots per explant were counted.

RESULTS AND DISCUSSION

Stock Explant Preparation

In the initial trials, the *in vitro* seed germination rate was 54% and it was improved up to 80% by treatment with GA_3. Seedlings grown up to 8 cm, consisting of an average of 4.6 nodes per shoot (data not shown), moreover no contamination was recorded for 4 weeks of the experimental period.

Nodal segments are recommended for *in vitro* propagation due to pre-existing meristems, which can develop into shoots maintaining clonal fidelity (Frabetti *et al.*, 2009). We evaluated three different strengths of MS medium on two to three nodal segments with and without leaves (~1cm petiole section remaining). On the 10th day of the experiment, both explants with and without leaves grew spontaneous roots on different strengths of (whole MS, ½ MS, ¼ MS) MS medium. The rooting rate was fairly high (53.3-100%) Table 1, but the resulting roots were thin and brittle. For most explants when roots grew, the overall plantlets shoots and leaves regenerated well but in certain situations, roots formed but no shoots or height growth ensued. In treatments 1 to 3 (Table 1), the percentage of regenerating explants ranged between 73.3% to 86.7%. Treatments 4 to 6 showed a low percentage of regenerating explants, ranging between 46.7% to 66.7% (Table 1).

To evaluate the effectiveness of different treatments, the regenerated nodes for each explant and measured the height of the shoots we quantified. The highest number of nodes per regenerated shoots was 11.1 with leaf explant in full MS media, while the lowest number of nodes per regenerated shoots was 3.9 without leaf explant in ¼ MS medium (Table 1). In conclusion, the explants with leaves showed more regenerative ability than those without leaves; additionally, when cultivated on full-strength MS medium, explants appeared to be the most suitable for stock material production (Figure 1). Shoot multiplication was not observed in any of the different strengths of the MS medium tested. Furthermore,shoot regeneration ability and length were closely linked to root formation (Table 1).

Shoot Multiplication

To achieve shoot multiplication, various plant growth hormones, specifically cytokinin were subsequently tested. Several researchers have used MS medium supplemented with plant growth regulators (PGR) in different concentrations for *L. barbarum* and *L. chinense* species. The full-strength MS medium supplemented with BAP showed superior results than the other media (Fira *et al.*, 2016; Silvestri *et al.*, 2018).

In the first experiment, five different cytokinin hormones BAP, TDZ, KIN, ZEA, and 2-iP at doses of 0.1 mg/L and 1 mg/L were utilized to stimulate shoot multiplication on nodal segments consisting of two to three nodes. Table 2 displays the explant regeneration rates, average number of shoots per explant,and callus formation for each treatment. Whereas previous results on the other *Lycium* species, MS media supplemented with BAP and TDZ had a negative effect on shoot multiplication, resulting in hyper-hydricity in all explants. Shoots induced on MS medium containing the rest of the cytokinins KIN, ZEA, and 2-iP had slightly higher regeneration rates ranging from 20% to 50%. In all treatments, a callus formed at the base of each explant (Figure 2) which prevented the induction of shoot multiplication. The number of nodes in each shoot was high, but the overall length of the shoot was shorter. Furthermore, newly multiplied shoots have grown from the base callus or the lateral nodes and separated one shoot by one into the new medium. Shorter explants were transplanted into the rooting medium and taller shoots were excised with 2 to 3 nodes and passaged to the new shoot multiplication medium.

According to the results of the first shoot multiplication experiment, the regeneration rate was the best in MS medium supplemented with 1.0 mg/L of KIN (Table 2). In addition, since the cytokinin alone had less effect on the proliferation of *L. truncatum*, the combination of cytokinin and auxin for the next experiments were tested using KIN (1 mg/L or 2 mg/L) in combination with NAA (0.1 mg/L) on MS medium. This experiment increased the rate of shoot regeneration to 100%, yielding 1.7 to 1.9 shoots per explant (Table 3 & Figure 2).

The Italian researchers reported that shoot growth on MS, DKW, QL, and WPM supplemented with BAP 0.5 mg/L had a substantial influence on media mineral composition, with

Table 1: Regeneration of shoots with or without leaves on different strengths of MS medium

Explants	Treatment	Medium strength	Rooting rate (%)	Regenerated explants (%)	Nodes/shoot (N)	Shoot length (cm)
With leaves	1	¼ MS	80.0±11.5	73.3±6.7	9.3±0.3	6.5±0.7
	2	½ MS	93.3±6.6	80.0±0.0	10.4±1.0	7.7±0.5
	3	Full MS	100.0	86.7±6.7	11.1±1.1	9.7±0.9
Without leaves	4	¼ MS	53.3±24.0	46.7±6.7	3.9±1.8	4.3±1.2
	5	½ MS	80.0±11.5	60.0±0.0	6.9±1.4	7.3±0.5
	6	Full MS	80.0±11.5	66.7±13.3	9.7±3.0	7.9±1.5

Table 2: Effects of MS medium supplemented with cytokinin and TDZ on shoot proliferation

Medium	PGR, mg/L	Regenerated explants (%)	Shoot/explants (N)	Callus formation
Control	-	76.7[a]	1.7±0.6[b]	-
MS	BAP, 0.1	0[c]	1.0±0.0[b]	+
	BAP, 1.0	0[c]	1.0±0.0[b]	+
	TDZ, 0.1	3.3±3.3[c]	1.1±0.1[b]	+
	TDZ, 1.0	3.3±3.3[c]	1.1±0.1[b]	+
	Kin, 0.1	36.7±3.3[b]	1.4±0.1[b]	+
	Kin, 1.0	50.0±8.6[b]	1.5±0.1[b]	+
	Zea, 0.1	36.7±8.0[b]	1.4±0.1[b]	+
	Zea, 1.0	36.7±6.2[b]	1.3±0.1[b]	+
	2iP, 0.1	33.3±8.4[b]	1.3±0.1[b]	+
	2iP, 1.0	20.0±0.0[c]	1.1±0.1[b]	+

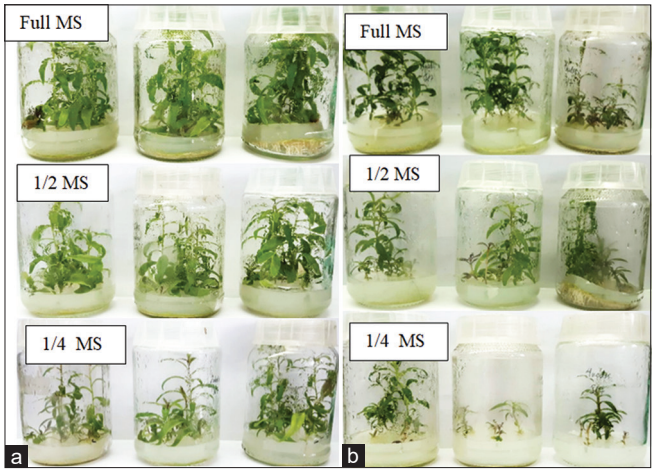

Figure 1: Regenerated explants with or without leaf (after 4 weeks). a) with leaf and b) without leaf

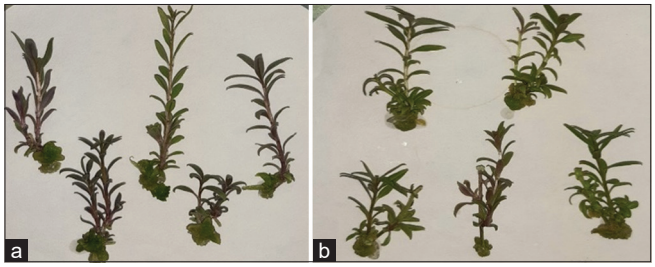

Figure 2: Effects of combined PGRs on shoot proliferation. a) Kin 1 mg/L + NAA 0.1 mg/Land b) Kin 2 mg/L + NAA 0.1 mg/L

the highest overall proliferation rates on the DKW medium (Silvestri *et al.*, 2018). Therefore, the same approach in our experiment to improve the multiplication rate were used. As a result, WPM had no significant effect as neither shoot regeneration nor shoot proliferation was observed. Mean shoots per explants were almost even on MS and DKW media ranging from 1.2 to 1.9 (Figure 3). Compared to other media, DKW showed significant shoot regeneration (86.6%), and shoot morphology and proliferation were abundant compared to other treatments (Table 4 and Figure 4). The mineral concentrations of the MS medium are too high for many woody species and increasing the number of subcultures provokes an increase of hyperhydricity, probably due to the ratio of NH_4^+ and NO_3^- of MS medium, a medium with high NH_4^+, which represents a great growth-limiting stress to plants (Silvestri *et al.*, 2018).

To improve shoot proliferation, a third experiment was conducted based on the results of first and second experiment showing that the combination of cytokinin and auxin positively affects shoot multiplication. Shoot regeneration and multiplication were achieved on DKW media and showed great results in the third experiment. Subsequently, DKW medium supplemented with 2 mg/L KIN in combination with 0.1 mg/L NAA showed highest result among the entire experiments. As a result, the regeneration of shoots of *L. truncatum* was 100% and shoots were 4.6 per explant (Figure 4). In conclusion, the regeneration and shoot multiplication was influenced by basal medium and the presence of cytokinin and auxin combination.

Rooting and Acclimatization

The rooting rate on hormone-free,different-strength MS mediums was very high (53.3-100%). However, the roots formed in the hormone-free medium were thin and brittle. Accordingly, different concentrations of auxins, IBA, IAA and NAA were used for strong root formation to facilitate acclimatization. Many authors have demonstrated that rooting takes place because of a series of physiological phases that are associated with changes in peroxidase activity and endogenous auxin concentrations, and often polyamine concentrations are used as markers of the rooting process (Rugini, 1992). As shown in Table 5, MS media supplemented with NAA at 0.1 mg/L showed the highest rooting rate of 80.4% among all treatments (Figure 5). Among the IBA treatments, a concentration of 1.0 mg/L showed the highest rooting rate of 73.3%. In reports dealing with other *Lycium* species, a possibility to induce shoot rooting with or without auxin treatment was observed. In most of the rooting stages different concentrations of IBA was used, as well as hormone-free half strength MS medium induced the root formation in certain cases as reported; IBA 0.3 mg/L - 82% (Ruta *et al.*, 2020), IBA 1 mg/L - 91.6% (Silvestri *et al.*, 2018), without PGR, *L. Barbarum* - 94% (Fira *et al.*, 2016), without PGR, *L. Ruthenicum* - 93.9% (Gao *et al.*, 2021).

Acclimatization or adapting plants in greenhouse/field conditions involves gradually moving to open-air conditions where the humidity is reduced and the light level is increased. This is a vulnerable stage for plantlet survival and can see large losses without proper acclimatization (Hazarika, 2003). Furthermore, contamination was one of the major obstructions to the survival rate. Therefore, the plantlets were transferred from *in vitro* to *ex vitro* in an aseptic condition in a laminar flow hood. *In vitro* conditions have high humidity and low light intensity. In order to reduce the stress induced in the plantlets, each plantlet was covered with polyethylene film to maintain high humidity and placed in an *ex vitro* culture room at 26±2 °C. After 10 days of transplanting, holes were poked into

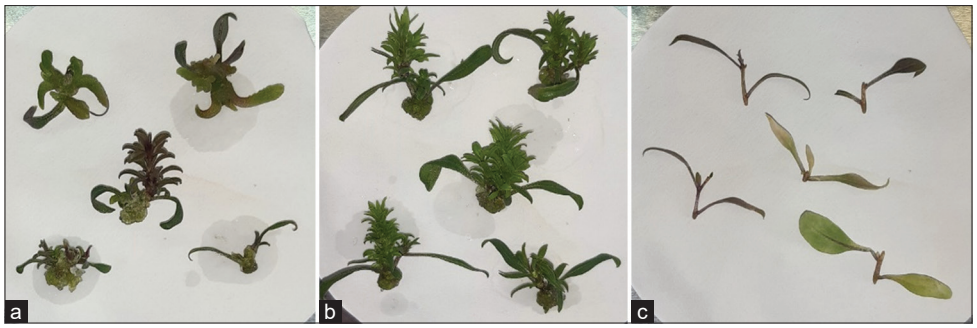

Figure 3: Effects of basal mediums on shoot proliferation. a) MS, b) DKW and c) WPM

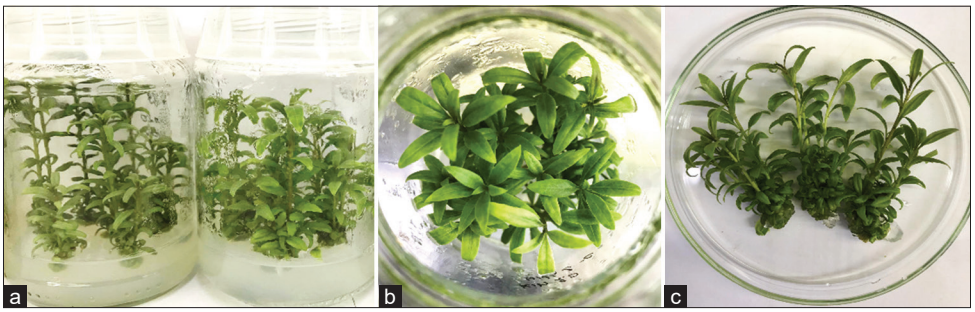

Figure 4: Effect of 2 mg/L Kin and 0.1 mg/L NAA in DKW basal medium on shoot proliferation

Figure 5: Plantlets of *Lycium truncatum* a) *in vitro* rooting, b) after transfer to soil and c) after 4 weeks of acclimatization

Table 3: Effects of MS medium supplemented with cytokinin and auxin on shoot proliferation

Medium	PGR, mg/L	Regenerated explants (%)	Shoot/explants (N)	Nodes/shoot (N)	Shoot length (cm)	Callus formation
Control	-	76.7[a]	1.7±0.6[b]	-	-	-
MS	Kin, 1.0+NAA, 0.1	100[a]	1.7±0.2[b]	7.1±2.0	2.7±0.5	+
	Kin, 2.0+NAA, 0.1	100[a]	1.9±0.3[b]	7.4±0.7	2.7±0.5	+

Table 4: The effect of basal media and PGRs onshoot multiplication

Nutrient medium	PGR, mg/L	Regenerated explants (%)	Shoot/explants (N)	Nodes/shoot (N)	Shoot length (cm)	Callus formation
Control (MS)	-	76.7± 0.0[a]	1.7± 0.6[b]	-	-	-
MS	BAP, 0.5	6.6± 4.2[c]	1.2± 0.1[b]	1.0± 0.0	-	++
DKW		86.6± 6.7[a]	1.9± 0.2[b]	4.2± 0.6	1.7± 0.5	+
WPM		0[c]	1.0± 0.0[b]	1.0± 0.0	-	-
DKW	Kin, 2.0 + NAA, 0.1	100[a]	4.6± 0.4[a]	6.5± 0.4	2.3± 0.1	+

the polyethylene film, gradually reducing the humidity. The polyethylene film was removed fully after 2 weeks. After 2-3 months the potted plantlets were transferred to the greenhouse and watered twice per week (Figure 5). The transplanting survival rate of *L. truncatum* plantlets was 91.7%.

Table 5: Auxin-related plant growth regulators effect on rooting of *Lycium truncatum*

PGR	mg/L	Rooting (%)	Root length (cm)
Control	-	56.7±3.1	8.9±2.3
IBA	0.1	63.3±0.6	11.2±1.2
	1.0	73.3±1.2	6.3±3.3
IAA	0.1	70.0±2.0	7.7±3.3
	1.0	60.0±2.7	8.1±4.3
NAA	0.1	80.4±0.6	11.1±2.3
	1.0	43.3±3.8	5.5±0.9

CONCLUSION

In this study, an effective micropropagation protocol was established for the Mongolian rare shrub of *L. truncatum* Wang, moreover, the results of this study will be effectively used for restoration after mining activity.

In conclusion, different concentrations of MS medium (full MS, ½ MS, ¼ MS) affect shoot regeneration and morphology differently. The explants grown on full MS medium thrived more than others. Furthermore, explants cultivated with leaves showed greater regenerative potential than those without leaves, therefore we chose the condition of explants with two to three nodes with leaves cultivating on complete MS medium for stock material preparation. However, when the explants recovered on the MS medium, no shoot multiplication was observed. To address this, various plant growth hormones, including cytokinin, were tested for their ability to promote shoot multiplication.

Among the tested five cytokinin, BAP and TDZ showed low regeneration activity which might be related to its synthetic characteristics. The addition of BAP hormone led to hyperhydricity in the explants' leaves (data not shown) and did not exhibit further growth. In contrast, the other three cytokinins demonstrated relatively higher activity in regeneration. Callus formation was observed at the base of each explant. The morphology of these regenerated with shorter internodes compared to the plants. Notably, the treatment with 1.0 mg/L KIN exhibited slightly higher regeneration activity and hence was chosen for further experiments in combination with auxin-related plant growth hormone. The newly proliferating shoots were higher on MS medium supplemented with 2.0 mg/L KIN and 0.1 mg/L NAA, and each shoot was excised and cultivated on a new medium for elongation.

After completing the initial screening and assessing the effects of plant growth hormones on shoot multiplication the influence of different basal media was examined. The DKW medium exhibited a 100% regeneration rate and the greatest multiplication rate 4.6 shoots per explant throughout the trial. In addition, the shoot morphology was improved, with longer lengths than those grown on MS medium. These explants can be excised into two to three nodal segments and effectively propagated. Proliferated shoots then rooted well on MS medium supplemented with 0.1 mg/L NAA, and successfully transplanted into the greenhouse.

To summarize, our research demonstrated that the multiplied explants on the DKW basal medium supplemented with 2.0 mg/L KIN and 0.1 mg/L NAA can be successfully transplanted *ex vitro* (grown in a greenhouse) and potential protocol for effective large-scale propagation of rare *L. truncatum*.

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Authors Contribution

Erdenetuul D, Selenge M, Uyanga B conducted all *in vitro* and acclimatization experiments. Kalaiselvi Senthil co-supervised and scripted the article. Oyunbileg Yu supervised the research project and provided the greatest intellectual contribution and Bolortuya U advised throughout the research process. Altantsetseg B supported by plant materials and project contribution.

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