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Establishment of Tissue Culture and Regeneration System in *Hippophae gyantsensis* Lian

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Abstract: *Hippophae gyantsensis* Lian is a pioneering tree species in Chinese forestry ecological engineering, known for its robust stress tolerance, water retention capacity, and soil improvement qualities. However, the lack of rapid nursery technology has been a significant impediment to the development of the *H. gyantsensis* industry. In the present study, we have successfully established a tissue culture regeneration system for *H. gyantsensis*. The most effective methods for seed disinfection, ensuring sterility in seedlings, were found to be 75% alcohol disinfection for 40 s and 10% sodium hypochlorite disinfection for 10 min. The best media tested for callus induction in cotyledons and hypocotyls of sterile seedlings were 1/3 MS + 0.5 mg/L KT + 0.75 mg/L NAA and 1/3 MS + 0.3 mg/L 6-BA + 1.5 mg/L IBA, respectively. As the explants, cotyledons yielded larger calli with a greater size and differentiation ability than hypocotyls. For the induction of indeterminate shoots and proliferation, the most suitable media were 1/3 MS + 0.5 mg/L IAA + 0.75 mg/L 6-BA and 1/3 MS + 1.0 mg/L 6-BA + 0.05 mg/L IBA + 0.1 mg/L KT, respectively. Lastly, the best worked rooting formulation was 1/4 MS + 0.3 mg/L IBA. This study marks a significant milestone in the establishment of a systematic tissue culture regeneration system for *H. gyantsensis*, which will facilitate the industrial rapid propagation of high-quality seedlings and provide the foundation for improvement through genetic transformation.

Keywords: *Hippophae gyantsensis* Lian; tissue culture regeneration; explant; media formulation

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1. Introduction

Hippophae gyantsensis Lian is a member of the genus *Hippophae* (seabuckthorn) in the family Elaeagnaceae [1], distributed in the riverbed gravel land or river floodplain at an altitude of 3500–3800 m exclusively in Tibet [2]. This heliophilous species is tolerant to cold, heat, salt, and drought with high soil adaptability, making it an excellent native tree for water retention, soil fixation, and reforestation of barren mountains [3]. As a non-leguminous woody nitrogen-fixing tree, it can form root nodules in symbiosis with *Frankia* to enrich the soil with nitrogen and enhance soil fertility [2], thus providing important ecological benefits. Furthermore, *H. gyantsensis* holds tremendous medicinal potential. Its roots, stems, leaves, flowers, and fruits contain flavonoids, vitamins, superoxide dismutase, and various other nutrients and active ingredients [4,5], which are capable of treating coronary heart disease, tumors, and other diseases [6], bringing it substantial value for both ecological and industrial applications.

Currently, the propagation of *H. gyantsensis* mainly relies on seedling and young branch cuttings [7,8]. The proportion of female plants propagated by seed is often lower than that of male plants, which is not in line with the intensive cultivation requirements and is unable to meet the social production demands. Furthermore, seabuckthorn explants have a limited range of options, with a diverse array of endophytic microorganisms that make thorough sterilization challenging. Through the adventitious regeneration system, small explant fragments can be utilized more easily for experimentation and propagation, without being constrained by the quantity of pre-existing buds. Combined with the potential of transformation for genetic improvement, a stable efficient, and rapid tissue culture propagation system for *H. gyantsensis* needs to be established.

There have been some reports on tissue culture of different species of seabuckthorn (*Hippophae rhamnoides* L.). The best explants for tissue culture of *H. rhamnoides* subsp. *sinensis* Rousi were the cotyledons of in vitro seedlings, and the highest shoot differentiation rate was achieved on 1/4 MS + 0.3 mg/L 6-BA + 0.002 mg/L NAA medium [9]. In the study of "YOUSHENG" of *H. rhamnoides* L. from Russia, the basal media for stem segments to induce adventitious shoots, calli, and roots were 1/2 MS, 1/3 MS, and 1/4 MS, respectively [10]. In the hybrid cultivar Julia (*H. r.* ssp *mongolica* × *H. r.* ssp. *rhamnoides*), it was found that TDZ significantly promoted the induction of adventitious shoots and the combination of TDZ and BA was able to promote shoot organogenesis [11]. In these seabuckthorns, it was found that under the same concentration, KIN induced somatic embryos better than BA, and the somatic embryogenesis and germination rates of cotyledons as explants were higher than those of leaves and hypocotyls [12]. Although the tissue culture regeneration system of many species of seabuckthorn has been established, none has been reported for *H. gyantsensis* to the best of our knowledge. Therefore, the present study explored the effects of different explants and different combinations of plant growth regulators on tissue culture regeneration based on aseptic seedlings cultivated from the seeds of *H. gyantsensis*, and we established a regeneration system to lay a foundation for the subsequent selection and improvement of *H. gyantsensis* resources.

2. Materials and Methods

2.1. Plant Material

The seeds of *Hippophae gyantsensis* Lian. were collected from one mother plant in Gyantse, Tibet, in 2022, and preserved in the laboratory of Nanjing Forestry University.

2.2. Cultivation of In Vitro Seedlings of H. gyantsensis

The seeds were thoroughly cleaned, immersed in warm water at 20~25 °C for 24 h, peeled off episperm, and then treated with different combinations of disinfection using 75% ethanol (Shandong Lierkang Medical Technology Co., Ltd., Dezhou, China) and sodium hypochlorite (4.5~5.0% active chlorine; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, CAS: 7681-52-9) (Table 1). The disinfected and drained seeds were inoculated on 1/2 MS medium (Murashige and Skoog medium (MS); Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China, CAS: HB8469-5), with 7.5 g/L agar (Guangzhou Saiguo Biotech Co., Ltd., Guangzhou, China, CAS: 9002-18-0). The size of the culture jars was 240 mL, the caliber was 6.5 cm, the diameter was 6.8 cm, and the height was 9.2 cm. About 50 mL of medium was distributed in the culture jars, and each bottle contained five seeds, for a total of 60 bottles. They were placed at 25 °C under a 16 h light/8 h dark cycle (LED light intensity of 2000 lx) in the room to obtain tissue culture seedlings for later study. After 20 days, the contamination rate (the number of contaminated seeds/the number of inoculated seeds) and germination rate (the number of in vitro seedlings/the number of seeds) of *H. gyantsensis* were counted. The experiment was repeated three times.

Table 1. Disinfection treatment of *H. gyantsensis*.

Treatment	Disinfection Method
A1	75% alcohol 30 s → rinse 3 times → 5% sodium hypochlorite 5 min → rinse 4 times
A2	75% alcohol 30 s → rinse 3 times → 5% sodium hypochlorite 8 min → rinse 4 times
A3	75% alcohol 40 s → rinse 3 times → 5% sodium hypochlorite 5 min → rinse 4 times
A4	75% alcohol 40 s → rinse 3 times → 5% sodium hypochlorite 8 min → rinse 4 times
A5	75% alcohol 40 s → rinse 3 times → 10% sodium hypochlorite 8 min → rinse 4 times
A6	75% alcohol 40 s → rinse 3 times → 10% sodium hypochlorite 10 min → rinse 4 times

2.3. Induction Culture of Callus

In the callus regeneration experiments, cotyledon and hypocotyl explants from 20-day-old seedlings germinated in vitro, were used. In the cotyledons, two cuts were made perpendicular to the main vein and the hypocotyls were cuts into 1–1.5 cm. Explants were inoculated into calli induction media with different combinations of plant growth regulators (cytokinin and auxin), including 6-benzylaminopurine (6-BA) or kinetin (KT), plus 3-indolebutyric acid (IBA), 1-naphthylacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) (Shanghai Macklin Biochemical Technology Co., Ltd., Shanghai, China) (Table 2). According to previous reports on callus induction in other *Hippophae* species [10,13,14], 1/3 MS was the best basal medium for most combinations, except that when 2,4-D was used alone, 1/4 MS worked better [9]. Therefore, 1/3 MS with 30 g/L sucrose and 6 g/L agar was used as the basal medium for the B1-B7 treatments, while 1/4 MS with 35 g/L sucrose and 7.5 g/L agar was used for the B8 and B9 treatments when 2,4-D was used alone. In each medium, 60 explants were inoculated (3 explants/jar). The cultures were placed in the dark at 24 °C in the culture room. The experiment was repeated three times. The induction rate (the number of explants forming callus tissue/the number of inoculated explants) of each treatment was recorded after 20 days.

Table 2. Different combinations of plant growth regulators for callus induction of *H. gyantsensis*.

Treatment	Plant Growth Regulators (mg/L)				
	Cytokinin		Auxin		
	6-BA	KT	IBA	NAA	2,4-D
B1	0.3	-	0.5	-	-
B2	0.3	-	1	-	-
B3	0.3	-	1.5	-	-
B4	0.3	-	-	0.5	-
B5	0.3	-	-	0.75	-
B6	-	0.5	-	0.3	-
B7	-	0.5	-	0.75	-
B8	-	-	-	-	0.3
B9	-	-	-	-	0.5

Note: Basal medium in treatments B1 to B7: 1/3 MS, 30 g/L sucrose, 6 g/L agar. Treatments B8 and B9: 1/4 MS, 35 g/L sucrose, and 7.5 g/L agar.

2.4. Induction and Proliferation of Adventitious Shoots

Six combinations of plant growth regulators, including 6-BA, IBA, IAA, KT, and NAA, were used in the treatment (Table 3). According to previous reports on callus induction in other *Hippophae* species [13,15,16], the combination of 6-BA (0.5 mg/L~0.75 mg/L) and IAA (0.5 mg/L) showed the best proliferation effect. And the concentration range was controlled from 0.1 to 0.5 mg/L for IBA, from 0.2 to 1.0 mg/L for KT, and from 0.004 to 0.2 mg/L for NAA. The calli were transferred to adventitious shoot induction media (1/3 MS with 30 g/L sucrose and 6 g/L agar as the basal medium). The cultures were placed in a 16 h light/8 h dark cycle at 24 °C in the culture room. Each bottle contained four callus pieces from different explants. The browning rate of the calli (the number of browned callus tissues/the number of total callus tissues) was calculated. Three replications of each

treatment were used in the proliferation culture, and the total number of calluses used per treatment was 96. After the adventitious shoots reached a height of about 1 cm, the shooting rate (the number of calli with induced adventitious shoots/the number of callus tissues) was measured after 60 days.

Table 3. Adventitious shoot differentiation and proliferation media for *H. gyantsensis*.

Treatment	Plant Growth Regulators (mg/L)				
	Cytokinin		Auxin		
	6-BA	KT	IAA	IBA	NAA
C1	0.5	-	0.5	-	-
C2	0.75	-	0.5	-	-
C3	1	-	0.5	-	-
C4	1	0.1	-	0.05	-
C5	1	-	-	0.5	-
C6	0.5	0.2	-	-	0.1

Note: C1 to C6 were based on 1/3 MS as the basal medium.

2.5. Rooting Induction of Adventitious Shoots

Different types and concentrations of 6-BA, IBA, and NAA were set up for treatment using 1/4 MS with 35 g/L sucrose and 6 g/L agar as the basal media (Table 4). The clumped seedlings of 2–4 cm in height and robust growth in the proliferation culture were selected, cut down singly, and transplanted into the root induction media. With ten bottles per treatment, three seedlings per bottle, and three repetitions, the total number of shoots used per treatment was 90. The rooting status of the adventitious shoots was observed, and the rooting rate (the number of adventitious shoots which induced roots/the number of adventitious shoots) was recorded after 40 days.

Table 4. Adventitious root differentiation and proliferation media for *H. gyantsensis*.

Treatment	Concentration of Plant Growth Regulators (mg/L)		
	Cytokinin	Auxin	
	6-BA	IBA	NAA
D1	-	0.5	0.3
D2	-	0.5	0.5
D3	0.3	0.5	-
D4	0.5	0.5	-
D5	-	0.3	-
D6	-	0.5	-

Note: D1 to D6 were based on 1/4 MS as the basal medium.

2.6. Statistical Analysis

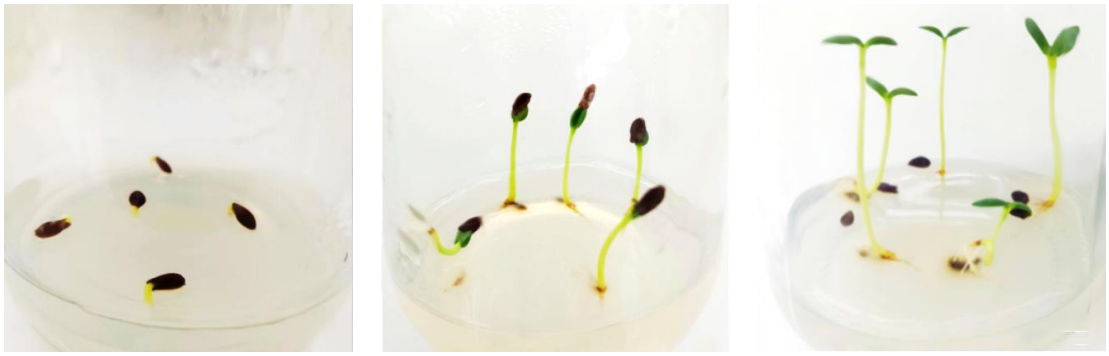
In this experiment, the data were subjected to analysis of variance and the significance of differences among the mean values was carried out using Duncan's multiple range test (DMRT) at $p < 0.05$ using SPSS software, version 19.0, and Microsoft Office Excel 2016. The results were expressed as the mean ± standard deviation (SD) of triplicates.

3. Results

3.1. Disinfection of Seeds for Germination of Hippophae gyantsensis

Seeds of *H. gyantsensis* Lian treated with different disinfection combinations were inoculated onto germination 1/2 MS medium. It was observed that the seeds sprouted after 3 days, the seed coats came off in about 15 days, and the cotyledons spread out in about 20 days (Figure 1). The rate of contamination-free seeds showed significant differences among the six different disinfection treatments, with a significant increase as the duration of disinfection increased (Table 5). The germination was not affected by the

different treatments since the uncontaminated seeds were all germinated at 100%. The best disinfection treatment combination was 75% alcohol disinfection for 40 s, followed by 10% sodium hypochlorite disinfection for 10 min, after which the treatment with 0% contamination could be observed (Table 5). All the seeds were germinated and the seedlings grew to 4–5 cm in 20 days. These seedlings of *H. gyantsensis* were obtained to be used as the starting material for the subsequent regeneration experiments.



(**a**) (**b**) (**c**)

Figure 1. The development process of in vitro germination of *H. gyantsensis*. (**a**) Sprouting at 3 days after incubation. (**b**) Seedlings at 15 days after incubation. (**c**) Seedlings at 20 days after incubation.

Table 5. Effects of different disinfection treatments on contamination of *H. gyantsensis*.

Treatment	Number of Seeds	Number of Contamination-Free Seeds	Contamination-Free Rate (%)
A1	50	41	82.00 ± 1.00 a
A2	50	42	84.00 ± 2.00 a
A3	50	45	90.00 ± 2.00 b
A4	50	45	90.00 ± 1.00 b
A5	50	48	96.00 ± 1.00 c
A6	50	50	100.00 ± 0.00 d

Note: A1 to A6 represent the treatment methods: (A1) 75% alcohol disinfection for 30 s and 5% sodium hypochlorite disinfection for 5 min; (A2) 75% alcohol disinfection for 30 s and 5% sodium hypochlorite disinfection for 8 min; (A3) 75% alcohol disinfection for 40 s and 5% sodium hypochlorite disinfection for 5 min; (A4) 75% alcohol disinfection for 40 s and 5% sodium hypochlorite disinfection for 8 min; (A5) 75% alcohol disinfection for 40 s and 10% sodium hypochlorite disinfection for 8 min; (A6) 75% alcohol disinfection for 40 s and 10% sodium hypochlorite disinfection for 10 min. The data presented were taken 20 days from the beginning of the incubation. Germination medium: 1/2 solid MS medium. Duncan's analysis was used, and different lowercase letters indicate the significant differences among the different treatments ($p < 0.05$, $n = 3$).

3.2. Callus Induction of H. gyantsensis

The cotyledons induced calli at about 10 days, after which the calli gradually proliferated and became larger along the incision site at about 20 days (Figure 2). The B1, B2, and B3 treatments, which were combinations of 6-BA and IBA, all had white-colored calli, and the callus induction rate increased with increasing IBA concentrations (Table 6). The calli of B4-B9 were all transparent-colored granules. B4 and B5 were combinations of NAA and 6-BA, and the calli browned slightly when the concentration of NAA was 0.5 mg/L. However, the growth of the calli decreased but did not turn brown when the concentration of NAA was reduced to 0.3 mg/L. B6 and B7 were combinations of KT and NAA, and these two combinations of cotyledons induced the best growth state of calli. As the concentration of NAA increased, the calli grew more, faster, and tighter without browning and had the potential to continue to differentiate. In addition, we found that cotyledons in the B1-B7 treatments produced a small number of adventitious roots with white fluff. B8 and B9 were the media with only the auxin 2,4-D added, which induced calli with fast growth and clumping at the initial stage, but with serious vitrification and browning, loose texture, and a very low differentiation rate at the later stage. In the case of the B8 medium with only 0.3 mg/L 2,4-D, the calli hardly differentiated at the later stage.

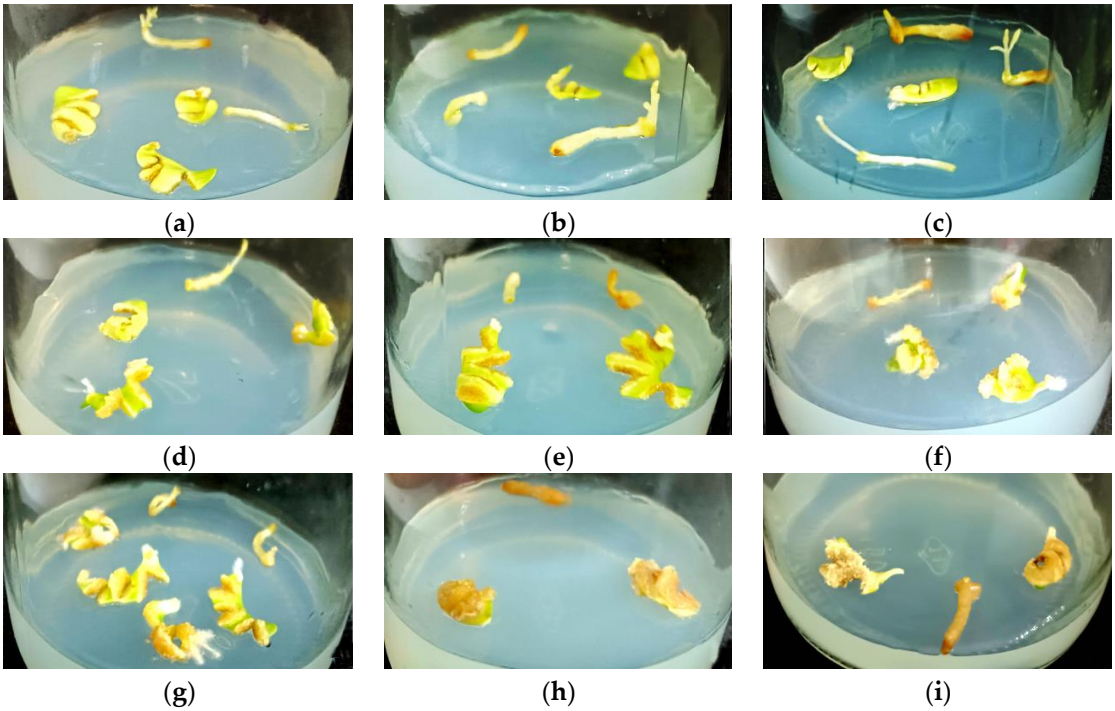


Figure 2. Calli of *H. gyantsensis* induced by different combinations of plant growth regulators. (**a**–**i**) Calli induced by B1–B9 media, respectively.

Table 6. Callus induction from the cotyledons of *H. gyantsensis* under different combinations of plant growth regulators.

Treatment	Days to Calli Appearing	Callus Induction Rates (%)	Development and Growth of Calli
B1	13	69.23 ± 10.14 c	Small amount
B2	12	90.00 ± 3.27 b	Small amount
B3	12	100.00 ± 0.00 a	Small amount, loose, transparent
B4	12	100.00 ± 0.00 a	Transparent color of callus at the incision site
B5	13	100.00 ± 0.00 a	Transparent-colored, dense, and slightly browned callus at the incision site
B6	13	100.00 ± 0.00 a	Callus at the incision site, transparent color
B7	7	100.00 ± 0.00 a	Best growth, dense, transparent granular
B8	8	100.00 ± 0.00 a	Loose, browning fastest, and no differentiation in later stages
B9	8	100.00 ± 0.00 a	Loose, browned and vitrified

Note: B1 to B9 represent different treatments: (B1) 0.3 mg/L 6-BA + 0.5 mg/L IBA; (B2) 0.3 mg/L 6-BA + 1.0 mg/L IBA; (B3) 0.3 mg/L 6-BA + 1.5 mg/L IBA; (B4) 0.3 mg/L 6-BA + 0.5 mg/L NAA; (B5) 0.3 mg/L 6-BA + 0.75 mg/L IBA; (B6) 0.3 mg/LNAA + 0.5 mg/L KT; (B7) 0.75 mg/LNAA + 0.5 mg/L KT; (B8) 0.3 mg/L 2,4-D; (B9) 0.5 mg/L 2,4-D. Data taken after 20 days. Different lowercase letters among treatments indicate significant differences according to Duncan's analysis ($p < 0.05$, $n = 3$).

The hypocotyls were obviously expanded in the B3 medium, and the calli showed a transparent state, with the best effect of proliferation and differentiation in the later stage. The phenomenon of inducing the calli of hypocotyls in B8 and B9 was the same as that of the cotyledons, with rapid growth in the early stage, and serious browning in the later stage, which might have led to the death of the explants directly. The B9 medium with the addition of 2,4-D at 0.5 mg/L showed the slight emergence of axillary buds but very slow growth in the later stages. Except for the B6 and B8 combinations, which did not have axillary buds sprouting during the callus induction, the axillary buds of all the combinations started sprouting at around 5 days (Figure 2; Table 7).

Table 7. Callus induction from the hypocotyls of *H. gyantsensis* under different combinations of plant growth regulators.

Treatment	Days to Callus Appearing	Average Shoot Height at 20 Days	Callus Induction Rates (%)	Status of Callus Growth and Development
B1	6	1	100.00 ± 0.00 a	Stems slightly expanded at both ends
B2	5	0.4	100.00 ± 0.00 a	Stem segments expanded
B3	5	1.7	100.00 ± 0.00 a	Stem segments expanded noticeably, sprouting quickly
B4	5	0.3	100.00 ± 0.00 a	Stem segments expanded and callus transparent
B5	4	0.2	100.00 ± 0.00 a	Stem segments expanded, easily browned, and callus transparent
B6	0	0	100.00 ± 0.00 a	Stems expanded at both ends and transparent
B7	2	0.2	100.00 ± 0.00 a	Stems slightly expanded at both ends and transparent
B8	0	0	100.00 ± 0.00 a	Initial stem segments visibly expanded, transparent, and browning fastest
B9	0	0	100.00 ± 0.00 a	Initial stem segments expanded, transparent, and browning fast

Note: B1 to B9 represent different treatment methods: (B1) 0.3 mg/L 6-BA + 0.5 mg/L IBA; (B2) 0.3 mg/L 6-BA + 1.0 mg/L IBA; (B3) 0.3 mg/L 6-BA + 1.5 mg/L IBA; (B4) 0.3 mg/L 6-BA + 0.5 mg/L NAA; (B5) 0.3 mg/L 6-BA + 0.75 mg/L IBA; (B6) 0.3 mg/LNAA + 0.5 mg/L KT; (B7) 0.75 mg/LNAA + 0.5 mg/L KT; (B8) 0.3 mg/L 2,4-D; (B9) 0.5 mg/L 2,4-D. The data presented were taken 20 days from the beginning of the callus induction. Duncan's analysis was used, and different lowercase letters indicate the significant differences among the different treatments ($p < 0.05$, $n = 3$).

The results show that both the cotyledons and the hypocotyls had a high callus induction rate. According to the time and rate of calli induction, the best medium for the cotyledon induction of *H. gyantsensis* calli was B7: 1/3 MS + 0.5 mg/L KT + 0.75 mg/L NAA, and for hypocotyl calli induction, it was B3: 1/3 MS + 0.3 mg/L 6-BA + 1.5 mg/L IBA. Compared to the hypocotyls, the cotyledons produced larger calli, and the number of cotyledons and yield were higher in the factory nursery process. Therefore, the cotyledons could be chosen as the explants in the tissue culture for the rapid propagation system of *H. gyantsensis*, which is more efficient and high-yielding.

3.3. Induction and Proliferation of Adventitious Shoots of H. gyantsensis

The calli were selected and transferred to the adventitious shoot induction medium, and the cotyledon and hypocotyl calli both grew further, appearing as light green and tightly clustered, leading to the formation of green adventitious shoot spots. This gradually developed into adventitious shoots with leaves (Figure 3a–f). This indicates that the six different treatments significantly affected the induction and proliferation of the adventitious shoots of *H. gyantsensis* (Table 8; Figure 3). C1, C2, and C3 were the combinations of 6-BA and IAA. When the 6-BA concentration was 0.75 mg/L, the hypocotyl calli of *H. gyantsensis* proliferated and differentiated the fastest, with the largest volume, the largest number of green shoots, and the highest proliferation coefficient (up to 52.6%). C4 and C5 compared the effects of the auxins IBA and KT, and it was found that the C4 medium with KT was characterized by faster and more differentiation of calli, relatively vigorous growth of adventitious shoots, and the lowest browning mortality rate in the C4 medium compared to that without KT. In C6, the adventitious roots continued to grow due to the addition of NAA, and the root tips were blackened at a later stage, thus reducing the rate of differentiation of the calli.

When the adventitious shoots increased to more than 1.5 cm, they were transferred to the proliferation medium (C4) and could grow into small shoots after 30 days (Figure 3g). Each hypocotyl-induced callus could produce 1–2 shoots, with 1 in most cases, while each cotyledon-induced callus could produce 3–5 shoots. Therefore, the best medium for inducing the adventitious shoot from calli was C2: 1/3 MS + 0.5 mg/L IAA + 0.75 mg/L

6-BA, and the best proliferation medium was C4: 1/3 MS + 1.0 mg/L 6-BA + 0.05 mg/L IBA + 0.1 mg/L KT.

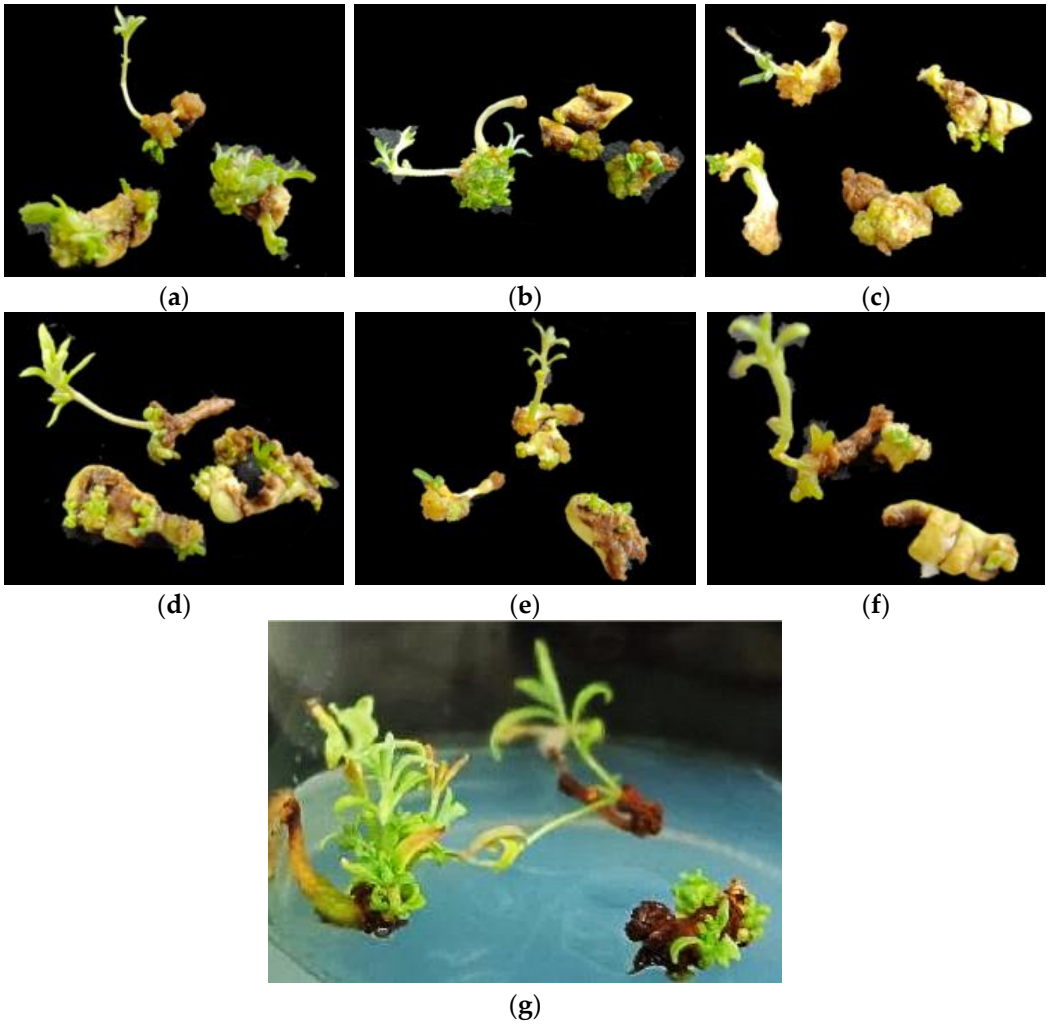


Figure 3. Induction and proliferation of adventitious shoots of *H. gyantsensis* under different treatments. (**a**–**f**) Adventitious shoots grown under the C1–C6 treatments, respectively. (**g**) Multiplication seedlings of *H. gyantsensis*.

Table 8. The effects of different combinations of plant growth regulators on the shoot differentiation from cotyledon- and hypocotyl-induced calli of *H. gyantsensis*.

Treatment	Shooting Rate (%)	Browning Mortality (%)	Growing Status
C1	28.60 ± 3.76 c	25.00 ± 4.58 a	Good growth of adventitious shoots
C2	52.60 ± 8.77 a	22.20 ± 1.93 b	Best differentiation of indeterminate buds of epiblast healing tissues, and the highest number of shoots.
C3	29.40 ± 7.65 c	19.00 ± 3.47 c	Average growth of adventitious shoots
C4	46.20 ± 7.34 b	16.70 ± 3.10 b	Suitable for proliferative culture, with many indeterminate bud spots and good growth in later stages
C5	45.60 ± 2.57 b	26.70 ± 1.39 a	Average growth of adventitious shoots
C6	24.10 ± 3.35 d	26.50 ± 3.29 a	Continued growth of adventitious roots and embryoid production from hypocotyl healing tissue

Note: Duncan's analysis was used, and the different lowercase letters indicate the significant differences among the different treatments ($p < 0.05$, $n = 3$). C1 to C6 represent the different treatment methods: (C1) 0.5 mg/L 6-BA + 0.5 mg/L IAA; (C2) 0.75 mg/L 6-BA + 0.5 mg/L IAA; (C3) 1.0 mg/L 6-BA + 0.5 mg/L IAA; (C4) 1.0 mg/L 6-BA + 0.5 mg/L IAA; (C5) 1.0 mg/L 6-BA + 0.05 mg/L IBA + 0.1 mg/L KT; (C6) 0.5 mg/L 6-BA + 0.2 mg/L KT + 0.1 mg/L NAA. The data presented were taken 60 days from the beginning of the adventitious shoot induction.

3.4. Induction and Proliferation of Adventitious Roots of H. gyantsensis

Vigorously growing shoots were inoculated into various rooting media, and significant differences in the rooting rate were observed (Table 9; Figure 4). In D1, D2, and D4 media, no roots were produced. In the D3 medium, white roots were observed emerging from the base of the shoots after 20 days. Although the developed root system appeared robust, the rooting process was slow, and there was evidence of proliferating callus tissue in the root zone. This phenomenon exerts inhibitory effects on the rooting induction process, leading to slow growth and gradually deteriorated condition of the resulting plantlet in the later stage, with yellowing and withering of the leaves, which did not meet the requirements for fast tissue culture propagation. In the D5 and D6 treatments, in which IBA was used, the rooting rate was as high as 77.8% when the IBA concentration was 0.3 mg/L, and roots emerged after 12 days. In the D5 treatment, two primary roots were differentiated per shoot on average, with a 1.5 cm root length achieved in 30 days. When the lateral roots were formed, compared to D6, the roots of D5 were longer and slimmer and the growth of the root system and the plantlets was optimal. Therefore, the most suitable medium for rooting induction was D5: 1/4 MS + 0.3 mg/L IBA.

Table 9. Effects of different plant growth regulators on rooting of *H. gyantsensis* shoots.

Treatment	Days to Root Formation	Rooting Rate (%)	Average Root Length (cm)
D1		0	0
D2		0	0
D3	20	50.00 ± 0.77 b	1.2
D4		0	0
D5	13	77.8 ± 0.23 a	1.5
D6	15	33.3 ± 0.48 c	0.8

Note: D1 to D6 treatments: (D1) 0.5 mg/L IBA + 0.3 mg/L NAA; (D2) 0.5 mg/L IBA + 0.5 mg/L NAA; (D3) 0.3 mg/L 6-BA + 0.5 mg/L IBA; (D4) 0.5 mg/L 6-BA + 0.5 mg/L IBA; (D5) 0.3 mg/L IBA; (D6) 0.5 mg/L IBA. Data taken after 40 days. In the rooting rate column, different lowercase letters indicate the significant differences among the treatments according to Duncan's analysis ($p < 0.05$, $n = 3$).

(**a**) (**b**) (**c**)

Figure 4. Rooting of *H. gyantsensis* shoots under different treatments: (**a**) D3 (0.3 mg/L 6-BA + 0.5 mg/L IBA), (**b**) D5 (0.3 mg/L IBA), and (**c**) D6 (0.5 mg/L IBA) treatments in Table 9, respectively.

4. Discussion

4.1. Cultivation of In Vitro Seedlings of H. gyantsensis

In the seabuckthorn tissue culture regeneration system, various explants can be chosen, including wild stem segments with axillary buds, stem tips, and water-cultivated leaves as explants [17]. Alternatively, the cultivation can begin from seed-grown in vitro seedings. Xu [9] and Yang [18] used *H. rhamnoides* in vitro seedlings to establish a tissue culture

system, which not only eliminated the obstacle of wild sampling leading to sterilization difficulties but also improved the tissue culture success rate. After disinfection with mercury dichloride solution, the seeds were inoculated into a 1/2 MS medium, resulting in a germination rate of 60%, with the seedlings being weak and exhibiting slow growth [18]. Additionally, following disinfection with 75% alcohol and mercury dichloride for 10 min, there were notable differences in the seed germination across the MS, 1/2 MS, and 1/4 MS culture media. Under identical cultivation conditions, the seeds exhibited the highest germination rate in the 1/2 MS medium at 16.67%, followed by 1/4 MS, with a germination rate of 13.33%. In contrast, the germination rate in MS was observed to be 3.33% [19].

Therefore, in this study, we cultivated in vitro seedlings of *H. gyantsensis* and used different tissues of the in vitro seedlings as the explants for tissue culture experiments, which greatly reduced the contamination rate and improved the experimental efficiency. For the seeds used, the best disinfection was 75% alcohol for 40 s and 10% sodium hypochlorite for 10 min; the seeds exhibited a remarkable 100% germination rate. In the in vitro seedling cultivation process of *H. gyantsensis*, 1/2 MS was used as the basic medium, and the massive element concentration was halved to be more suitable for seed germination, which is consistent with the studies of Liu [19] and Zheng [20].

4.2. Effects of Different Explants on the Callus Induction of H. gyantsensis

There have been many reports on *Hippophae* L. tissue culture regeneration using different explants. Axillary buds, stem segments, and leaves of plants have been selected as explants for research, but there has not been any report on *H. gyantsensis* [15]. Lv [21] discovered that the germination rate of stem tip explants consistently exceeded 90% in the culture medium of 1/2 B_5 + 0.5–1.0 mg/L 6-BA + 0.3~0.5 mg/L IAA. The study revealed that apical shoot explants in the culture medium of 1/2 MS + 0.3–0.5 mg/L KT + 0.03–0.05 mg/L NAA achieved an axillary bud induction rate of 85% [17]. In another study, Liu [22] inoculated in vitro seedings segments in 1/2 MS + 0.5–1.0 mg/L 6-BA + 0.5 mg/L IAA, and the callus induction rate exceeded 91.3%. Liu [23] found that the most suitable culture medium for callus induction in *H. rhamnoides* subsp. *Sinensis* and *H. rhamnoides* subsp. *Mongolica* consists of 1/3 MS + 6-BA 0.5 mg/L + IBA 1.0 mg/L, with an induction rate reaching 75.9%.

In this study, the cotyledons and hypocotyls of the in vitro seedlings of *H. gyantsensis* were selected as the explants for the experiment. The results show that both explants formed calli in about 10 days at a high rate. Except for the cotyledons on the 1/3 MS medium supplemented with 0.3 mg/L 6-BA and 0.5 mg/L IBA, and 0.3 mg/L 6-BA and 1.0 mg/L IBA, all reached 100%. But the cotyledon calli were larger in size and more capable of differentiation, and the following adventitious shoots grew robustly and vigorously compared to the shoots differentiated from the hypocotyl calli. Therefore, the cotyledons of the in vitro seedlings are more suitable as explants for callus induction in the *H. gyantsensis* tissue culture regeneration system. It is worth noting that the somaclonal variation can be obtained through this indirect organogenesis [24], which should be considered in the process of propagation and breeding.

4.3. Effects of Different Plant Growth Regulators on the Tissue Culture of H. gyantsensis

In this study, it was found that the cotyledons and hypocotyls of in vitro seedlings of *H. gyantsensis* formed calli quickly in medium with only 2,4-D, but at the same time, browning followed soon and could not be differentiated. This result is in agreement with the conclusions of Li [14] and Xu [9]. In the study on "YOUSHENG" of *Hippophae rhamnoides* L. from Russia [10], it was found that when IAA was 0.2 mg/L and 6-BA was 1 mg/L or 0.5 mg/L, the callus induction rate of explants was high, but they all browned and died at 20 days. When the concentration of 6-BA was less than 0.5 mg/L, the calli were in good condition. Therefore, in our experiment, the 6-BA concentration was controlled at 0.3 mg/L when inducing calli, which effectively inhibited browning.

A study on *Hippophae rhamnoides* 'Real gifted one' using stem segments with dormant buds as explants found that the best medium for proliferation and callus induction was 1/3 MS + 0.75 mg/L 6-BA + 0.5 mg/L IAA, and the induction rate was as high as 91% [25], which is consistent with our results. In addition, we found that the proliferation and differentiation rate of the callus decreased when the concentration of 6-BA was greater than 0.75 mg/L. Hybrids of seabuckthorn grew best at WPM + 0.6 mg/L BA + 0.03 mg/L IBA, and the rooting rate can reach 100% [26]. In our study, we tried the combination of 1/3 MS with 1.0 mg/L 6-BA and 0.05 mg/L IBA and found that the medium with a reduced IBA concentration combined with KT showed the best proliferation. Also, the combination of 6-BA, KT, and NAA (C6) was tried, and the results show that embryoids were produced in the hypocotyl near the cotyledon end.

The rooting of seabuckthorn varieties 'Ze liang' and 'Xiang yang' with 1/2 B5 + 0.15–0.18 mg/L 6-BA + 0.2 mg/L IBA and 1/2 B5 + 0.5 mg/L KT + 0.03–0.07 mg/L NAA were preferred; the rooting rates were 90% and 86.67%, respectively [27]. The seabuckthorn variety 'Real gifted one' is better with 1/2 B5 + 0.1 mg/L 6-BA + 1.0 mg/L NAA, and a rooting rate of 85% was reached [28]. In this study, two combinations of IBA and NAA were designed, and it was found that none of the seedlings of *H. gyantsensis* rooted, but all of them rooted in the medium with only IBA, which indicate that IBA is more suitable for the root growth of *H. gyantsensis*. In the formulation of 1/4 MS + 0.3 mg/L 6-BA + 0.5 mg/L IBA, the root system of *H. gyantsensis* also grew, but the root growth was slow, and the plant proliferation phenomenon affected the normal growth of the root system, so the most suitable media for the root induction of *H. gyantsensis* was 1/4 MS + 0.3 mg/L IBA. In order to improve the rooting rate, Xu soaked the bases of regenerated *Hippophae rhamnoides* subsp. *sinensis* Rousi plants in the rooting agent solution for 15 min and then transferred them to the rooting medium, which not only improved the rooting rate but also shortened the rooting time significantly and developed the root system [9]. Therefore, further study can focus on the most suitable rooting agent solution and soaking time for *H. gyantsensis* to promote root growth.

5. Conclusions

To establish an efficient regeneration system for tissue culture, an experiment was carried out with *H. gyantsensis* as the material under a variety of plant growth regulators types and concentrations. This study established the best disinfection method for sterile seedlings. The best culture media were determined for calli, adventitious shoots, and adventitious roots using the cotyledons and epicotyls of the sterile seedlings as explant materials. In general, this is the first time the tissue culture regeneration system of *H. gyantsensis* has been established, which lays the foundation for the subsequent genetic improvement of *H. gyantsensis* and provides a reference for the nursery factory.

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