



## Construction of Plant Regeneration System of *Glycyrrhiza mongolicum* and *Glycyrrhiza Xinjiang*

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**Abstract** In order to establish the plant regeneration system of *Glycyrrhiza mongolicum* and *Glycyrrhiza Xinjiang*, this study used hypocotyls, leaves, cotyledons and stems to inducing callus and regenerated plant, and it screen out the most suitable explants and the best medium for plant regeneration. The experimental results showed that MS+2.0 mg/L NAA+2.0 mg/L 6-BA was the best medium for callus induction and plant regeneration. Among these, hypocotyls and cotyledons are the best explants for callus induction. The tissue induction rate is higher than 90%. The rapid propagation system established in this study can provide the theoretical reference and practical guidance for the breeding and production of fine *Glycyrrhiza uralensis* Fisch. varieties.

**Keywords** *Glycyrrhiza uralensis* Fisch.; Sterile seedlings; Callus; Regenerated plants

*Glycyrrhiza uralensis* Fisch. is a plant of the genus *Glycyrrhiza* in the leguminous family, with the reputation of "ten directions and nine herbs". Its medicinal part is the root, its medicinal properties are mild, its nature is sweet, and it has medicinal values such as moisturizing the lungs and relieving cough, invigorating the spleen and qi (Chen et al., 2016; Jiang et al., 2017, Chemical Industry Times, 31(7): 25-28.; Li and Li, 2019). *G. uralensis* extract is also a good sweetener, it is widely used in the food industry (Karaođul et al., 2016), skin disease treatment (Song, 2019, Chinese Medicine Modern Distance Education of China, 17(8): 52-54) and tumor treatment (Wang et al., 2019) and other fields. It is a tonic Chinese herbal medicine (Liu et al., 2020). *G. uralensis* is widely distributed in many regions of China, mostly growing in arid and desert semi-desert regions, among which the *G. uralensis* is most widely distributed in Ningxia, Gansu, Xinjiang and Inner Mongolia (Liu et al., 2012; Wang Q. et al., 2018, Grass-Feeding Livestock, (2): 52-56). As an important bulk herbal medicine, *G. uralensis* has always been in great demand (Li et al., 2018). Because of its important medicinal value and extremely high ecological value, *G. uralensis* has always attracted much attention. However, due to overexploitation for a long time, the current wild resources are facing brown, and the limited cultivated land area greatly restricts the planting of *G. uralensis* and the breeding and seed collection of excellent varieties. The natural cultivation period of *G. uralensis* is long, and the seed circulation of *G. uralensis* is large and susceptible to natural disasters and mixed provenance and species, which leads to uneven seedling emergence and different quality of medicinal materials of *G. uralensis*. It is difficult to ensure the germplasm resources of *G. uralensis*, leading to a sharp decline in the quality and yield of *G. uralensis* (Zhao et al., 2011; Liu et al., 2013; Zhou et al., 2012, Journal of Anhui Agricultural Sciences, 40(19): 10065-10066). Therefore, it is urgent to explore the technology of rapid and efficient breeding of *G. uralensis*.

So far, many plants have used tissue culture technology to establish seedling breeding systems, such as *Epimedium pubescens* Maxim. (Fu et al., 2019), *Astragalus membranaceus* (Fisch.) Bunge. (Guo et al., 2020) and *Nicotiana tabacum* L. (Wang et al., 2017). Among them, the tissue culture of *G. uralensis* has been studied. The use of tissue culture technology to establish a rapid propagation system of *G. uralensis* is beneficial to increase the propagation speed of *G. uralensis*, the extraction of effective ingredients and the preservation of good varieties (Li et al., 2013). At present, some scholars have established the foundation for tissue culture (Yang et al., 2014). Some

scholars have found that *G. uralensis* explants can form callus under the induction of different hormones, and have screened the optimal culture medium for different explants to induce callus (Zhao and Lin, 2017); Previous studies have found that the hypocotyl at different explants induction rates is conducive to the induction of callus (Fan et al., 2009). Callus has the ability to develop into complete plants, so whether the callus can be subcultured and developed into regenerated plants is the key to achieving plant mass reproduction. At present, Although the tissue culture of *G. uralensis* has been reported, the rapid propagation and conservation of *G. uralensis* have not been reported. Among them, the tissue culture and rapid propagation system of *Glycyrrhiza glabra* L. have not been reported, so this study further optimized the aseptic and rapid propagation system of *G. glabra* in the local areas (Inner Mongolia and Xinjiang) based on the previous research on the rapid propagation of *G. uralensis* tissues, in order to speed up the *G. uralensis* sterile breeding, maintain good varieties of medicinal properties, thus promote the industrialization development of *G. uralensis*, and provide the theoretical basis for the breeding efficiency of *G. uralensis* and the preservation, development and utilization of the superior varieties.

## 1 Results and Analysis

### 1.1 Sterile seedling induction rate and pollution rate

The seeds of *G. mongolicum* and *G. Xinjiang* were inoculated into sterilized MS basic medium respectively after being treated with 8% sodium hypochlorite solution. During the culture of the sterile seedling, the growth, induction rate and pollution rate of the two *G. uralensis* were observed and counted (Table 1). After 30 days of culture, sterile seedlings from the seeds were obtained (Figure 1).

### 1.2 Callus induction rate of different tissues of *G. mongolicum*

The hypocotyls, leaves, cotyledons and stems of *G. mongolicum* were cultured in callus induction medium with different hormone ratios (NAA and 6-BA) (Figure 2). After 15 days of culture, light green loose callus grew at the incisions of the explants, and both ends of cotyledons and stems gradually expanded (Figure 3). The results show (Table 2) that the induction rate of stem and hypocotyl was higher in MS+0.5 mg/L NAA+0.5 mg/L 6-BA hormone induction medium; the induction rate of cotyledon and hypocotyl was higher in MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA hormone induction medium, and the callus formed by cotyledon and hypocotyl grew well.

Table 1 The induced and polluted rates of *G. mongolicum* and *G. Xinjiang*

<i>G. uralensis</i>	Growth of sterile seedling			
	The total number of seeds	Number of germinated seeds	Induction rate of sterile vaccine (%)	Pollution rate of sterile vaccine (%)
<i>G. mongolicum</i>	63	54	85.7	3.1
<i>G. Xinjiang</i>	63	52	82.5	1.6

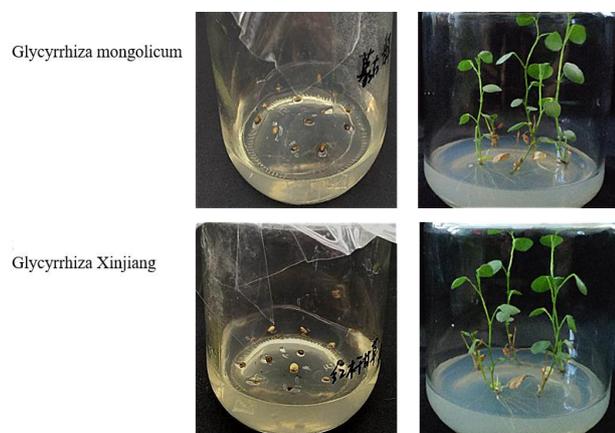


Figure 1 Acquisition of sterile seedlings

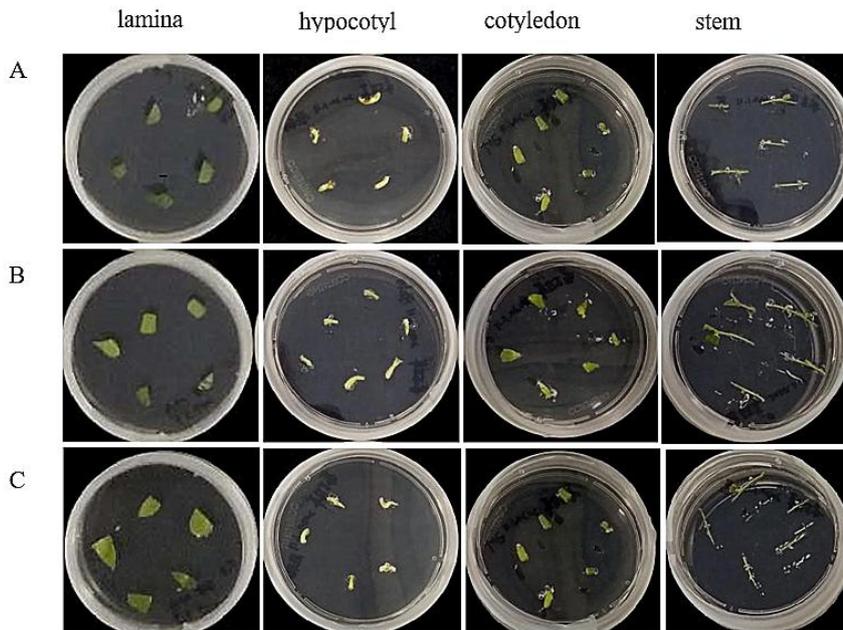


Figure 2 Callus induction from different explants of *G. mongolicum*

Note: A: MS+0.5 mg/L NAA+0.5 mg/L 6-BA; B: MS+1.0 mg/L NAA+1.0 mg/L 6-BA, C: MS+2.0 mg/L NAA+2.0 mg/L 6-BA

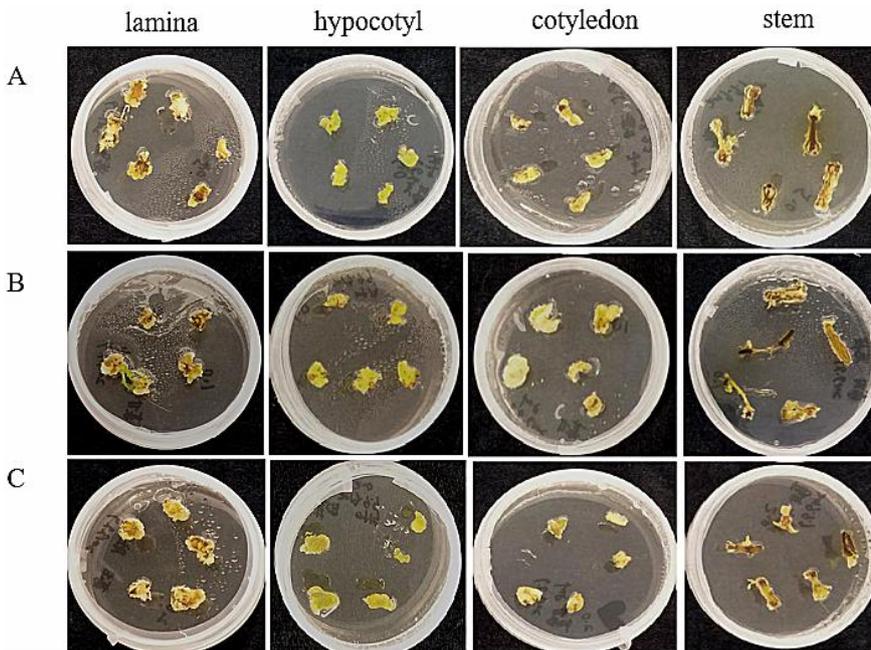


Figure 3 Hormone induction of different ratio of *G. mongolicum*

Note: A: MS+0.5 mg/L NAA+0.5 mg/L 6-BA; B: MS+1.0 mg/L NAA+1.0 mg/L 6-BA, C: MS+2.0 mg/L NAA+2.0 mg/L 6-BA

Table 2 Callus induction rates of different explants of *G. mongolicum*

Explant	Hormone-induced medium		
	MS+0.5 mg/L NAA+0.5 mg/L 6-BA (%)	MS+1.0 mg/L NAA+1.0 mg/L 6-BA (%)	MS+2.0 mg/L NAA+2.0 mg/L 6-BA (%)
Cotyledon	69.2	93.3	92.9
Leaf	60	35.7	60
Stem	87.5	61.1	65
Hypocotyl	100	100	100

### 1.3 Callus induction rate of different tissues of *G. Xinjiang*

The hypocotyls, leaves, cotyledons and stems of *G. Xinjiang* were cultured in callus induction medium with different hormone ratios (NAA and 6-BA) (Figure 4). After 15 days of culture, light green loose callus grew at the incisions of the explants, and both ends of cotyledons and stems gradually expanded (Figure 5). The results (Table 3) show that the induction rate of stem and cotyledon was higher in MS+0.5 mg/L NAA+0.5 mg/L 6-BA hormone ratio induction medium; the induction rate of cotyledon and hypocotyl was higher in MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA hormone induction medium, and the callus formed by cotyledon and hypocotyl grew well.

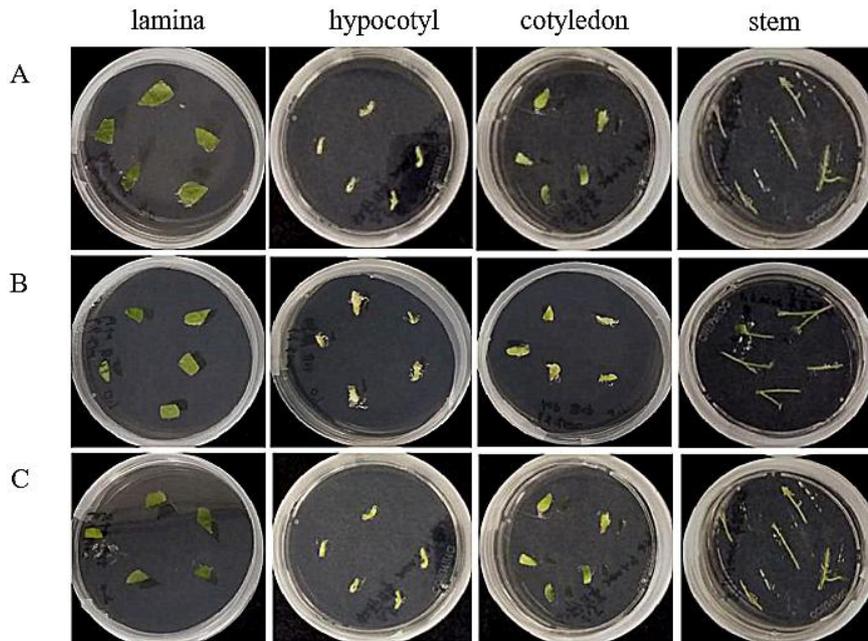


Figure 4 Callus induction of different explants of *Glycyrrhiza* Xinjiang

Note: A: MS+0.5 mg/L NAA+0.5 mg/L 6-BA; B: MS+1.0 mg/L NAA+1.0 mg/L 6-BA; C: MS+2.0 mg/L NAA+2.0 mg/L 6-BA

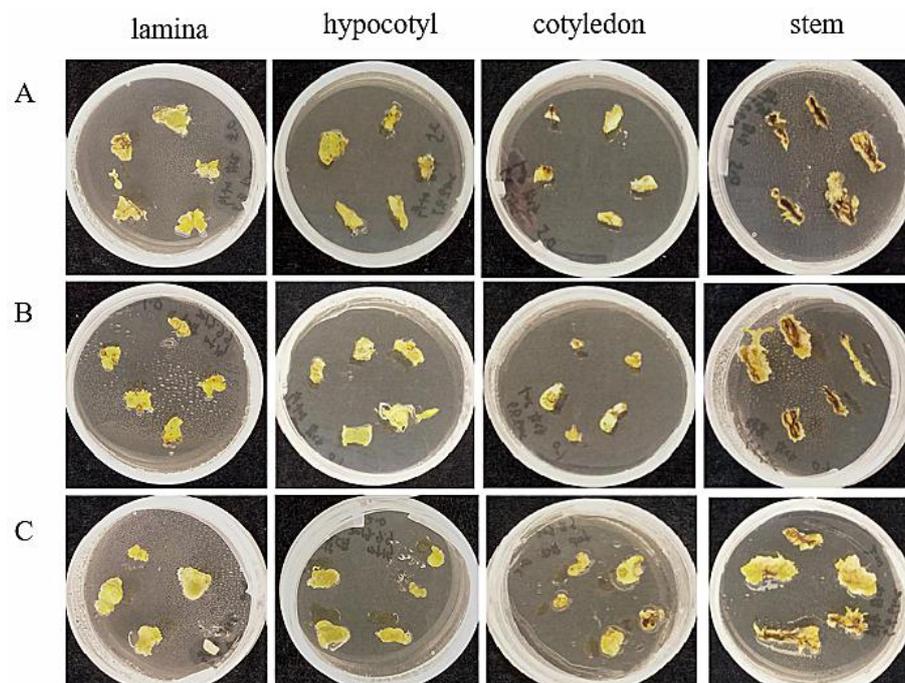


Figure 5 Hormone induction of Different ratio of *G. Xinjiang*

Note: A: MS+0.5 mg/L NAA+0.5 mg/L 6-BA; B: MS+1.0 mg/L NAA+1.0 mg/L 6-BA; C: MS+2.0 mg/L NAA+2.0 mg/L 6-BA

Table 3 Callus induction rates of different explants of *G. Xinjiang*

Explant	Hormone-induced medium		
	MS+0.5 mg/L NAA+0.5 mg/L 6-BA (%)	MS+1.0 mg/L NAA+1.0 mg/L 6-BA (%)	MS+2.0 mg/L NAA+2.0 mg/L 6-BA (%)
Cotyledon	93.3	80	92.3
Leaf	62.5	62.5	91.7
Stem	70	50	62.5
Hypocotyl	60	100	100

#### 1.4 Regeneration plants of *G. mongolicum* and *G. Xinjiang*

The callus induced from cotyledons and hypocotyls of *G. mongolicum* and *G. Xinjiang* were transferred into MS +0.5 mg/L NAA+0.5 mg/L 6-BA, MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA induction medium. After about 20 days of culture, it can be seen that there are buds on the surface of callus, after the buds grew three true leaves, they can be transferred to MS basic medium and can take root in the process of growth. Finally, two kinds of *G. uralensis* regenerated plants were obtained (Figure 6). The differentiation rate of the regenerated plants varies with the medium containing different hormone ratios (Table 4). The results showed that the differentiation rate of regenerated plants in MS+2.0 mg/L NAA+2.0 mg/L 6-BA medium was higher than that in another hormone ratio medium.

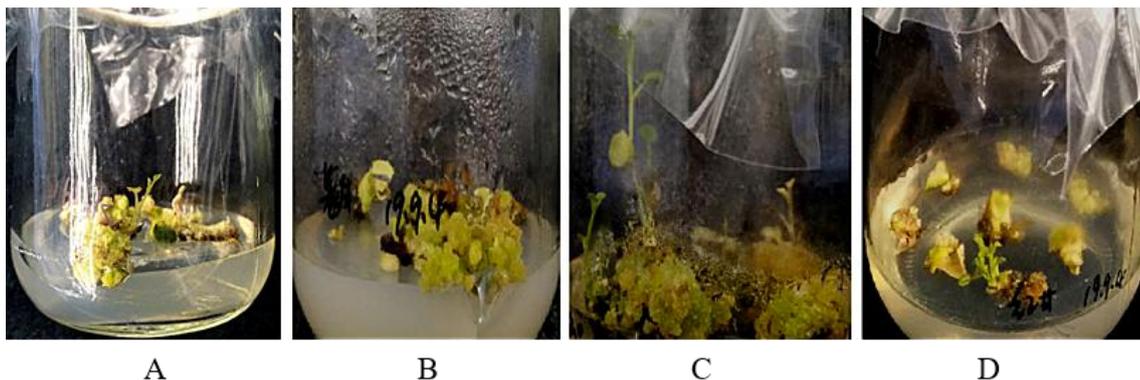


Figure 6 Regenerated plants of *G. mongolicum* and *G. Xinjiang*

Note: A,B: *G. mongolicum*; C,D: *G. Xinjiang*

Table 4 Plant differentiation rate of *G. mongolicum* and *G. Xinjiang*

<i>G. uralensis</i>	Hormone-induced medium	Number of transferred callus	Number of differentiable callus	Differentiation rate of regenerated plants (%)
<i>G. mongolicum</i>	MS+0.5 mg/L NAA+0.5 mg/L 6-BA	19	5	26.3
	MS+1.0 mg/L NAA+1.0 mg/L 6-BA	20	4	20.0
	MS+2.0 mg/L NAA+2.0 mg/L 6-BA	20	18	90.0
<i>G. Xinjiang</i>	MS+0.5 mg/L NAA+0.5 mg/L 6-BA	20	2	10.0
	MS+1.0 mg/L NAA+1.0 mg/L 6-BA	20	3	15.0
	MS+2.0 mg/L NAA+2.0 mg/L 6-BA	18	17	94.4

## 2 Discussion

With the increasing consumption of *G. uralensis*, wild resources are facing exhaustion. Although the artificial planting area of *G. uralensis* is gradually expanding, the current production of *G. uralensis* materials is difficult to meet the market demand (Gu and Wang, 2020). Therefore, the use of in vitro rapid propagation technology can accelerate the supplement of *G. uralensis* resources. At present, the rapid propagation system has been studied in plants. For example, 9 kinds of proportioning hormones and sucrose were added to the MS basic medium to study the callus induction and rooting of *Mandevilla sanderi* and the rapid propagation system of *Mandevilla sanderi* was established (Zhang Y.Y. et al., 2020, <https://kns.cnki.net/kcms/detail/46.1068.S.20200820.1749.013.html>);

Optimizing the rapid propagation system of *Lycium ruthenicum* to increase its yield and quality (Li et al., 2020) and exploring the effect of plant growth regulators on the callus and rooting induction of *Lilium lancifolium* Thunb. to establish its rapid propagation system (Zhao et al., 2020). In recent years, there have been many studies on the in vitro culture and callus induction of *G. uralensis* and *G. inflata* Batal., but the rapid propagation system of *G. uralensis* is less. Predecessors used hypocotyls, stems, cotyledons and radicles of *G. uralensis* as explants to study the induction of callus from various explants with different hormone ratios, it was found that the hypocotyl had the highest callus induction rate and the hypocotyl was the best explant for callus induction (Zhao et al., 2011). The root of *G. uralensis* was used as explant to induce callus and subculture and it was found that the root of 10 days old was suitable for callus induction (Ma et al., 2018). In previous studies that the hypocotyl axis and cotyledon of *G. inflata* were used as explants to induce callus, and the optimal medium for callus induction was MS+1.5 mg/L NAA+0.5 mg/L 6-BA and MS+2.5 mg/L NAA+0.5 mg/L 6-BA (Liu et al., 2010). Therefore, this study is based on the predecessors' induction of plant callus and construction of regeneration system to construct a rapid propagation system of *G. glabra* in order to improve the yield and quality of *G. uralensis*.

In this experiment, the hypocotyls, leaves, cotyledons and stems of different explants of *G. mongolicum* and *G. Xinjiang* can form callus, but different concentrations of hormone treatment on different explant have influence on the degree of callus formation. In MS+0.5 mg/L NAA+0.5 mg/L 6-BA induction medium, the induction rate of stem and hypocotyl of *G. mongolicum* was higher, and that of cotyledon and stem of *G. Xinjiang* was higher; In MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA induction medium, the induction rate of cotyledon and hypocotyl of *G. mongolicum* was higher than stem and leaf, the induction rate of cotyledon and hypocotyl of *G. Xinjiang* was higher than that of stem and leaf, and the cotyledon and hypocotyl of the two *G. uralensis* were in good growth state. The callus was transferred to the induction medium and continued to be cultured to gradually form regenerated plants. The cotyledons and hypocotyls of *G. mongolicum* and *G. Xinjiang* formed regenerated plants in MS+2.0 mg/L NAA+2.0 mg/L 6-BA medium, and the plant differentiation rate was high. The results showed that the best hormones for callus induction and plantlet regeneration was MS + 2.0 mg / L NAA + 2.0 mg / L 6-BA medium and cotyledon and hypocotyl could be the best explants for callus induction and plantlet regeneration. In this study, the same medium was used for callus induction and subculture. Both explants can form callus and the induction rate was high. Continue to cultivate and develop into regenerated plants, but in the process of callus induction, because callus is easy to brown, the induction medium should be changed frequently.

Compared with the traditional natural culture of *G. uralensis*, the materials obtained by the sterile seeding method in this study were more feasible to induce callus and were not affected by pollution, which greatly improved the induction rate. The acquisition of callus provides a theoretical basis for the establishment of the rapid propagation system of *G. uralensis*. The establishment of a rapid propagation system of *G. uralensis* can not only increase the propagation speed of seedlings, but also benefit the preservation and development of excellent varieties. It can fundamentally solve the contradiction between market demand and yield and quality and it is of great significance to the breeding and breeding of excellent varieties of medicinal plants, resource protection and sustainable development. In addition, the use of different explants to induce callus and extract the effective components is one of the effective ways to develop and utilize the medicinal value of *G. uralensis*, it is also an important field of modern Chinese medicine research, which has certain academic value and research significance.

### 3 Materials and Methods

#### 3.1 Materials

Seeds of *G. mongolicum* and *G. Xinjiang* (both purchased from Anguo seed market).

#### 3.2 Sterile seedling induction

The seeds of *G. mongolicum* and *G. Xinjiang* were soaked in self-deionized water for about 24 h, then the water was poured out, the seeds were left and put on the sterilized culture dish. Pour 8% sodium hypochlorite solution (purchased from Xilong Science Co., Ltd.) to soak the seeds for 20 min. During this period, shake the Petri dish,

and then rinse with sterile water for 7 times and put the seeds on the filter paper to absorb the water. Finally, the seeds were inoculated on the newly sterilized MS solid medium with sterilized tweezers and the temperature was 25°C and the humidity was 30%.

### 3.3 Callus induction

The sterile seedlings with good growth condition after 30 days of culture were used as materials, the explants including hypocotyls, leaves, cotyledons and stems were cut from sterile seedlings cultured for one month in a super clean working table and they were placed on the callus induction medium (MS+0.5 mg/L NAA+0.5 mg/L 6-BA, MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA). The callus induction medium used in this experiment was MS as the basic medium. The temperature was 25°C, the humidity was 30% and the light intensity was 3500 lx. The number of inoculated explants of *G. mongolicum* and *G. Xinjiang* (Table 5; Table 6) were taken. The callus induction and growth of different explants were observed, and the callus induction rate was counted.

Table 5 The number of four explants inoculated with *G. mongolicum*

Explant	Number of explants inoculated		
	MS+0.5 mg/L NAA+0.5 mg/L 6-BA	MS+1.0 mg/L NAA+1.0 mg/L 6-BA	MS+2.0 mg/L NAA+2.0 mg/L 6-BA
Cotyledon	13	15	14
Leaf	15	14	15
Stem	16	18	20
Hypocotyl	6	5	6

Table 6 The number of four explants inoculated with *G. Xinjiang*

Explant	Number of explants inoculated		
	MS+0.5 mg/L NAA+0.5 mg/L 6-BA	MS+1.0 mg/L NAA+1.0 mg/L 6-BA	MS+2.0 mg/L NAA+2.0 mg/L 6-BA
Cotyledon	15	15	13
Leaf	16	16	12
Stem	20	20	16
Hypocotyl	5	5	5

### 3.4 Induction of regenerated plants

The callus was transferred to the medium containing different hormone ratio (MS+0.5mg/L NAA+0.5mg/L 6-BA, MS+1.0 mg/L NAA+1.0 mg/L 6-BA and MS+2.0 mg/L NAA+2.0 mg/L 6-BA) for subculture, and the growth was observed and the differentiation rate of the regenerated plants was statistically analyzed.

#### Authors' contributions

Gao Xinxin and Tian Yu are the experimental designer and executor of this study, and complete the data analysis and the writing of the first draft of the paper; Chen Guoliang and Wu Jiawen participate in the experimental design and analysis of the experimental results; Bai Zhenqing is the designer and person in charge of the project, guiding the experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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