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Rapid Establishment of a Regeneration System for *Astragalus membranaceus Mongholicus*

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ABSTRACT

Background: *Astragalus membranaceus Mongholicus* (Astragalus) is one of the most widely used medicinal plants in the world. It is a traditional Chinese medicine plant that has been used to treat various human diseases and conditions, such as inflammation, gastrointestinal bacterial infections and malignant tumors. With the expansion of artificial planting areas, the demand for high-quality Astragalus seedlings is also increasing. The traditional seedling-raising methods of direct seeding and induced callus have problems such as a long growth period, unstable genetic traits, strict sampling and rooting difficulties. Therefore, it is very important to multiply the excellent germplasm by optimizing the tissue culture regeneration system.

Methods: In this study, Astragalus field cultivated plants, which are easily available, were used as materials and the method of tissue culture cuttings was used to select the direct inoculation method of stem segments as explant materials to establish a plant regeneration system of Astragalus and the best sampling period, sampling site and optimal medium for regeneration of field cultivated plants were screened.

Result: The best sampling period for plant regeneration of Astragalus in the field was about 40 d after the emergence of the 3-year-old Astragalus and the rooting rate of the regenerated plants was 100.00%. The best sampling site is the middle section of the stem and the regeneration system using the middle section of the stem as the explant is better than using the top and bottom of the stem as the explant and the rooting rate of the regenerated plants is 100.00%; the best medium for the regenerated plants is N22: 1/2MS+1 mg/L NAA and T28: 1/2MS+1 mg/L ABT, the rooting rates were 76.67% and 96.67%, respectively. The regenerated plants established with the stems of the field cultivated plants of Astragalus as explants were all able to induce seedlings after 1 week and the regenerated seedlings start to take root after 3 weeks of growth and can be transplanted and propagated after 6 weeks of culture. The regeneration system established in this study by direct cuttings from cultivated Astragalus stems is more rapid and efficient, breaking through the difficulties of limited access to material and long growth period of regenerated seedlings in the traditional regeneration system and providing technical support for the conservation and utilization of wild resources of Astragalus and rapid expansion of new varieties and germplasm conservation.

Key words: *Astragalus membranaceus Mongholicus*, Cultivation of plants, Stem segment, Tissue culture, Regeneration plants.

INTRODUCTION

Astragalus membranaceus Mongholicus is a perennial herb of the Leguminosae family. The secondary metabolites in their roots have significant pharmacological effects and are widely used in the international medical industry (Li *et al.*, 2022; Yin *et al.*, 2019). Astragalus possesses numerous excellent characteristics, such as high medicinal value, edible value, strong ornamental performance and a wide range of applications (Li *et al.*, 2023; Wei *et al.*, 2023). Pharmacological research and clinical trials of modern medicine have confirmed that the active ingredients in Astragalus combined with chemotherapy can effectively reduce the probability of cancer spread (Li *et al.*, 2020). Astragalus is widely planted in the Inner Mongolia region of northern China and is one of the typical medicinal materials grown in the grasslands of Inner Mongolia (Qin *et al.*, 2013). The plant of Astragalus is tall, the height can reach more than one meter and the main root is thick and long (Yang *et al.*, 2020). Influenced by the international medical industry, the demand for raw materials of Astragalus has been increasing and the planting scope of artificial cultivation has also expanded and the planting area under Astragalus has been rapidly increasing worldwide in the last decade (Bi *et al.*, 2020). Therefore, the demand for high-quality Astragalus seedlings is also increasing.

In recent years, with the gradual scarcity of wild resources and the continuous expansion of artificial cultivation area, the preservation of excellent germplasm

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resources and the breeding of high-quality varieties of Astragalus are particularly important. Because Astragalus is a highly self-incompatible plant, it mainly reproduces offspring by cross-pollination. The genetic variation background is complex and the excellent traits of the parent plants cannot be completely inherited to the offspring (Wang *et al*., 2017). Therefore, the establishment of a stable and efficient tissue culture regeneration system is the basis for cultivating excellent varieties of Astragalus. Tissue culture technology is a rapid way to protect and utilize plant resources and expand excellent germplasm (Amirova *et al*., 2022; Yao *et al*., 2022; Gu *et al*., 2022). At present, the study of the tissue culture regeneration system of Astragalus is mainly to obtain regenerated plants after dedifferentiation and redifferentiation of different types of explants. This method of indirect differentiation regeneration method to obtain regenerated plants takes a longer time and has a lower induction rate. The difficulty of rooting high-quality Astragalus tissue culture regenerated seedlings has greatly limited the breeding process of this species (Wang *et al*., 2017; Tian *et al*., 2022). Therefore, improving on the existing tissue culture means and systematically screening out the best method for the rapid propagation system of Astragalus is conducive to shortening the cultivation period of excellent germplasm resources and producing excellent Astragalus germplasm on a large scale.

In this study, the field cultivated plants of Astragalus were used as materials and the stem segments of the cultivated plants of Astragalus at different growth stages were used for tissue culture and cutting propagation. The number and growth rate of adventitious buds and adventitious roots of regenerated seedlings under different medium and different hormone concentration ratios were determined. The effects of different tissue culture factors on the formation of regenerated seedlings of Astragalus were analyzed to screen out the optimum culture conditions for the propagation of Astragalus in the field. Establishing a fast and efficient regeneration system of cultivated plants for the selection of excellent varieties of Astragalus to provide a technical basis for propagation and protection and utilization of wild resources.

MATERIALS AND METHODS

The stems of Astragalus were collected from the experimental site of Inner Mongolia Agricultural University, Hohhot, Inner Mongolia, China. From 2020 to 2022, it is sown in April each year and transplanted in the second year. Sampling was started 20 days after emergence in 2022 to observe the asexual reproduction efficiency of stem cuttings of 1-year-old, 2-year-old and 3-year-old plants. The specific information of the tissue culture cuttage explant materials selected in this study (Table 1). Due to the high degree of lignification at the lower end of the stem segment of the 2-year-old and 3-year-old plants at the late stage of sampling (60 d after emergence of seedlings), the lower end of the stem segment was discarded during propagation.

Selection of explants

In May 2022, the aboveground branches of 1-year-old, 2-year-old and 3-year-old plants of Astragalus were cut and soaked in sterile water. The upper, middle and lower segments of each branch were separated in a ratio of 1 : 1 : 1 and all leaves on the stem segments were pruned. After washing with running water for 30 minutes, they were fished out and transferred to an ultra-clean bench for use. First, the stem segments were soaked in anhydrous ethanol for 1 min and rinsed with sterile water for 2 times. Second, they were soaked in 75 % alcohol for 1 min and rinsed with sterile water for 2 times. Then, they were soaked in 0.1% mercuric chloride for 5 min and rinsed with sterile water for 3 times. Finally, the sterilized filter paper was used to dry the water. Before inoculation, the upper end of the stem segment was quickly cut off with a willow blade, so that a stem node was

Table 1: Sampling information of Astragalus explants under different sowing years.

Sowing year	Sampling years	Growth pattern	Sampling time	Sampling location
2020.04	Year 3	Three-year-old: transplanted in 2021 and sampled in the 2nd year after transplanting	20 d after emergence of seedlings	Stem top, middle and lower segments
			40 d after emergence of seedlings	Stem top, middle and lower segments
			60 d after emergence of seedlings	Stem top, middle section
2021.04	Year 2	Two-year-old: transplanted in 2022 and sampled in the year of transplanting	20 d after emergence of seedlings	Stem top, middle and lower segments
			40 d after emergence of seedlings	Stem top, middle and lower segments
			60 d after emergence of seedlings	Stem top, middle section
2022.04	Year 1	One-year-old: sampling in the year of sowing 2022	-	-
			-	-
			60 d after emergence of seedlings	Stem top, middle and lower segments

retained at a distance of 5-8 mm from the upper incision, the incision at the lower end of the stem segment was a 45° oblique incision and the incision at the lower end was retained at 15-20 mm to the stem node. The trimmed stem segments were cut into pre-sterilized MS medium, so that the lower end of the stem node was flush with the surface of the medium and cultured under the conditions of light time of 16 h/d, constant temperature of (25±1)°C, humidity of 50-60% and light intensity of 3000 lx (Fig 1).

Hormone proportion

Add 20 g/L sucrose and 7.5 g/L agar to the MS medium and then add an appropriate amount of 1 mol/L NaOH solution to adjust the pH value of the medium solution to 5.8. Different concentrations of indole acetic acid (IAA), indole butyric acid (IBA), gibberellin (GA3), 1-naphthylacetic acid (NAA) and rooting powder (ABT) were added to induce rooting of regenerated seedlings. The hormone ratio is shown in Table 2. Sixty stem segments were inserted on the medium with each hormone ratio and repeated three times.

Statistics of growth rate and rooting rate of regeneration system

The pollution rate, adventitious bud induction rate, adventitious root induction rate and growth rate of regenerated seedlings of Astragalus were counted and determined daily. Photographs were taken and the induction and growth of adventitious roots at the wound of regenerated seedlings under different growth years and different hormone ratios were observed. The adventitious roots breaking through 2 mm at the incision were regarded as rooting and the root growth rate of regenerated seedlings was counted. The calculation formula is as follows:

$$\text{Contamination rate (\%)} = \frac{\text{Number of contaminated explants}}{\text{Total number of explants inoculated}} \times 100\%$$

$$\text{Adventitious bud induction rate (\%)} = \frac{\text{Number of explants capable of inducing buds}}{\text{Total number of explants inoculated}} \times 100\%$$

The adventitious roots are regarded as rooting when they break through 2 mm at the incision point and the root growth rate is counted. Calculation formula is as follows:

$$\text{Adventitious root induction rate (\%)} = \frac{\text{Number of explants capable of rooting}}{\text{Total number of explants inoculated}} \times 100\%$$

Data statistics and analysis

All data and pictures were collated using Excel 2019, SAS9.0 and PowerPoint 2021. The adventitious bud induction rates and adventitious root induction rates were analyzed using analysis of variance (ANOVA, $p<0.05$).

RESULTS AND DISCUSSION

Selection of explants

In this study, we found that the induction rate of adventitious shoots from one-year-old Astragalus stem segment cuttings was low at 50.00% and adventitious roots could not be

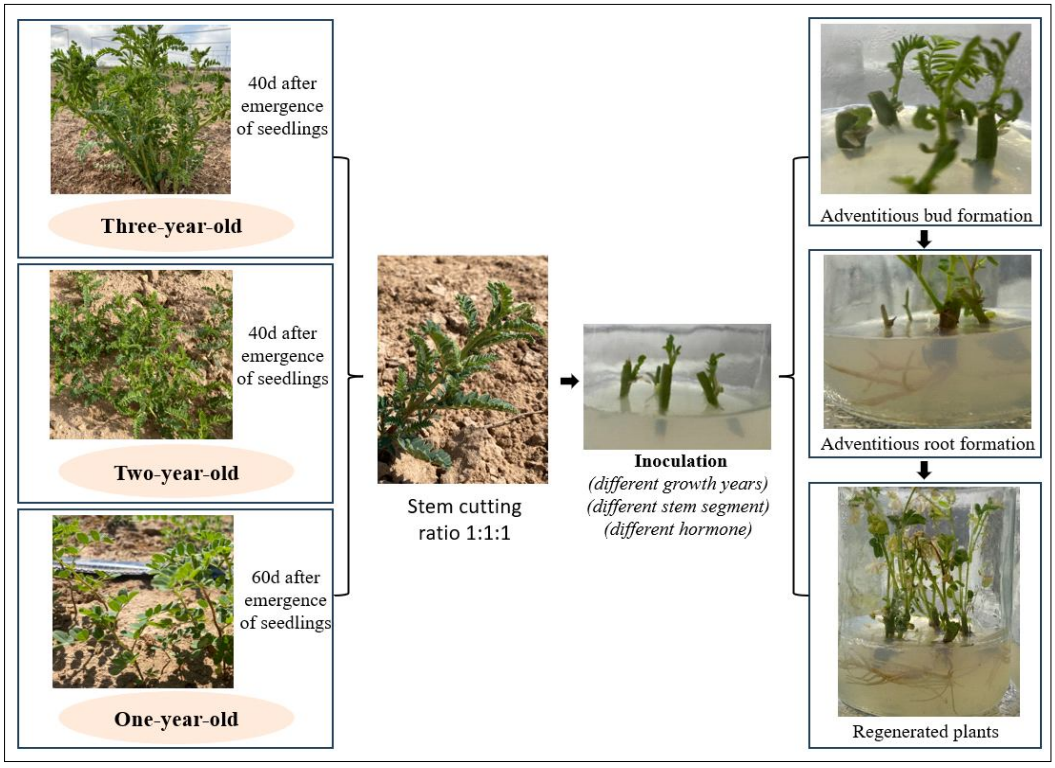


Fig 1: Astragalus tissue culture regeneration process.

Table 2: Configuration of different hormone concentrations profiles in stem segment cuttage medium of Astragalus.

Hormone type	Medium type	Number	Hormone addition (mg/L)	Number	Hormone addition (mg/L)	Number	Hormone addition (mg/L)
IAA	MS	A1	1	A2	5	A3	10
	1/2MS	A4	1	A5	5	A6	10
IBA	MS	B7	1	B8	5	B9	10
	1/2MS	B10	1	B11	5	B12	10
GA3	MS	G13	1	G14	5	G15	10
	1/2MS	G16	1	G17	5	G18	10
NAA	MS	N19	1	N20	5	N21	10
	1/2MS	N22	1	N23	5	N24	10
ABT	MS	T25	1	T26	5	T27	10
	1/2MS	T28	1	T29	5	T30	10

Table 3: Induction rate of adventitious buds and adventitious roots of Astragalus stem explants during different growth years and different sampling parts.

Sowing year	Sampling years	Sampling time	Adventitious bud induction rate %	Adventitious root induction rate %
2020.04	Year 3	20 d after seedling emergence	93.33±0.05 ab	10.00±0.10 fg
		40 d after seedling emergence	100.00±0.10 a	100.00±0.10 a
		60 d after seedling emergence	83.33±0.05 bc	66.67±0.05 b
2021.04	Year 2	20 d after seedling emergence	90.00±0.10 ab	0.00±0.00 g
		40 d after seedling emergence	96.67±0.05 a	46.67±0.05 c
		60 d after seedling emergence	76.67±0.05 c	26.67±0.05 de
2022.04	Year 1	-	-	-
		-	-	-
		60 d after seedling emergence	50.00±0.10 d	0.00±0 g
Sowing year	**Sampling years**	**Sampling location**	**Adventitious bud induction rate %**	**Adventitious root induction rate %**
2020.04	Year 3	Stem top segment	83.33±0.05 bc	16.67±0.05 ef
		Stem middle segment	100.00±0.10 a	100.00±0.00 a
		Stem lower segment	96.67±0.05 a	30.00±0.10 d

Note: Different lowercase letters in the same column represent significant differences (P<0.05).

induced. The induction rate of adventitious shoots was above 70% in both, two-year-old and three-year-old Astragalus. The mid-stem cuttings at 40 days after emergence have the best induction rate of adventitious buds, with the best induction rate of 100.00% and 96.67% and the highest induction rate of adventitious buds in the middle and lower sections of three-year-old Astragalus stems, which were more than 13.34% higher than the top segment. Therefore, both two-year-old and three-year-old Astragalus stem segment media cuttings could induce adventitious roots and the best induction of adventitious roots could be achieved in test tube seedlings cut 40 d after the emergence of three-year-old Astragalus, which could reach 100.00%. Adventitious roots could also be induced from stem cuttings taken 60 d after seedling emergence in three-year-olds and 40 d after seedling emergence in two-year-olds, but the induction rates were lower, 66.67% and 46.67%, respectively. The results showed that the induced rates of adventitious buds and adventitious roots were significantly higher than those of other treatments (P<0.05) using the mid-sections of the stems of three-year old Astragalus plants inoculated on the medium at 40 d after seedling emergence, both of which were 100.00% (Table 3).

Effects of exogenous hormones on the induction rate and growth rate of adventitious buds

The middle cuttings of Astragalus stem segments were selected and inoculated into 30 different exogenous hormone media. New buds were induced from the cuttings about 1 week after inoculation and the adventitious bud induction rate and contamination rate of stem cuttings were observed, counted and the growth rate of new buds was mensurated. The contamination rate of stem cuttings of Astragalus was less than 12% and the induction rate of adventitious buds was more than 30%. Among them, the induction rates of adventitious buds were significant under different ratios of IAA (A1- A6) and IBA (B7-B12) hormones and MS medium lower than other hormone types (P<0.05), all below 55%. GA3 (G13-G18), NAA (N19-N24) and ABT (T25-T30) three hormones with different MS medium ratios

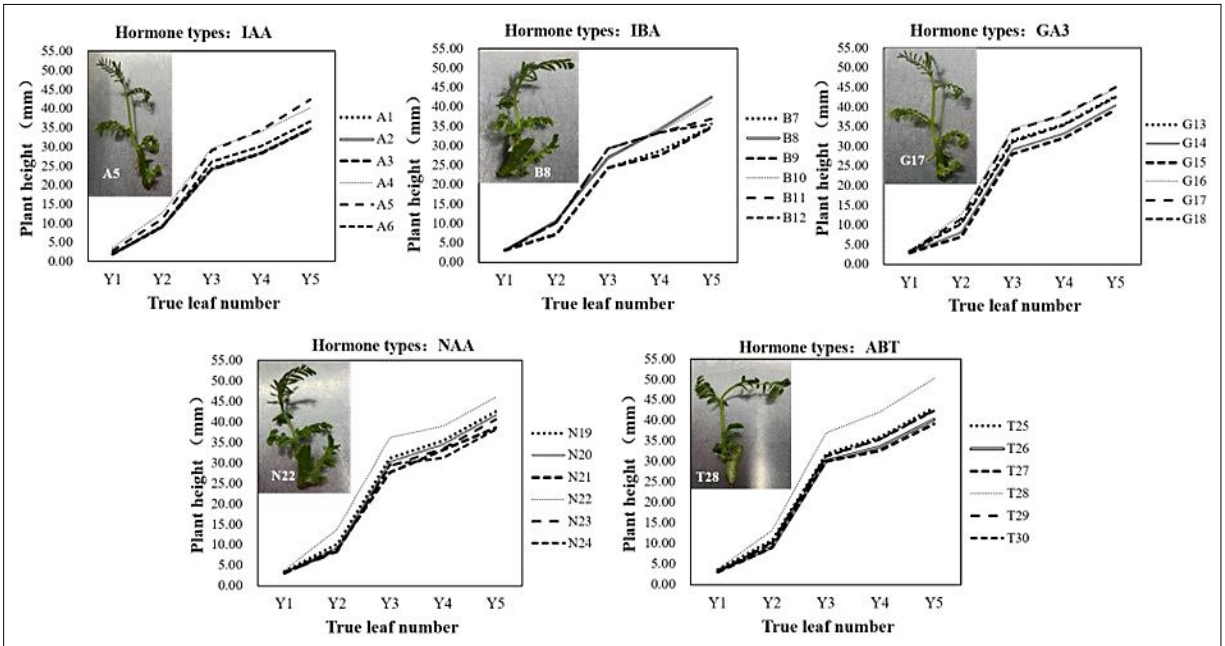
had higher induction rates of adventitious buds, when the three hormone concentrations were 1 and 5 mg/l, the induction rate of adventitious buds was above 60%. The induced rate of adventitious buds was significantly higher in medium type 1/2 MS and hormones GA3 (G16), NAA (N22) and ABT (T28) at a concentration of 1 mg/l than the other treatments (P<0.05), which were 88.33%, 93.33% and 96.67% respectively (Table 4).

After cultivating for 1 week, the first true leaf was grown and the leaves of Astragalus with the hormone ratio (T28: 1/2 MS + 1 mg/L ABT) were slightly larger (Fig 2). The stem-cutting tissue culture method can induce adventitious buds in the medium with different hormone concentrations. Two weeks after inoculation, the fifth true leaf grew, the thickness of the cotyledons increased, the first four true leaves were fully expanded, the leaf color gradually became darker, the leaf area became larger and the plant height of the regenerated seedlings had grown to the maximum before inducing adventitious roots. According to the results of adventitious buds induction, it was found that the stem explants of Astragalus could induce five true leaves in the medium of five different hormone concentration ratios, but the growth rates of the regenerated seedlings were quite different. When the hormone types were IAA and IBA, the

Table 4: Adventitious bud induction rate and contamination rate of Astragalus stem segment cuttage under different hormone concentrations.

No.	Adventitious bud induction rate %	Contamination rate	No.	Adventitious bud induction rate %	Contamination rate	No.	Adventitious bud induction rate %	Contamination rate
A1	45.00±0.05 ij	11.67%	A2	43.33±0.03 jk	8.33%	A3	46.67±0.03 hi	10.00%
A4	48.33±0.03 hi	10.00%	A5	46.67±0.03 hi	10.00%	A6	50.00±0.00 ghi	8.33%
B7	51.67±0.08 ghi	6.67%	B8	45.00±0.05 ij	6.67%	B9	40.00±0.05 jk	8.33%
B10	55.00±0.05 fg	10.00%	B11	48.33±0.06 hi	10.00%	B12	35.00±0.05 k	11.67%
G13	83.33±0.10 bc	8.33%	G14	63.33±0.03 ef	8.33%	G15	50.00±0.10 ghi	6.67%
G16	88.33±0.03 ab	10.00%	G17	70.00±0.05 de	11.67%	G18	71.67±0.06 de	5.00%
N19	81.67±0.06 bc	6.67%	N20	68.33±0.03 e	6.67%	N21	53.33±0.03 gh	8.33%
N22	93.33±0.03 a	5.00%	N23	71.67±0.03 de	5.00%	N24	65.00±0.05 e	8.33%
T25	81.67±0.03 bc	6.67%	T26	70.00±0.09 de	8.33%	T27	53.33±0.03 gh	5.00%
T28	96.67±0.03 a	8.33%	T29	78.33±0.03 cd	5.00%	T30	63.33±0.08 ef	6.67%

Note: Different lowercase letters represent significant differences (P<0.05).



Note: A1-T30 in the legend respectively represent the different concentration ratios of five hormones. See Table 2 for the specific hormone ratios. Abscissa Y1-Y5 indicates the first true leaf growing to the fifth true leaf. The photo of cuttings in the figure shows the state when the regenerated seedlings grow the fifth true leaf under the optimal concentration of each hormone (After 2 weeks of inoculation).

Fig 2: The growth rate of adventitious bud from stem explants of Astragalus under different hormone ratios.

Table 5: Adventitious root induction rate of Astragalus after stem segment cuttage under different hormone ratios.

Number	Adventitious root induction rate %	Number	Adventitious root induction rate %	Number	Adventitious root induction rate %
A1	0.00±0.00 j	A2	0.00±0.00 j	A3	0.00±0.00 j
A4	0.00±0.00 j	A5	0.00±0.00 j	A6	0.00±0.00 j
B7	0.00±0.00 j	B8	0.00±0.00 j	B9	0.00±0.00 j
B10	0.00±0.00 j	B11	0.00±0.00 j	B12	0.00±0.00 j
G13	16.67±0.05 gh	G14	10.00±0.10 i	G15	3.33±0.05 ij
G16	23.33±0.05 ef	G17	13.33±0.15 hi	G18	10.00±0.10 i
N19	53.33±0.05 c	N20	13.33±0.11 hi	N21	6.67±0.11 ij
N22	76.67±0.05 b	N23	26.67±0.05 de	N24	10.00±0.10 i
T25	60.00±0.10 c	T26	23.33±0.17 fg	T27	10.00±0.10 i
T28	96.67±0.05 a	T29	33.33±0.05 d	T30	13.33±0.11 hi

Note: Different lowercase letters represent significant differences (P<0.05).

Table 6: The growth rate of stem explants of Astragalus on three optimal media G16, N22 and T28.

Hormone types	Appearance time of the true leaf (d)					Root length (mm)						
	First piece	Second piece	Third piece	Fourth piece	Fifth piece	2 mm	4 mm	6 mm	8 mm	10 mm	15 mm	35 mm
G16	6d	9d	12d	17d	21d	24d	27d	30d	33d	37d	39d	50d
N22	6d	8d	10d	15d	18d	21d	24d	26d	28d	30d	32d	43d
T28	5d	7d	9d	13d	16d	19d	21d	24d	25d	27d	30d	40d

growth rate of the regenerated seedlings was slower and the plant height of the regenerated seedlings at the five-leaf stage (Y5) was all below 45.00 mm. The regenerated seedlings grew faster when the hormone types were G17: 1/2MS + 5 mg/L GA3, N22: 1/2 MS + 1 mg/L NAA and T28: 1/2 MS + 1 mg/L ABT and the highest plant height of 50.33 mm was obtained for the five-leaf stage (Y5) cuttings when the hormone type was T28. The results indicated that the optimal medium for adventitious bud induction was G17, N22 and T28 when the cultivation plant stems of Astragalus were used as the explant for tissue culture cutting.

Effects of exogenous hormones on induction rate of adventitious roots

Statistical analysis of adventitious root induction showed that IAA (A1-A6) and IBA (B7-B12) don't seem to induce adventitious roots (Table 5). Interestingly, the seedlings stop growing after the five-leaf stage and begin to wither until death (Fig 3 IV \ V). Hormones GA3 (G13-G18), NAA (N19 - N24) and ABT (T25-T30) were mixed with different MS mediums in the ratios, the inoculation of stem segments of cultivated plants of Astragalus can induce adventitious roots at the incision at the bottom of the stem segment (Fig 3 I \ II \ III). The induced rate of adventitious roots of GA3 (G13-G18) were significantly lower than that of other treatments (P<0.05), which were all below 25%; the induced rate of adventitious roots of NAA (N19-N24) and ABT (T25-T30) were significantly higher than that of other treatments (P<0.05) and the rate of adventitious roots induced by hormone concentration of 0.1mg/l (N19/N22/T25/T28) were all above 50%. The best adventitious root induction rate were 96.67% when the medium type was 1/2MS and the hormone concentration were 1 mg/l of ABT (T28).

Acquisition of stem segment regeneration system for cultivated Astragalus

The tissue culture of Astragalus has high requirements on inoculation materials and it is usually necessary to use sterile seedlings cultured from high-quality seeds for inoculation (Yao *et al.*, 2022; Zhu *et al.*, 2019). However, the most abundant tissue culture resources on Astragalus plants are the fresh and vigorous growing branches of the field cultivated plants as well as the older branches with a high degree of lignification. Since the cultivation of sterile seedlings is time-consuming and the plants that can reach the inoculation conditions are limited, it often takes a lot of time for indoor cultivation. Therefore, in order to improve the reproduction coefficient, this study attempted to establish a regeneration system of Astragalus with vigorous green branches in the field. Experiments showed that stem segments cut from green branches were able to take root well (Fig 3, Fig 4) and the induction rate of adventitious roots was up to 96.97%. Stem segment tissue culture induces the formation of adventitious roots first at the lower incision during root formation (Fig 4 A), which plays an important role in the absorption of water and nutrients for regenerated seedlings and also creates a good environment for root development (Wu *et al.*, 2016; Wang *et al.*, 2016). As can be seen from Fig 4, adventitious roots were formed from the outer circle of the incision, indicating that the making of the wound contributes to the formation of an adventitious roots at the lower end of the stem segment. At the same time, green branches with one stem node are rooted better.

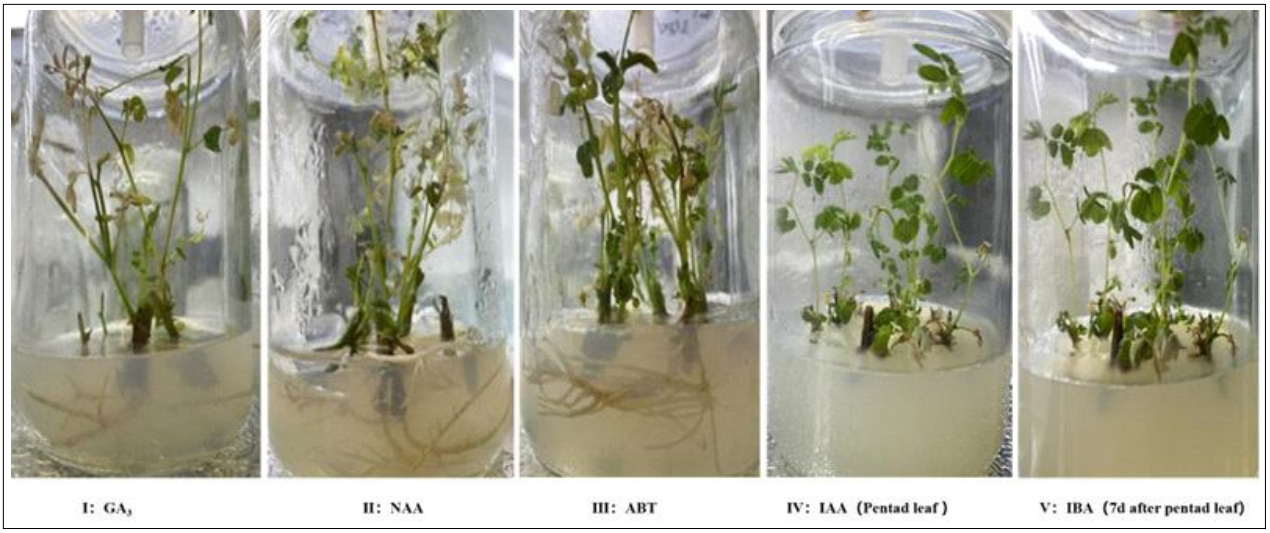


Fig 3: Adventitious root induction of Astragalus stem plant under different hormone ratios.

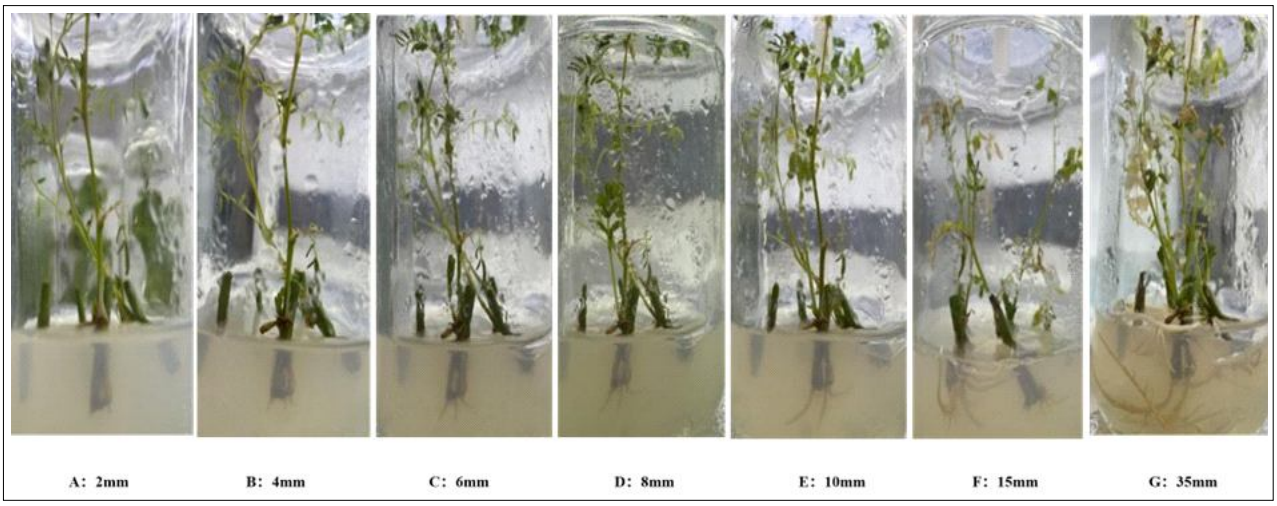


Fig 4: Root growth status of regenerated plants from stem explants of Astragalus cultivated in the field.

After culturing for 2-3 weeks, the test-tube seedlings grew to the fifth true leaf and the growth of the leaves was stagnant (Table 6). The incision at the bottom of the stem cutting begins to expand, the color of the stem becomes darker and the degree of lignification becomes higher, 3-5 adventitious roots were induced at the oblique incision of the stem segment (Fig 4 A). The adventitious root induction rate after inoculation of Astragalus explants was calculated and the growth rate of adventitious roots was measured. It can be seen that the adventitious root grows the fastest when cultured for 4-7 weeks and the root system grows rapidly by more than 20 mm within 15d (Fig 4 G). After 40 d of culture, test-tube seedlings grown from stem segment inoculation were obtained. When the hormone ratio was (T28: 1/2 MS + 1 mg/L ABT), the roots of the regenerated seedlings from the stems of Astragalus grow more vigorously and faster than the regenerated seedlings with other hormone ratios (Fig 3 III).

CONCLUSION

In this study, MS medium was used as the substrate and the branches with a stem node on the field cultivated plants of Astragalus were used as explants. After adding appropriate concentrations of hormones (NAA and ABT), the survival coefficient of this method was higher. With the increase of growth years, the induction rate of adventitious buds and adventitious roots of Astragalus stem segments after cutting on different hormone concentration medium also increased. The longer the growth years, the greater induction rate of adventitious roots and the greater possibility of survival of regenerated seedlings. The stem segments of 3-year-old Astragalus were used as explants and cuttaged on 1/2MS medium (hormone ratio of N22: 1/2MS + 1 mg/L NAA and T28: 1/2MS + 1 mg/L ABT). The induction rate of adventitious roots and adventitious buds of regenerated seedlings could reach 100.00%. Since this experiment successfully used tissue culture technology to achieve the

establishment of a green stem segment regeneration system from field-cultivated plants, the sampling season can be extended from spring to summer, which greatly extends the period of tissue culture sampling and the amount of inoculation material is large. The rooting period of regenerated seedlings obtained by this method is short and the stem segments of asexual propagation raw materials in the field are large and easy to obtain, which improves the propagation coefficient of Astragalus clones and the survival rate of regenerated seedlings and has important practical application value.

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Conflict of interest

The authors declare that they have no competing interests.

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