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Research Article

In vitro regeneration of *Haloxylon ammodendron*

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Abstract

Haloxylon ammodendron (C.A.Mey) Bunge is one of the important species of arid desert vegetation in China, and it is also an ideal material for studying plant stress resistance, which plays an extremely important role in protecting desert ecosystems and preventing land desertification. However, there are relatively few studies on the regeneration of the fusiform, and the genetic transformation system has not been successfully reported, which restricts the in-depth study of the molecular mechanism of shuttle propagation and stress resistance. In the present study, the seeds, hypocotyls, cotyledons, cotyledon nodes, terminal buds and fixed buds were used as explants, and a set of tissue culture and plant regeneration system was established by inducing adventitious buds, adventitious bud rooting and transplanting. The results showed that amongst different *H. ammodendron* explants, i.e., seeds, hypocotyls, cotyledons and cotyledon nodes, the last induced budding effect was better. The optimal medium for inducing clandine buds by cotyledon segment differentiation is 0.5 mg· L^{-1} NAA+0.5 mg· L^{-1} 6-BA induced budding rate was high, reaching 61.90%, rooting medium was 1/2 MS+1 mg· L^{-1} NAA +1 mg· L^{-1} IBA + 1 mg· L^{-1} IAA with a rooting rate of 50%. The results of this study will provide a theoretical basis for the genetic transformation of *H. ammodendron*.

Keywords: adventitious buds; root induction; organogenesis; *ex vivo* regeneration; *H. ammodendron*

Introduction

Haloxylon ammodendron is a small perennial tree of the genus Haloxylon in the family Amaranthaceae, widely distributed in desert areas of Asia and Africa, and is a major building block of desert vegetation. In China, *H. ammodendron* is mainly distributed in Xinjiang, Inner Mongolia, Qinghai, Gansu and Ningxia provinces and regions; *H. ammodendron* is a small perennial tree, sometimes shrub-like, which is unique to desert areas drought tolerance (when the soil moisture content is 1.3~2.6%, it can still grow normally), high temperature resistance, salinity resistance (salt resistance critical range can reach 4% ~ 6%), wind erosion resistant plants (Kung *et al.,* 1979). It is a typical C4 species of high drought tolerance and salt tolerance (Casati *et al*., 1999), having the morphological and physiological characteristics of a super-arid plant. To adapt to the environment of extreme soil water scarcity and intense transpiration at high temperatures, *H.*

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ammodendron degenerates into scale-like, juicy green shoots ('assimilated shoots') with high salt content, which can be used as preferred fodder for livestock and is a woody fodder in desert arid areas (Kazuo et *al*., 2000; Xu *et al*., 2007; Wu *et al*., 2019).

H. ammodendron forests play an important role in protecting the security of the oasis by fixing a large amount of drifting sand around the oasis and have a high ecological value in combating desertification (Su *et al*., 2007; Maina *et al.,* 2015). The natural desert vegetation (known as "desert forest") formed by *H. ammodendron* as the dominant species provides a suitable breeding environment for nearly 200 species of desert plants, and also provides a habitat and breeding place for many desert animals and insects, which is an important place for biodiversity conservation in desert plain areas, and has important ecological and economic value in mitigating desertification, maintaining regional ecological balance and protecting the ecological environment.

Under natural conditions, *H. ammodendron* is reproducing extensively by seeds, which have a short germination retention period and are prone to interspecific variation and intergenerational degeneration. The heterogeneity of its germplasm and limited germination shelf life, limit the natural renewal and development of *H. ammodendron*. The high degree of lignification and thin bast of assimilated branches of *H. ammodendron* is one of the reasons why it is difficult to form adventitious roots. Using relatively young and tender branches for cuttings, the spike rot is also serious, resulting in cuttings rooting cannot continue, and using histogenic seedling technology can overcome these shortcomings. The use of in vitro culture technology allows not only to obtain new plantings and varieties, but also to obtain the metabolic substances required by humans. Still, the plant tissue culture technology has a high reproduction factor and can provide a large number of seedlings in a short period of time. Tissue culture of woody plants has always had problems such as low reproduction coefficient, difficulty in rooting and serious pollution. However, with the development of time, a variety of regeneration systems for fruit trees and forest trees have been successfully established, for example, Zhang *et al.* (2014) used apple leaves as explants to obtain regenerated plants. Bertsouklis *et al.* (2023) studied *in vitro* propagation of *Juniperus oxycedrus* adult native plants, examining the effects of seasonal explant collection (spring, summer, winter), different culture media, and cytokinin types. There are also citrus (Jardak *et al*., 2020), fir (Hu *et al*., 2017) and pine (Kim *et al*., 2014) that have successfully established regeneration systems. Some economically important species have been widely used in plantation production, such as poplar (Chan *et al.,* 2016) and eucalyptus (Fernando *et al*., 2016). Yu-Qing *et al.* (2018) used leaves of *Zenia insignis* Chun as explants to obtain regenerated fertile plants through guaired tissue induction, adventitious shoot induction, and rooting. In view of this, the present study was conducted to investigate the shoot induction and adventitious shoot rooting of *H. ammodendron* weeds, hypocotyls, cotyledons, cotyledon nodes, terminal buds and fixed buds as experimental materials, with a view to providing a theoretical basis for genetic transformation of *H. ammodendron*.

Materials and Methods

Seed treatment and acquisition of sterile seedlings

Picked uniform and full seeds of *H. ammodendron* weeds (harvested from Gurbantunggut Desert in northern Jimsar County, Changji Prefecture, Xinjiang. -20 °C for storage), add 75% (v/v) ethanol, shake for 3 min, discard the liquid; rinse with sterile water 3 times, shake for 3 min; finally soak the seeds with sodium hypochlorite for 10 min, rinse with sterile water 6 times; soak in sterile water overnight, cultured on MS solid medium the next day to germinate, and obtain sterile seedlings of *H. ammodendron* after 35 d (Figure 1).

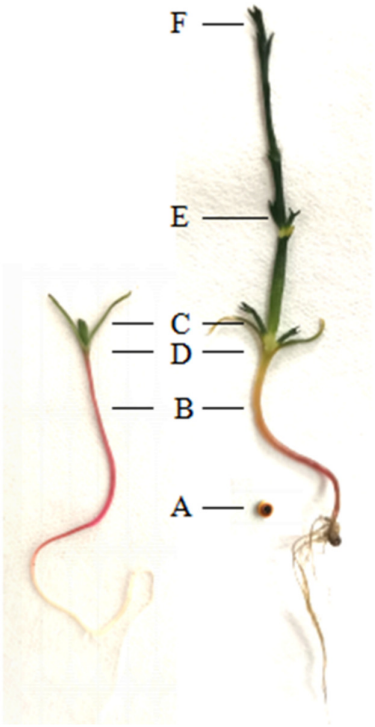

Figure 1. *H. ammodendron* sterile seedlings (Note: A: seed B: hypocotyl C: cotyledon D: cotyledon nodes E: fixed bud F: terminal bud)

Induction of indeterminate buds

When the sterile seedlings grew to 3-5 cm, the hypocotyl, cotyledons, cotyledon nodes, staple buds and terminal buds of about 0.5-1 cm in size were rapidly cultured into the germination induction medium, three bottles of each combination of different hormone concentrations, and seven explants were inoculated in each bottle. Germination was observed and recorded 10 days after inoculation. Germination percentage = (number of explants forming shoots/number of inoculated explants) × 100%. (See Results and Analysis section for details of hormone combinations)

Three hormones IBA (Indole-3-butyric acid), NAA (1-naphthal acetic acid), and IAA (Indole acetic acid) were added to the basic medium of 1/2 MS, and four hormone concentration gradients of 1, 2, 3, and 4 mg L^{-1} were orthogonally designed for the induction of adventitious roots in 16 treatments. After the adventitious shoots induced by the *H. ammodendron* nodes grew about 0.5~1 cm in size, they were put into the induction rooting culture flasks; three replicate controls were made for each treatment, and about 7 explants were placed in each culture flask. After 35 d of incubation, the growth status was observed and the rooting rate was counted. Rooting percentage (%) = number of rooted plants/number of inoculated explants × 100%. The data were processed with Microsoft Excel 2010 software. (See Results and analysis section for details of hormone fractions).

Refining seedlings for transplanting

The regenerated seedlings with well-developed root systems and more robust growth were opened in the artificial climate chamber, cling film was applied, small holes were poked in the cling film, and the seedlings were refined for about one week, then the regenerated seedlings were removed from the culture bottles, the medium attached to the root surface was rinsed with sterile water, transplanted into sterilized vermiculite: charcoal: sand = 1:1:1, and placed in the culture room for cultivation.

Results

Effect of different hormone concentration ratios on the induction of adventitious shoots

From the results of the experiment, it can be seen that *H. ammodendron* seeds, cotyledon nodes, fixed buds and terminal buds can be induced to germinate, but hypocotyl and cotyledons did not germinate (Table 1). During the experimental observation, it was found that seeds and cotyledons emerged preferentially within 14 d, while fixed and terminal buds emerged one after another after 14 d. Since the material of fixed and terminal buds was difficult to obtain, although the seeds could also induce buds, it could be seen from the pictures that the quality of the buds was not as good as that of the indeterminate buds induced by the cotyledon nodes, and for the consideration of subsequent rooting, the most suitable explants for the induction of buds were determined to be the cotyledon nodes (Figure 2).

The cotyledonary nodes were induced in treatment 11 with a higher rate of 61.90% at MS + 0.5 mgL^{-1} NAA + 0.5 mgL^{-1} 6-BA. The induced shoots had vigorouw growth and dark green color. In treatment 3, cotyledonary nodes did not induce buds when no phytohormones were added, thus showing that phytohormones play a very important role in cotyledonary node induction of buds. The germination rate of cotyledon nodes in treatment 8 and 13, MS+1 $mg\cdot L^{-1}$ IBA+1 $mg\cdot L^{-1}$ KT was 14.5%, MS+1 $mg\cdot L^{-1}$ NAA+1 $mg\cdot L^{-1}$ 6-BA was 23.81%, which indicated that the combination of NAA and 6-BA and IBA and KT hormones was at a concentration of 1 mgL^{-1}, it is not suitable as a medium for cotyledon nodes, inducing adventitious buds. The budding rate of cotyledonary node in treatment 5 and 15 was the same, second only to treatment 11, and the budding rate reached 57.14%. In the combination with high germination rate, 6-BA was found to be involved, indicating that 6-BA played a role in the process of inducing adventitious budding in the *H. ammodendron* cotyledonary node. Germination of cotyledon nodes started within 14 d. Adventitious shoots were vigorous, had normal leaf color, usually appeared in compact clusters, and it was difficult to quantify the number of regenerated shoots, with a few vitrification seedlings, but no deformed shoots.

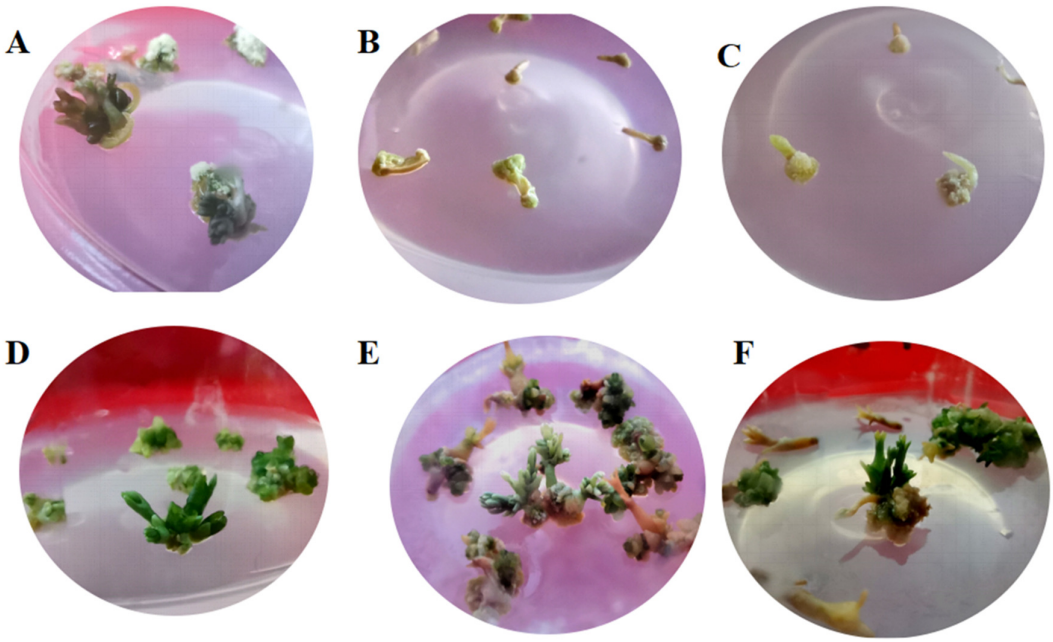

Figure 2. Induction of germination from six exosomes of *H. ammodendron* (35d)
(Note: A: seed B: hypocotyl C: cotyledon D: cotyledon nodes E: fixed bud F: terminal bud)

Table 1. Effect of adding different hormone concentration ratios on germination induction of six explants (% germination induction rate)

Treatment code	Media type	A seed	B hypocotyl	C cotyledon	D cotyledonary nodes	E fixed bud	F terminal bud
1	MS+0.5 mg·L^{-1} 6-BA+1 mg·L^{-1} KT	76.01ab	0	0	33.33bcde	47.62ab	28.57ab
2	MS+1 mg·L^{-1} 6-BA+0.5 mg·L^{-1} KT	61.9abc	0	0	42.86abcde	33.33ab	23.81ab
3	MS	42.86bc	0	0	0f	0b	0b
4	MS+0.5 mg·L^{-1} IBA+1 mg·L^{-1} 6-BA+1 mg·L^{-1} KT	47.62bc	0	0	47.62abcd	23.81ab	33.33ab
5	MS + 0.5 mg·L^{-1} IBA + 0.5 mg·L^{-1} 6-BA	61.91abc	0	0	57.14ab	38.1ab	23.81ab
6	MS + 0.5 mg·L^{-1} IBA + 0.5 mg·L^{-1} KT	47.62bc	0	0	52.38abc	0b	19.05ab
7	MS+1 mg·L^{-1} IBA+1 mg·L^{-1} 6-BA	71.43abc	0	0	28.57cde	4.76ab	23.81ab
8	MS+1 mg·L^{-1} IBA+1 mg·L^{-1} KT	47.62bc	0	0	14.5ef	0b	0b
9	MS+1 mg·L^{-1} IBA+0.5 mg·L^{-1} 6-BA+0.5 mg·L^{-1} KT	38.1c	0	0	47.62abcd	33.33ab	14.29ab
10	MS+0.5 mg·L^{-1} NAA+1 mg·L^{-1} 6-BA+1 mg·L^{-1} KT	71.43abc	0	0	42.86abcde	33.33ab	52.38a
11	MS+0.5 mg·L^{-1} NAA+0.5 mg·L^{-1} 6-BA	71.43abc	0	0	61.9a	52.38a	33.33ab
12	MS + 0.5 mg·L^{-1} NAA + 0.5 mg·L^{-1} KT	61.9abc	0	0	52.38abc	23.81ab	9.52b
13	MS+1 mg·L^{-1} NAA+1 mg·L^{-1} 6-BA	66.67abc	0	0	23.81de	0b	0b
14	MS+1 mg·L^{-1} NAA+1 mg·L^{-1} KT	66.67abc	0	0	33.34bcde	47.62ab	23.81ab
15	MS+1 mg·L^{-1} NAA+0.5 mg·L^{-1} 6-BA+0.5 mg·L^{-1} KT	85.71a	0	0	57.14ab	28.57ab	33.33ab

Note: different letters indicate significant differences at $P \leq 0.05$

Effects of different concentrations of IBA, NAA and IAA on the rooting of regenerated plants

The results of 1/2 MS as the basal medium with different concentrations of IAA, IBA, and NAA were used to induce rooting of adventitious shoots in the culture, and the results are shown in Table 2 below. From the test results, it can be seen that the highest rooting rate of adventitious shoots in 1/2 MS + 1 mg· L^{-1} IBA + 1 mg· L^{-1} NAA + 1 mg· L^{-1} IAA medium was 50%, and the average root length was 6.96 mm. In the course of the experiment, It was found that the overall rooting was better when 1/2 MS was used as the basal medium than when MS was used as the basal medium, so 1/2 MS was used as the basal medium for subsequent adventitious shoot induction rooting. Although the rooting rate was higher when 1/2 MS was used as the basal medium, the effective number of roots was lower, and during the rooting process, most of the adventitious shoots in the medium would gradually disappear, leaving only a large number of healed and adventitious roots produced. After the main roots had lignified and a certain number of fibrous roots had grown, the caps of the histoponic bottles were left open for a small period of time; after 1~2 d, the caps were left open for another period of time to allow the regenerated seedlings to gradually adapt to the natural environment. After one week of refinement, the root medium of the regenerated seedlings was rinsed with sterile water and transferred into sterilized vermiculite: charcoal: sand = 1:1:1. To maintain a certain temperature and humidity, the nursery pots for transplanting regenerated seedlings were covered in multiple layers using cling film.

Based on the above results and the observation during the experiment, it is concluded that the seeds, cotyledons, fixed buds and top buds can induce budding, and the cotyledons have the best bud-inducing effect, and the most suitable medium for cotyledons to induce budding is MS+0.5 mg· L^{-1} NAA+0.5 mg· L^{-1} 6-BA, the germination rate was 61.9%, and the budding was successively after 14 days. The optimal medium for inducing adventitious bud rooting is 1/2 MS+1 mg· L^{-1} NAA +1 mg· L^{-1} IBA + 1 mg· L^{-1} IAA, with a rooting rate of 50%, took root after 14 days, and the *H. ammodendron* regeneration system of organogenesis was successfully established.

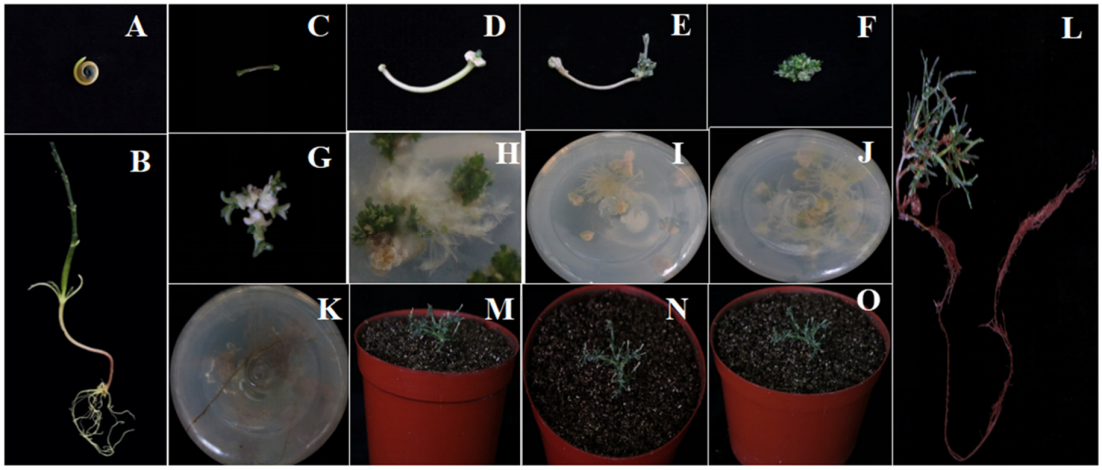

Figure 3. Flow chart of organogenesis regeneration system of H. ammodendron
(Note: A: H. ammodendron weed; B: sterile seedling; C: Cotyledon nodes; D~G: cotyledon node induced shoot; H~K: adventitious shoot induced rooting; L: H. ammodendron regenerated seedling; M~O: transplant)

Table 2. Effects of adding different concentrations of hormones on the rooting of regenerated plants with 1/2 MS as the base medium

Treatment code	IBA $(mg{\cdot}L)^{-1}$	NAA $(mg{\cdot}L)^{-1}$	IAA $(mg{\cdot}L)^{-1}$	Rooting rate (%)	Average root length(mm)	Growth
1	2	2	4	8.5b	0.75b	After the formation of healing wounds, the buds fade away, with roots but no buds
2	2	1	2	0b	0b	After the formation of healing wounds, the buds fade away, with roots but no buds
3	4	1	4	0b	0b	After the healing wound is formed, the buds fade away.
4	3	2	1	18.5ab	3.5ab	After the healing wound is formed, the buds fade away.
5	3	1	3	8.5ab	1.9ab	More roots, shorter, more healing, fewer shoots, slower growth
6	1	3	4	14.5ab	4.17ab	More roots, slender, fewer shoots, more healing tissue
7	1	1	1	50a	6.96a	Many roots, good growth condition, more healing tissue, forming a complete plant
8	1	4	2	36.5ab	1.52b	Many roots, slender, with roots and shoots, slow-growing
9	3	4	4	33.5ab	1.3b	After the formation of healing wounds, the buds fade away, with roots but no buds
10	4	2	3	47a	1.36b	Few slender roots, no buds, many healing wounds, slow growth
11	4	2	2	36ab	1.87ab	After the formation of healing wounds, the buds fade away, with roots but no buds
12	4	4	3	0b	1.23b	After the formation of healing wounds, the buds fade away, with roots but no buds
13	2	4	1	40.5ab	2.39ab	After the formation of healing wounds, the buds fade away, with roots but no buds

14	3	3	2	12.5ab	1.56b	After the formation of healing wounds, the buds fade away, with roots but no buds
15	4	3	1	8.5ab	1.97ab	After the healing wound is formed, the buds fade away.
16	2	3	3	24.5ab	1.9ab	Buds disappeared, only healing wounds remain, roots few, slender

Discussion

The use of plant tissue culture techniques to obtain regenerated plants directly or indirectly through the somatic embryo pathway or organogenesis pathway is the basis for biotechnological breeding, protoplast culture, somatic cell hybridization and gene transformation research. In this study, the induction of adventitious buds was carried out using *H. ammodendron* seeds, hypocotyls, cotyledons, cotyledon nodes, fixed buds and terminal buds as explants, and it was found that *H. ammodendron* seeds, cotyledon nodes, fixed buds and terminal buds could be induced to produce buds, but hypocotyls and cotyledons did not produce buds. The cotyledonary nodes showed fast and high germination rate when germination was induced. Due to the long time for the formation of definitive and terminal buds in *H. ammodendron* sterile seedlings, the source of material was limited and time-consuming; the cotyledon nodes induced better quality of germination, and the cotyledon nodes were finally identified as the best explants for germination induction. Yasmin *et al*. (2003) showed that the types and proportions of different exogenous plant hormones have a significant effect on the regeneration of the same plant. 6-BA favours cell division and affects organ differentiation, and in agreement with Chen and Gao reported (Dabauza *et al*. 1977), a mixed fraction of 6-BA and growth hormone was found to increase the rate of germination induction in *Plumbago auriculata*. Higher 6-BA concentrations strongly inhibited shoot elongation, resulting in little or no production of large shoots. This finding is consistent with the cotyledon explants of kale type oilseed rape (Xiaolan *et al*. 2001). This may be because the plant has reached a specific balance between internal and external hormones and the hormone receptors are saturated (Miedema, 1984; Pasqualetto *et al*., 1986; Leshem *et al.,* 1988; Liu *et al*., 2008) found that high levels of cytokinins can cause vitrification in in vitro cultures of phytoplankton with high water content, respectively. However, there was no significant vitrification of adventitious shoots in the present study. The specific cause of this problem deserves further investigation.

In addition, it was found in the pre-test pre-experiment that the *H. ammodendron* regeneration seedlings could be rooted when MS and 1/2 MS were used as the base medium, but it was found that the growth of explants on 1/2 MS medium was better than MS during the test observation, so in this experiment, we chose 1/2 MS as the base medium for rooting, and the rooting rate could reach when IBA, IAA & NAA were added to 1/2 MS 50%. During the rooting phase, IBA, IAA, and NAA are the three most used hormones. The effects of IBA on rooting have been shown to be more effective than other plant growth regulators (Amiri and Elahinia, 2011). In addition, the addition of NAA had some promotion effect on the growth of adventitious roots. This may be due to the appropriate concentration of growth hormone acting on the cells, which facilitates the binding to ATPase on the plasma membrane. This acidifies the cell wall environment and some unstable hydrogen bonds are easily broken, allowing the molecular structures of cell wall polysaccharides to interweave. The cell wall tends to relax, making it easy for the cells forming adventitious roots to break through (Li *et al*., 2009). Therefore, it is necessary to investigate the optimal concentration for rooting. In addition, many studies have shown that IAA can promote root formation and growth, such as banana and bergamot (Miilion *et al*., 2015; Jose *et al*., 2015). Although the root rate of this graduate student is high, most adventitious shoots will disappear in the later culture, and only calluses and adventitious roots will appear, which is guessed to be related to the concentration of plant hormones, and subsequent experiments also need to adjust the concentration of plant hormones to induce rooting. Browning, vitrification, and contamination are relatively common and poorly controlled phenomena in in vitro plant culture. Vitrification is a physiological lesion in

which the regenerated shoots are translucent and have an abnormal morphological appearance. The majority of vitrification seedlings are adventitious shoots from stem tips or stem cut cultures, and usually the recovery percentage of vitrification seedlings is very low, and vitrification seedlings still form in succession cultures. In this experiment, the occurrence of vitrification seedlings was less frequent at the induction stage of shoot emergence, and it was observed that the occurrence of vitrification seedlings was reduced accordingly by decanting the residual water in the culture flasks.

In summary, this study establishes a system for *H. ammodendron* regeneration by organogenesis. The method will provide a basis for the application of *H. ammodendron* ex vivo regeneration and genetic transformation.

Conclusions

It plays an extremely important role in protecting desert ecosystems and preventing desertification. Ex vivo reproduction is an effective method for species conservation and restoration. In this study, six explants of the *H. ammodendron* were used to establish an ex vivo regeneration system of the shuttle by inducing adventitious buds, adventitious bud rooting and transplanting. The optimal medium for inducing clandine buds in cotyledon nodes differentiation is 0.5 mg· L^{-1} NAA+0.5 mg· L^{-1} 6-BA induced budding rate was high, reaching 61.90%, rooting medium was 1/2 MS+1 mg· L^{-1} NAA +1 mg· L^{-1} IBA + 1 mg· L^{-1} IAA with a rooting rate of 50%. This study will lay a solid technical foundation for in-depth study of the molecular mechanism of drought resistance and cultivation of excellent germplasm.

Authors' Contributions

Conceptualization: Hua Zhang and Bo Wang; Data curation: Ping Wang, Lingjuan Man and Li Ma; Formal analysis: Ping Wang, Lingjuan Man and Li Ma; Investigation: Ping Wang, Lingjuan Man and Li Ma, Jiaxin Qi; Methodology: Yanping Ren and Cong Chen; Supervision: Ping Wang, Lingjuan Man and Li Ma; Visualization: Lingjuan Man and Ping Wang; Writing - original draft: Ping Wang; Writing - review and editing: Hua Zhang and Zhengpei Yao. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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