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Establishment of Rapid Propagation System of *Lycium Ruthenicum* Murr Tissue Culture

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Abstract *Lycium ruthenicum* is a perennial shrub of Lycium in Solanaceae, which is an important plant for improving the environment of desert greening. The sterile seedlings of *Lycium ruthenicum* were inoculated in callus induction medium MS-1 (MS+0.5~1 mg/L NAA+1.5~2.5 mg/L 6-BA+30 g/L sucrose + 8 g/L agar, pH5.2) for about 10 days. After 40 days, the callus was separated from the explants and placed in MS-2 medium (MS + 0.5～1 mg/L NAA + 0.5~1.4 mg/L 6-BA + 30 g/L sucrose + 6 g/L agar, pH5.2) to induce adventitious buds. Finally, the buds were inoculated in MS medium to take root. Rooting in MS medium about 20 days later, the seedlings grew up, each seedling had 3-4 roots, and then carried out seedling refining and transplanting. This method of callus induction is due to obtaining callus directly from sterile seedlings of *Lycium ruthenicum*. And it shorts the time of tissue culture, reduces the cost of culture and improves the efficiency of culture in *Lycium ruthenicum*.
Keywords *Lycium Ruthenicum* Murr; Tissue culture; MS medium

Lycium ruthenicum, one of the most important drought and salt resistant desert pioneer species, is a perennial thorny shrub of *Lycium ruthenicum* in *Solanaceae*. In the world, many *Lycium ruthenicum* species are found in South America, South Africa, southwest North America, Mediterranean, Asia, and there is a native species of *Lycium ruthenicum* in Australia (Levin et al., 2007). In China, it is mainly distributed in special environments, such as Tibet, Helan Mountain in Ningxia, Eastern Qinghai, Northern Shanxi, Northern Xinjiang, Neiling, river bed and sand beach, etc. and it is resistant to low temperature (Liu et al., 2018).

Wild *Lycium ruthenicum* is rich in nutrients, as well as Calcium, Magnesium, Copper, Zinc, Manganese, Iron, Lead, Nickel, Cadmium, Cobalt, Chromium, Potassium, Sodium, Lithium, Phosphorus, Titanium, Vanadium, Aluminum, Boron, Silicon and other metal elements (Qi et al., 2019). *Lycium ruthenicum* is also rich in anthocyanins, which has antioxidant and anti-aging medicinal value. *Lycium ruthenicum* is a combination of economic value, ecological value, medicinal value and health value. How to fully and reasonably develop and utilize *Lycium ruthenicum* resources is an important issue that needs urgent attention (Hao et al., 2016).

Plant tissue culture technology is an asexual propagation technology based on cell totipotency. It has the advantages of small space occupation, no regional and seasonal restrictions, short culture cycle, rapid propagation in a short time, no variation, maintaining all genetic characteristics of female parent, low investment and high economic benefits. Once it comes out, it was rapidly applied in flowers, crops and forest seedling production. So far, researchers have used different *Lycium ruthenicum* tissues as explants to induce callus, including twigs (Hao et al., 2005), seeds (Chen et al., 2018), tender stems (Sun et al., 2016), mature leaves (Sun et al., 2016; Yang et al., 2016), cotyledons (Wang et al., 2016), hypocotyls (Zheng et al., 2017). With the help of plant tissue culture technology, the cultivation method of regeneration seedlings of *Lycium ruthenicum* was established to produce large-scale seedlings of *Lycium ruthenicum*, so as to meet the needs of high quality, high yield and industrialization of *Lycium ruthenicum*.

RAPD, SRAP, cpDNA and SSR were used to analyze the genetic diversity and structure of *Lycium ruthenicum* (Zhang et al., 2001; Liu et al., 2012; Chen et al., 2014b; Chen and Zhong, 2014). Liu analyzed the genetic diversity of 14 endangered wild *Lycium ruthenicum* populations in Northwest China, and found that the genetic diversity frequency of *Lycium ruthenicum* in this region was very high using sequence related amplified polymorphic markers, which was of great significance for the protection and breeding of endangered species (Liu et al., 2012). Zhao used 18 pairs of simple sequence repeat (SSR) markers to analyze the genetic diversity and population structure of 139 *Lycium ruthenicum* materials (Zhao et al., 2010). Chen found that the differentiation time of different lineages of *Lycium ruthenicum* was consistent with the rapid uplift period of Qinghai Tibet Plateau and the formation and expansion time of desert in northern China (Chen et al., 2014b). Chen identified 1913 up-regulated genes and 536 down-regulated genes in *Lycium ruthenicum* by RNA sequence analysis (Chen et al., 2014a). Liu used virus induced gene silencing (VIGS) technology to silence the endogenous genes of *Lycium ruthenicum* for gene function analysis (Liu et al., 2014). Xue cloned and obtained the key flowering gene *FT* (*Flowering Locus T*) of *Lycium ruthenicum* and *Lycium barbarum*. They are mainly expressed in leaves, which is helpful for the follow-up study of related functional genes (Xue et al., 2015).

In this study, callus was directly induced from sterile seedlings of *Lycium ruthenicum*, and regenerated plants were obtained quickly and effectively, which greatly shortened the time of tissue culture, and laid the foundation for the next step of rapid obtaining *Lycium ruthenicum* plants.

1 Results and Analysis

1.1 Obtaining differentiated seedlings of wild *Lycium ruthenicum*

Firstly, the seeds of *Lycium ruthenicum* were disinfected, and then the sterilized seeds were transferred to the MS ordinary medium plane. After about 14 days, the aseptic seedlings grew into seedlings, and the aseptic seedlings with good growth were selected for subsequent experiments. Then, the well-developed sterile seedlings of *Lycium ruthenicum* were transferred to MS-1 (callus induction medium), waiting for the growth and development of callus (Figure 1A; Figure 1B; Figure 1C).

After about 21 days, the callus grew rapidly and grew to a callus with a diameter of about 10 mm, which could be peeled off and placed on MS-2 (induction differentiation medium) (Figure 1D); then the callus continued to grow and grew to a diameter of about 15 mm about 30 days after peeling off (Figure 1E), and then grew to a diameter of about 20 mm about 40 days after peeling off. In addition, the state of callus changed from hard green to soft yellow green (Figure 1F); then about 60 days after peeling from callus, about 3 subcultures (each subculture cycle is about 20 days), differentiated buds appeared (Figure 1G).

Then the differentiated buds continued to grow for about 20 days (Figure 1H), and the well-developed buds were stripped off and inserted into MS ordinary medium to induce rooting (Figure 1I); after the buds continued to grow for about 20 days, they would grow into seedlings, and each seedling had 3-4 roots, which could be used for the next transplanting. The following is the specific time of the changes of tissue cell state of *Lycium ruthenicum* (Table 1).

1.2 Seedling refining and transplanting of *Lycium ruthenicum*

Firstly, the seedlings of *Lycium ruthenicum* with good growth condition were selected, the cap of culture bottle was opened, and the seedlings were adapted to normal temperature and light for 2~3 days. Then put 1:2 ratio of fine sand and nutrient soil matrix into the flowerpot, and add appropriate amount of farmyard manure, mix and fit. Soaked the flowerpot in water to make it wet from bottom to top, then selected the seedlings that grew well after refining, selected 2 seedlings to plant in one pot, and covered them with plastic film to keep moisture. After waiting for 3 days, opened a corner of the preservative film, opened half of it in 3 days, and removed all the preservative film in 3 days. When the seedlings survived and developed well, divided them into pots, one plant in each pot, and do a good job in water and fertilizer management. The survival rate of *Lycium ruthenicum* seedlings was over 85%.

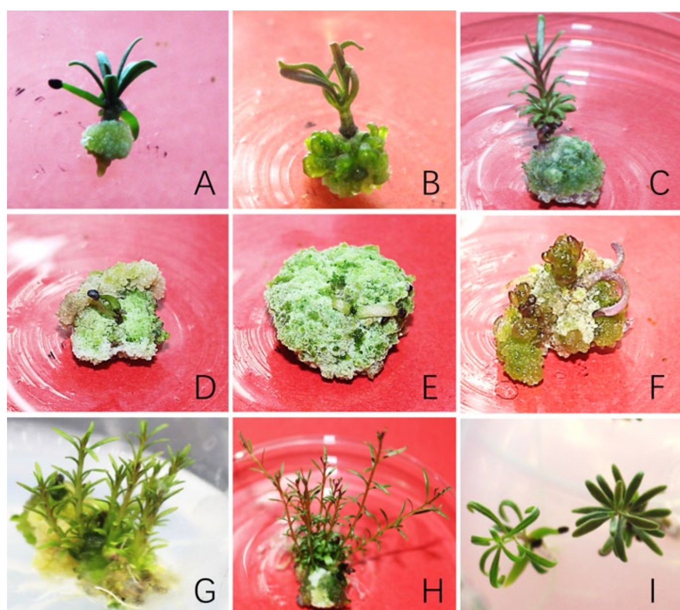

Figure 1 Plant regeneration system process of *L. Ruthenicum* Murr

Note: A: callus development after aseptic seedlings growing into MS-1 medium for about 7 days; B: callus development after aseptic seedlings growing into MS-1 medium for about 14 days; C: callus development after aseptic seedlings growing into MS-1 medium for about 21 days; D: callus developing for about 21 days after callus was peeled; E: callus developing for about 30 days after callus was peeled; F: callus developing for about 40 days after callus was peeled; G: many shoots emerged from one plate of callus for about 60 days after callus was peeled; H: continuously developing young seedlings on one plate of callus; I: rooting of the seedlings

Table 1 Tissue cell state change schedule of *Lycium ruthenicum*

Initiation state of tissue cells in *Lycium ruthenicum*	Termination state of tissue cells in *Lycium ruthenicum*	Time interval
Sterilized seeds were planted on MS medium	The aseptic seedlings grew well	Fourteen days
Well aseptic seedlings were transferred to MS-1 medium	Callus with diameter of about 10 mm were induced	Twenty-one days
Callus with diameter of about 10 mm were peeled and transferred to MS-2 medium	Callus continued to grow until differentiated buds appeared	Sixty days (subculture 3 times), 20 days each time
Differentiated buds appeared	Well growth buds were peeled from the callus	Twenty days
Well growth buds were peeled and transferred to MS medium	The seedlings continued to grow and produced 3-4 roots, which were used for further transplanting	Twenty days

2 Discussion

This study provided a rapid method to obtain regeneration seedlings of *Lycium ruthenicum*. Callus was directly obtained from the sterile seedlings of *Lycium ruthenicum*. The specific explant part was the contact surface between the root and stem of *Lycium ruthenicum* and the upper surface of the culture medium. It was particularly easy to produce callus, and grew rapidly. The callus state changed from green to yellow green, from hard to soft, and in a short period of time the callus would germinate multiple buds, then grew into seedlings, and obtained multiple differentiated plants within a period of time. Therefore, the callus differentiation efficiency of *Lycium ruthenicum* was higher. Compared with the traditional callus induction through the leaves, stems and roots of seedlings, it greatly shortened the time of tissue culture, greatly reduced the cultivation cost of *Lycium ruthenicum*, and effectively improved the cultivation efficiency. The survival rate of regenerated seedlings was as high as 85%.

By adding 0.5~1.0 mg NAA and 1.5~2.5 mg 6-BA to each liter of MS medium, it was easier to obtain differentiated callus, and the callus formation rate was up to 90%. The rooting rate could be as high as 97% without adding any hormone, and the root growth rate was faster. Each seedling grew at least three roots, and the roots were strong and the root hairs were dense. In this study, the method of rapid regeneration of *Lycium*

ruthenicum had the characteristics of good genetic stability, low cost, short seedling period, high reproduction coefficient and simple operation.

3 Materials and Methods

3.1 Disinfection of seeds and obtaining of aseptic seedlings

The seeds of *Lycium ruthenicum* were soaked in 70 v% (volume percent) alcohol for 3~7 min, then disinfected with 0.1 wt% (mass percent) mercuric chloride solution for 10~20 min, and then washed with sterile water for 2~3 times. Sterilized seeds were placed in MS medium, and aseptic seedlings could be obtained after 8~12 days of germination.

3.2 Callus induction

The aseptic seedlings were transplanted into MS-1 medium (MS+0.5~1 mg/L NAA+1.5~2.5 mg/L 6-BA+30 g/L sucrose+8 g/L agar, pH 5.2) to induce callus. The culture conditions were as follows: temperature 23℃~27℃, light 12 h/D, medium pH 5.0~5.5, culture 35~45 D.

3.3 Obtain of adventitious buds

The callus of step 3.2 was separated from the explants and placed in MS-2 medium (MS+0.5~1 mg/L NAA+0.5~1.4 mg/L 6-BA+30 g/L sucrose+6 g/L agar, pH 5.2) to induce adventitious buds. The culture conditions were as follows: temperature 23℃~27℃, light 14 h/D, medium pH 5.0~5.5, proliferation culture for 3~5 generations.

3.4 Root induction

The adventitious buds of step 3.3 were transplanted into the MS medium to induce rooting, and the regeneration seedlings of *Lycium ruthenicum* were obtained; the culture conditions were as follows: temperature 23℃~27℃, light 14 h/D, culture time 17~22 D. The obtained rooting seedlings were transferred into the greenhouse for at least 2 weeks.

Authors' contributions

Wang like is the executor of the experimental design and research; Wang like and Zhong Huiping complete the data analysis and write of the first draft of the paper; Mao shanguo, Yang Ping, Wu Yunwen, He Ying and Chen Quanzhan participate in the experimental design and the analysis of the experimental results; Wang like is the designer and director 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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