Research on key techniques of tissue culture of Qinghai Rhubarb

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Abstract. Rheum palmatum L. is a perennial herb of the Rheum genus Polygonaceae. It is a traditional Chinese herbal medicine which has extremely high medicinal value. Rhubarb has scarce seeds in subtropical regions, and natural planting conditions have limitations. In recent years, the demand of rhubarb has increased, and the means of sowing and raising seedlings can no longer meet the needs of the market. Traditional sowing and propagation methods have limitations such as long cycle and low reproduction coefficient. Tissue culture technology can shorten the reproduction cycle that increase the reproduction coefficient and maintain the excellent characteristics of the parents, which is an effective way to promote the large-scale and commercial production of fine varieties of rhubarb. The results of the study showed that: (1) Analysis of the characteristics of Qinghai Rhubarb seeds. (2) Seed sterilization treatment. (3) Primary culture medium selection. (4) Callus induction. (5) Callus proliferation culture. (6) Hypocotyl callus bud differentiation. (7) Rooting culture. (8) Qinghai Rhubarb tissue culture seedling transplantation. Using the method of the present invention to establish a Qinghai Rhubarb in vitro culture aseptic propagation system, high-quality Qinghai Rhubarb seeds as explants and different hormone medium ratios, Qinghai Rhubarb hypocotyl induction rate reached 90%, and the proliferation coefficient reached 4.3. The survival rate of transplanting tissue cultured seedlings reaches 95% which can improve the reproduction efficiency of Qinghai Rhubarb, and provide theoretical basis and technical support for subsequent research on transgenic and genetic improvement. It can also provide a basis for medicinal value for the systematic research and commercial production of Qinghai Rhubarb.

1. Introduction

Rheum palmatum L. is a perennial herbaceous plant belonging to the genus Rheum in the Polygonaceae family. It is a traditional Chinese medicinal material with special effects which has been cultivated for many years in China and is mainly produced under natural environmental conditions in plateau areas such as Qinghai and Tibet. Its types mainly include Qinghai Rhubarb, Tanggute Rhubarb and Medicinal Rhubarb, which have extremely high economic value and medicinal value. Rhubarb was first published in the "Shen Nong's Materia Medica" in the Han Dynasty[1]. In the Eastern Han Dynasty, several rhubarb prescriptions were recorded in the works "Treatise on Febrile Diseases" and "The Synopsis of the Golden Chamber" which are still in use today. The dried roots and rhizomes of rhubarb have bitter taste and coldness which have a wide range of medicinal effects. The 2015 edition of the Chinese Pharmacopoeia described its effects as "purge down and attack accumulation, clear heat and fire, cool blood and detoxify, expel blood stasis and relieve menstruation, relieve dampness and relieve yellow"[2]. In ancient times, rhubarb was often used as a plaster for external use or water decoction for oral administration. Nowadays, rhubarb prescriptions have significant effects in the
treatment of common clinical diseases such as pediatrics, surgery, and gynecology, as well as acute, critical, severe and difficult diseases, including gastrointestinal stagnation, carbuncle swelling and furuncle, promoting blood circulation to remove stasis and damp-heat jaundice[3]. With the development of the pharmaceutical industry, the demand for rhubarb increases continuously. Due to the long breeding cycle and low reproduction coefficient of Qinghai wild rhubarb, supply exceeds demand. In recent years, artificial cultivation of rhubarb has been the main production method in Qinghai. However, traditional methods of sowing and breeding have certain limitations. For example, it is difficult to collect pure seeds, the quality of seeds is uneven, mixing and hybridization lead to degradation of Qinghai Rhubarb germplasm, traditional propagation methods require high regional environment and cultivated land, propagation is relatively low, mass production is difficult, and the output cannot meet market demand. In recent years, some progress has been made in the establishment of the aseptic system of rhubarb and the regeneration of callus. However, there have been few in-depth studies on rhubarb tissue culture system. Plant tissue culture technology separates plant organs or tissues and sterilizes them. The method is to inoculate plant tissues on artificially prepared nutrient media to obtain callus or regenerate complete plants to obtain its medicinal or economic value[4]. Due to the totipotency of plant cells, plant tissues can be cultured in vitro, and the genetic information that can develop into complete plants can be expressed, so that regenerated plants with the same genetic information as the parental traits can be obtained in a short time[5]. This research is aimed at carrying out a comprehensive and systematic tissue culture to accelerate the rapid artificial breeding process of Qinghai Rhubarb. In this study, the Qinghai Rhubarb seeds donated by the Chenshan Botanical Garden Institute of the Chinese Academy of Sciences were used to induce, proliferate and differentiate the hypocotyls, cotyledons, and leaves of the rhubarb to establish an aseptic system which will provide a theoretical basis for in-depth research.

2. Qinghai Rhubarb Explant Disinfection Research

2.1 Disinfection treatment of explants
Take QHDH2 rhubarb fruits, most of which are oblong, with 3-winged achenes, and a few are 4-winged. The seed coat is gray-brown, the surface is smooth and hairless, and it is shrunken, with umbilicus of different lengths. After the wings are removed, the inside of the fruit can be seen as a single seed, which is dark brown. Rinse with sterile water 3 times, soak in water for 3 hours, after removing the wings, rinse with sterile water for later use. The pretreated rhubarb seeds were sterilized with 75% alcohol for 20 seconds, rinsed with sterile water 2-3 times, and then soaked in 0.5%, 1%, 5% HgCl2 for 6, 8 and 10 minutes for sterilization (3 sterilizations deal with). During the soaking period, the container is often shaken to make the explant fully contact with the HgCl2 solution. Rinse the sterilized explants with sterile water for 4 to 5 times, and use sterile filter paper to absorb water on the surface for later use. During inoculation, insert the seed germ into MS medium on a clean bench. Inoculate 5 bottles per treatment and 4 explants per bottle, repeated 3 times. Observe and record once every 5 days, and count the pollution rate, browning rate and germination rate after 10 days of inoculation.

2.2 Analysis of disinfection treatment results

| Number | Disinfection time(min) | Sodium hypochlorite (%) | Number of seeds | Browning rate (%) | Pollution rate (%) | Germination rate (%) |
|--------|------------------------|-------------------------|-----------------|-------------------|-------------------|---------------------|
| 1      | 6                      | 0.5%                    | 20              | 15                | 50                | 30                  |
| 2      | 8                      | 0.5%                    | 20              | 10                | 55                | 70                  |
| 3      | 10                     | 0.5%                    | 20              | 5                 | 25                | 65                  |
| 4      | 6                      | 1%                      | 20              | 5                 | 20                | 80                  |
| 5      | 8                      | 1%                      | 20              | 5                 | 20                | 80                  |
| 6      | 10                     | 1%                      | 20              | 10                | 20                | 90                  |
| 7      | 6                      | 5%                      | 20              | 10                | 10                | 80                  |
3. Qinghai Rhubarb callus induction

3.1 Processing method
Select 6-BA, KT, NAA 3 hormones and their different concentrations to design the experiment (Table 4) to investigate the best hormone combination for induction of callus from Rhubarb palmatum. Take the above-mentioned one-month-old sterile seedlings, take the hypocotyls, cotyledons, and leaves as explants and inoculate them on MS solid medium containing hormones, count the induction rate, repeat 3 times, and screen out the most suitable callus induction. After tissue culture hormone ratio, the callus induction of rhubarb cotyledons, leaves and other explants under optimal conditions was investigated.

3.2 Qinghai Rhubarb callus induction results

Table 2. Callus induction results of Qinghai Rhubarb hypocotyls, cotyledons and leaves.

| Hormone | Concentration of hormone (mg/L) | Explants | Number of explants | Number of callus | Induction frequency (%) |
|---------|--------------------------------|----------|--------------------|-----------------|------------------------|
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(1.0mg/L)KT+(1.0mg/L)NAA | Hypocotyl 10 1 10.00 | Cotyledons 20 4 20.00 | Leaves 20 3 15.00 |
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(1.5mg/L)KT+(1.0mg/L)NAA | Hypocotyl 9 1 11.11 | Cotyledons 20 1 5.00 | Leaves 20 2 10.00 |
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(2.0mg/L)KT+(1.0mg/L)NAA | Hypocotyl 9 1 11.11 | Cotyledons 20 1 5.00 | Leaves 20 3 15.00 |
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(1.0mg/L)KT+(0.5mg/L)NAA | Hypocotyl 10 2 20.00 | Cotyledons 15 2 13.33 | Leaves 15 1 6.67 |
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(1.5mg/L)KT+(0.5mg/L)NAA | Hypocotyl 10 1 10.00 | Cotyledons 10 0 0 | Leaves 15 2 13.33 |
| 6-BA, KT, NAA | (1.0mg/L)6-BA+(2.0mg/L)KT+(0.5mg/L)NAA | Hypocotyl 10 2 20.00 | Cotyledons 15 1 6.67 | Leaves 15 1 6.67 |
| 6-BA, KT, NAA | (2.0mg/L)6-BA+(1.0mg/L)KT+(1.5mg/L)NAA | Hypocotyl 5 0 0 | Cotyledons 15 2 13.33 | Leaves 15 1 6.67 |
| 6-BA, KT, NAA | (2.0mg/L)6-BA+(1.5mg/L)KT+(1.5mg/L)NAA | Hypocotyl 9 1 11.11 | Cotyledons 15 1 6.67 | Leaves 15 3 20 |
| 6-BA, KT, NAA | (2.0mg/L)6-BA+(2.0mg/L)KT+(1.5mg/L)NAA | Hypocotyl 9 1 11.11 | Cotyledons 15 2 13.33 | Leaves 15 4 26.67 |
| 6-BA, KT | (1.5mg/L)6-BA+(1.5mg/L)KT | Hypocotyl 9 0 0 | Cotyledons 15 1 6.67 | Leaves 15 2 13.33 |
| 6-BA, NAA | (1.5mg/L)6-BA+(1.5mg/L)NAA | Hypocotyl 19 13 68.42 | Cotyledons 15 9 60.00 | Leaves 30 20 66.67 |
| KT, NAA | (1.5mg/L)KT+(1.5mg/L)NAA | Hypocotyl 10 1 10.00 |
4. Proliferation, differentiation and rooting of Qinghai Rhubarb hypocotyls

4.1 Hypocotyl callus induction
Take the hypocotyl of the sterile plantlet and inoculate it into the MS induction medium. The temperature of the light incubator was 20±1°C, and the culture was dark for 7 days. After 15 days, the hypocotyls showed yellow-green callus, and the induction rate reached 80%. The induction medium is: MS4.43g/L+6-BA1.5mg/L+NAA0.5mg/L+agar 3g/L+sucrose 30g/L, and the PH is 5.8 (the same below).

4.2 Proliferation and culture of hypocotyl callus
The hypocotyl callus was transplanted to MS proliferation medium. After 20 days, the hypocotyl was dark green callus with a growth rate of 3.5. The proliferation medium is: MS4.43g/L+6-BA 0.5mg/L+NAA 1.5g/L+agar 3g/L+sucrose 30g/L.

4.3 Hypocotyl callus bud differentiation
Transplant the hypocotyl callus to the bud differentiation medium, and induce multiple green buds after 15 days. The bud differentiation medium is MS4.43g/L+6-BA1.5mg/L+NAA0.5mg/L+agar 3g/L+sucrose 30g/L.

4.4 Rooting culture
Cut the sterile buds into individual plants, each with 2 to 3 leaves, and transplant them to rooting medium. The temperature of the light incubator: 20±1°C, light culture for 16h/d, dark culture for 8h/d. After 14 days, take roots to obtain Qinghai Rhubarb tissue culture seedlings with green leaves and good growth. The rooting medium is MS3.32g/L+agar 5g/L+sucrose 15g/L.

4.5 Transplanting Qinghai Rhubarb tissue-cultured seedlings
Transplant the rooted seedlings into a flower pot equipped with nutrient substrate soil, the pH of the soil substrate is 6.5-7.0, and the ratio is substrate soil: vermiculite: perlite (1:1:1), room temperature 25°C, light 12h/d, spraying Huaduoduo No. 1 once a week, 2000 times dilution, survival rate of more than 85% after transplantation 30 days.

Table 3. Three experimental data of callus of Qinghai Rhubarb.

| Example | Example 1 | Example 2 | Example 3 |
|---------|-----------|-----------|-----------|
| Callus induction rate | 90% | 87% | 80% |
| Callus proliferation | 4.3 | 4.0 | 3.5 |
| Survival rate of transplanted seedlings | 95% | 90% | 85% |
5. Conclusion
This study established a comprehensive and systematic tissue culture aseptic system through the induction, proliferation, bud differentiation and tissue culture seedling transplantation of rhubarb hypocotyls, cotyledons and leaves, in order to promote the rapid artificial propagation of Qinghai Rhubarb and in-depth research provide a theoretical basis.

![Figure 1. Different periods of somatic embryogenesis of Chinese herb rhubarb.](image)

Note: A: Qinghai Rhubarb seeds; B: Disinfected seeds; C: Hypocotyl callus; D: Bud differentiation; E: Cotyledon callus; F: Petiole callus; G: Embryonic callus; G: Incomplete regeneration seedlings; H: Rhubarb transplanted seedlings

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