Studies on callus induction of *Valeriana officinalis* under different disinfection methods and photoperiod conditions

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Abstract. Using tender leaves of *Valeriana* as explants, 70% ethanol and 0.1% mercury as disinfectants, the effects of different disinfection methods and photoperiod on callus induction of *Valeriana* were studied. The results show that when C3 group was used to treat explants, the pollution rate and browning rate are lower, the callus induction rate was the highest. When the photoperiod was 0h/d, the healing time was the shortest, and the callus quality was the best in the experimental group, which was more conducive to the next step. The optimal disinfection combination is to soak the explants in 70% ethanol for 30 s and then soak them in 0.1% mercuric chloride for 7 min. The optimum photoperiod is 0h/d.

1. Introduction
*Valeriana officinalis* L. is a perennial herb of *Patrinaceae* and *Valeriana*, which mainly distributes in northeast and southwest of China. Its rhizomes are medicinal [1]. *Valeriana* has the functions of calming mind, dispelling wind, relieving spasm, and curing injury caused by falls and beats [2]. *Valeriana* has low sexual reproductive efficiency and exceedingly slow growth. Natural habitat destruction and uncontrolled excavation lead to the rapid reduction of wild resources and bring difficulties to the production of *Valeriana*. Rapid propagation of excellent varieties of *Valeriana* by biotechnological means has become an inevitable trend [3]. In the timely distribution of *Valeriana* plants, the types of culture medium, hormone concentration and types are mainly involved, which have been confirmed in stem organogenesis and somatic embryogenesis experiments of leaf explants of *Valeriana* [4]. The effects of explant disinfection and photoperiod on callus induction in the process of tissue culture of *Valeriana* were studied to provide a theoretical basis for further development and utilization of high-quality varieties of *Valeriana*.

2. Materials and methods

2.1. Experimental materials
Cultivate *Valeriana* indoors and use new tender leaves as explants. All reagents required for the experiment were of analytical pure.
2.2. Instruments and equipment
The multifunctional ultra-clean operating platform, RTOP-268Y Intelligent incubator, LED white light lamps, LDZM-80KCS pressure steam sterilizer, Shanghai Hengping FB124 electronic balance, TES-1339 high precision photometer.

2.3. Method

2.3.1. Preparation of culture medium and culture conditions
The callus induction medium of *Valeriana* was MS+6-BA5.0mg/L+NAA0.2mg/L, sterilized at 121°C for 20 minutes. The incubation temperature was (21±2) °C.

2.3.2. Disinfection and inoculation of explants
The disinfection method uses 70% ethanol and 0.1% mercuric chloride as disinfectants. This experiment designed different combinations of disinfection times. The setting of disinfectants and disinfection combinations is detailed in Table 1.

After the explants were washed and put into the test-bed, 70% ethanol (0s, 10s, 30s, 60s) was soaked, then 0.1% mercury chloride was disinfected (0 min, 4 min, 7 min, 9 min), and rinsed them three times with sterile waters. Leaves were cut into small pieces with an area of (1 cm ×1 cm) and inoculated on callus induction medium under sterile conditions. Four explants were grafted into each tissue culture bottle, and ten bottles were treated with one treatment. The culture conditions were as follows: (21±2) °C, the illumination intensity was 2000lx, and the illumination time was 8 hours a day. The first day was after inoculation. The growth contamination and browning death of explants were observed continuously during the inoculation period, and the related data were recorded.

Table 1. Different disinfectants and their treatment time combinations

| Disinfection time of 70% Ethanol /s | Disinfection time of 0.1%mercuric chloride/min |
|-------------------------------------|---------------------------------------------|
| 0                                  | A1                                          |
| 10                                 | B1                                          |
| 30                                 | C1                                          |
| 60                                 | D1                                          |
| 4                                  | A2                                          |
| 7                                  | A3                                          |
| 9                                  | A4                                          |
| 4                                  | B2                                          |
| 7                                  | B3                                          |
| 9                                  | B4                                          |
| 4                                  | C2                                          |
| 7                                  | C3                                          |
| 9                                  | C4                                          |
| 4                                  | D2                                          |
| 7                                  | D3                                          |
| 9                                  | D4                                          |

2.3.3. Study on the culture conditions of callus.
The illumination conditions are designed to be four photoperiods, which are 0 h/d, 8 h/d, 12 h/d and 16 h/d, respectively. The explants were inoculated on the same medium with ten bottles in each group and repeated three times. The illumination intensity was 2000lx under the condition of temperature (21±2) °C, recording the callus induction rate, and callus induction time. After 35 days of inoculation, the texture, color and state of callus were observed.

2.3.4. Data analysis. After ten days of inoculation, counting the number of polluted explants and calculating the rate of polluted explants. After 20 days, the mortality of callus was counted. After 25 days, the number of callus was counted, and the rate of callus induction was calculated. The data were analyzed by SPSS software (Duncan's method).

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Pollution\ \text{rate} = \frac{\text{The number of contaminated explants}}{\text{Total number of inoculated explants}} \times 100\% \quad (1)
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Mortality\ \text{rate} = \frac{\text{The number of dead explants}}{\text{Total number of inoculated explants}} \times 100\% \quad (2)
\]

\[
\text{Callus\ induction\ rate} = \frac{\text{The number of callus induction}}{\text{Total number of inoculated explants}} \times 100\% \quad (3)
\]
3. Results and analysis

3.1. Effect of disinfection reagent and time on Valeriana explants

As can be seen from Table 2, there are significant differences in the sterilization effect of different disinfection combinations. Treatment group C had the best disinfection effect in the experimental group, of which C3 had the highest leaf callus rate (35.83±3.82)%). The treatment effect of group D was poor, and the mortality rate of explants browning reached (58.33±5.20)%. Treatment group A had the worst disinfection effect, with the highest explants contamination rate (99.17±1.44)% and the highest survival rate only. It was (17.50±2.50)%. Therefore, the combination of C3 treatment is a suitable way to disinfect the leaves of Valeriana.

Table 2. Effects of different disinfection treatments on callus induction

| Combination | Number of explants inoculated | Explant contamination/% | Explant mortality/% | Callus induction rate of explants/% |
|-------------|-------------------------------|-------------------------|--------------------|-----------------------------------|
| A1          | 40                            | 99.17±1.44a             | 0.83±1.44c         | 0.00±0.00c                        |
| A2          | 40                            | 74.17±3.82b             | 21.67±1.44b        | 4.16±2.89b                        |
| A3          | 40                            | 59.17±3.82c             | 26.67±6.29b        | 14.17±5.20a                       |
| A4          | 40                            | 39.17±1.44d             | 40.00±6.61a        | 17.50±2.50ab                      |
| B1          | 40                            | 65.83±3.82a             | 20.00±2.50c        | 14.17±3.82b                       |
| B2          | 40                            | 50.83±3.82b             | 22.5±2.50c         | 26.67±5.20a                       |
| B3          | 40                            | 43.33±6.29b             | 33.33±3.81b        | 23.33±8.04ab                      |
| B4          | 40                            | 30.00±2.50c             | 45.00±5.00a        | 25.00±4.33ab                      |
| C1          | 40                            | 64.17±5.20a             | 23.33±2.88c        | 12.50±2.50c                       |
| C2          | 40                            | 45.83±5.20b             | 25.00±2.50c        | 29.16±3.82b                       |
| C3          | 40                            | 30.83±2.89c             | 33.00±5.20b        | 35.83±3.82a                       |
| C4          | 40                            | 19.17±1.44d             | 47.5±2.50a         | 33.33±1.44ab                      |
| D1          | 40                            | 55.00±5.00a             | 27.5±2.50c         | 19.16±3.82b                       |
| D2          | 40                            | 35.83±1.44b             | 35.83±1.44b        | 28.33±1.44a                       |
| D3          | 40                            | 25.83±1.44c             | 55.00±5.00a        | 19.17±5.20b                       |
| D4          | 40                            | 17.50±2.50d             | 58.33±5.20a        | 24.17±3.82ab                      |

Note: Using Duncan method, different letters in the same column show significant differences. (p<0.05)

3.2. The effect of photoperiod on callus formation of Valeriana

The time when the callus amount reached 50% was taken as the starting time. As shown in Table 3, the starting order of the four photoperiod callus was 0 hours of light per day > 8 hours of light per day > 12 hours of light per day > 16 hours of light per day. There were significant differences in callus induction rate of explants under different photoperiod conditions. The callus induction time was the shortest, and the induction rate was the highest when the photoperiod was 0 h/d, and the highest was (81.50±4.04)%. Under 16 h/d photoperiod, the induction rate was only (52.08±8.01)%). The callus induction rate from high to low was 0 h/d > 8 h/d > 12 h/d > 16 h/d, which indicated that light was not conducive to callus formation of Valeriana.

Table 3. Effects of different photoperiod on callus formation of Valeriana Leaves

| Treatment group | Callus appearance time/ (day) | Callus induction rate/ (%) | Treatment group |
|-----------------|--------------------------------|----------------------------|-----------------|
| 0 hours light per day | 9                             | 81.50±4.04a                | 0 hours light per day |
| 8 hours light per day | 13                            | 65.57±5.10b                | 8 hours light per day |
| 12 hours light per day | 15                            | 56.55±6.27b                | 12 hours light per day |
| 16 hours light per day | 20                            | 52.08±8.01c                | 16 hours light per day |

Note: adapting Duncan method, different letters in the same column show significant differences. (p<0.05)
3.3. The effect of photoperiod on callus quality of Valeriana.

In this study, explants can induce callus under four photoperiods. The callus induced by photoperiod of 0 h/d was light yellow and loose, with fine granules and fast growth rate (Fig. 1A). The later stage of 8h/d illumination induction, the callus was light green and looser, and the growth rate was faster (Fig. 1B). The callus obtained by photoperiod of 12 h/d was bright green with dense middle and sparse surroundings. The callus obtained at 16 h/d of photoperiod was dark green, solid, with tumorous protuberances on the surface, and the callus grew slowly and browned (Fig. 1D). When callus induction was carried out, light culture for 0 h/d had the shortest callus induction time, and the quality of callus was high, so it was not easy to brown. Light culture for 16 h/d resulted in slow induction speed, poor quality of callus and easy browning, which indicated that dark culture was beneficial to the formation of callus of Valeriana.

![Fig. 1 Effects of different photoperiod on Callus quality.](image)
A photoperiod 0 h/d; B photoperiod 8h/d; C photoperiod 12 h/d; D photoperiod 16 h/d

4. Discussion and conclusion

4.1. The effect of disinfection methods on callus induction

In the process of plant tissue culture, explant disinfection is the first step to establish a plant regeneration system. Commonly used explant disinfection reagents are sodium hypochlorite, hydrogen peroxide, ethanol, mercury, and so on [5]. Sodium hypochlorite has strong sterilization effect and easy removal, but its alkalinity is very strong. It is toxic to tender plant tissues and unsuitable for sterilization. The killing effect of hydrogen peroxide on plant explants is better, and the killing rate is lower, but it has strong toxicity and needs to be recovered after use. Ethanol has a mild germicidal effect and can be disinfected for a longer time to enhance the disinfection effect. In this study, 70% ethanol and 0.1% mercuric chloride were used to disinfect the explants of Valeriana leaves. When the disinfection time of ethanol increased from 0 s to 60 s, the contamination rate decreased from (99.17 ±1.44)% to (55.00± 5.00)%. The disinfection time of mercury ascending from 0 min to 9 min decreased the contamination rate from (99.17±1.44)% to (39.17±1.44)% , and the mortality increased from (0.83±1.44)% to (40.00 ±6.61)%. This indicated that the prolongation of disinfection time would increase the toxicity to cells and increase the death rate of explants. Because the use of a single disinfectant sometimes fails to achieve good results, the use of two disinfectants combined disinfection has become a common way of tissue culture disinfection, disinfection time control and disinfectant selection are the key factors [6].
4.2. The effect of photoperiod on callus formation of Valeriana.
Photoperiod is one of the critical factors affecting plant growth and development. Different plants, genotypes and explants also have different needs and performances for light conditions [7]. When studying the effect of light conditions on callus induction of valeriana, it was found that 24-hour light and 24-hour dark culture were not conducive to the formation of valeriana callus. In this experiment, the time of callus induction was the shortest when the photoperiod was 0 h/d. After seven days, leaf edge began to discolor and expand. After nine days, callus formation gradually occurred, and the highest induction rate was 81.5%. This is consistent with the conclusion drawn by Khosh M experiment that darkness is beneficial to the induction of Rose callus and apple leaves and cotyledons callus [8-9]. The longest time of callus emergence was 16 h/d in light culture. The edge of valerian leaves expanded gradually after 16 days of inoculation, and the callus formed gradually after 20 days. The rate of callus induction was only (52.08±8.01)%%. At the same time, the number of Callus Browning deaths increased significantly. We speculate that light stimulates the expression of some genes related to browning in the somatic cells of Valeriana officinalis [10], but whether this effect is direct or indirect remains to be studied.

4.3. Effect of photoperiod on callus quality
Under different photoperiod treatments, explants differed not only in the time of callus induction and the rate of induction, but also in callus quality [11]. The callus induced under 16 h/d photoperiod was turbid and grew slowly. The subculture of this callus under normal conditions is mostly unable to propagate due to browning death. It was not suitable for the next step of proliferation and differentiation experiment. The callus induced by 12 h/d photoperiod was yellow-green in color, compact in texture and slower in growth rate, which was more suitable for further proliferation and differentiation. The dark treatment-induced callus of Valeriana had a light yellow and transparent color, loose texture, fast growth, high induction rate and low browning rate, and strong ability of subculture and proliferation and regeneration.

In summary, the most suitable disinfection method for callus induction was 70% ethanol 30s+ 0.1% mercury chloride 7 min, and the optimum photoperiod was 0h/d with young leaves of Valeriana as explants, which provided theoretical guidance and data basis for rapid tissue culture and propagation of Valeriana.

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