Effects of Phytohormone Compatibility on Callus Growth of Rosa rugosa tunb

Wenbo Chang¹, Xue Wang¹, Ying Lan¹, Meini Wang¹, Bin Wen¹*, Lan Li²*

¹College of Science, Jiamusi University, Jiamusi City, Heilongjiang Province, 154007, China
²School of Data Science and Software Engineering, Wuzhou University, Wuzhou City, Guangxi Province, 543000, China

*Co-Corresponding author’s e-mail: 20888372@qq.com; lljmsu@163.com

Abstract. To obtain superior quality callus and to regenerate root system of Rosa rugosa tunb and improve culture efficiency, this experiment used a multi-factor orthogonal test method to establish a sterile system with young leaves of Rosa rugosa tunb as experimental materials and different concentrations of hormones in each stage. Explore the effects of plant callus growth. The results showed that the optimal disinfection method of explants was 70% alcohol 30s+0.1% HgCl₂ 8min. At this time, the browning rate was 46.70%, and the infection rate was 10.00%, which was 14.40% and 72.67% lower than that before disinfection. [Primary medium]: MS+1.5 mg/L 6-BA+3.0 mg/L NAA+3% sucrose+0.9% agar, the induction rate at this time is 75.00%, an increase of 62.50% compared with no added hormone. [Proliferation medium] MS+2.0mg/L 6-BA+0.2mg/L NAA+3% sucrose+0.9% agar, the callus diameter reached 2.49cm after proliferation, the growth was strong, and the callus was highly available. [Rooting medium] MS+0.4mg/L IBA+0.5g/L AC+3% sucrose+0.9% agar, the rooting rate is 75.00%, the root system is long and the growth is robust.

1. Introduction
Aromatic plants are a general term for cultivated plants and wild plants that have the aroma and can provide aromatic oils, and mainly include plants such as chamomilla, lavender (Lavandula angustifolia Mill.), and rose (Rosa rugosa)[1]. Extracting essential oils from flowers, stems and fruits of aromatic plants by molecular distillation and microwave extraction [2]. They are not only non-toxic but also have many curative effects. The main components are phenols, aldehydes, ketones, alcohols, and aromatic compounds[3]. The refined essential oils play a significant role in the production of natural flavors and have many uses in the daily necessities, pharmaceuticals and tobacco industries[4]. The study found that plant essential oils have low toxicity, safety, pollution-free, and strong antibacterial activity, which has caused widespread concern [5]. The treatment of fruits and vegetables with essential oils can effectively inhibit the activity of surface molds, thereby reducing the enzyme activity in fruits. The intensity of physiological activity undertakes a role in preserving freshness[6]. With the rapid development of China’s national economy, and the increasing living standards, the demand for material products is increasing. Studies have shown that the extractible essential oils in aromatic plants are rare, and the oil yield of Anthemis nobilis is only 1.7%. The maximum price per 100mL can reach 65 dollars, which means the demand for raw materials is great, but most of the aromatic plants such as chamomile, lavender and rose have to face the problem of low temperature and unsuitable growth in winter [7]. Evaporation, reducing nutrient consumption in the body, and
passing through a protective adaptation in winter, it is subject to seasonal restrictions, and the supply of raw materials is insufficient. *Rosa rugosa tunb* belongs to the genus *Rosaceae* shrub flower, distributed in northern North China, Northeast China, and Russia, ability to grow normally below the minimum ground temperature of -40℃. Its petals can be used to make a variety of products such as rose tea, raised sauce, and rose wine, etc [8-9]. It is also a homologous rose of medicine and food. Its plant is rich in essential oils, and it is an indispensable raw material for high-grade cosmetics after refining. Since the breeding method of *R. rugosa tunb* belongs to asexual reproduction, new individuals can be directly formed from a part of the mother. Therefore, most researchers' research on reproductive techniques mainly focuses on cuttings and ramets, but the raw materials are less during breeding, the coefficient is too low, the speed is slow, and the Coronavirus is easily infected, resulting in a decrease in plant reproduction rate or death. The use of plant tissue culture technology not only enables a large number of raw materials with consistent biological properties, but also achieves efficient production[10]. Therefore, to alleviate the shortage of raw materials, this study uses the young leaves of *R. rugosa tunb* as materials to screen the disinfection method through plant tissue culture technology, explore the optimal growth state of the callus at each stage and the optimum medium, and establish an efficient breeding system[11]. And apply it to agricultural production, which in turn promotes the development of the *R. rugosa tunb* cultivation market[12].

2. Materials and methods

2.1 Experiment material

Test materials: The origin of the test materials in this study is in Xinjiang and was introduced into the Science and Technology Park of Jiamusi University. The young leaves of *R. rugosa tunb* with no pests and diseases and good growth conditions were selected as experimental materials.

Main drugs: 6-BA (6-Benzylaminopurine), NAA (1-naphthyl acetic acid), agar, sucrose, mercuric chloride, absolute ethanol.

The medium used in this study was MS as the basic medium, with an additional concentration of 0.9% agar, 3% sucrose, and different concentrations of phytohormone, and adjust the pH of the medium to 5.8-6.0 which was autoclaved at 121℃ for 20 min. The culture conditions were temperature 20±5℃, light intensity 2000lx, and illumination time 10-12 h/d.

2.2 Experiment method

2.2.1 Disinfection of explants. The healthy growing leaves were selected and immersed in distilled water containing 2-3 drops of surfactant for 15-20 min. After the dipping, the leaves were wrapped with clean four-layer gauze and rinsed with running water for 20 min to rinse off the excess foam. In the clean bench, firstly treat 10s, the 20s, 30s with 70% alcohol, rinse 3-4 times with sterile water, and then treat with 0.1% HgCl₂ for 0min, 3min, 5min, 8min, 10min and 12min respectively. Wash the bacteria water 3-4 times, use the filter paper to absorb the residual water on the leaves, cut into a 0.5cm² sheet structure, and inoculate MS+1.0mg/L NAA+0.1mg/L 6-BA+3% sucrose + 0.9% agar in the medium. Ten bottles were processed per combination, 3 explants were processed per bottle, and repeated three times. After 20 days of culture, the browning rate and the bacterial infection rate were observed and recorded.

2.2.2 Screening of callus induction medium. The sterilized leaves were cut off from the tip and petiole, and cut into 1cm² and surrounded by incisions. The leaves were inoculated into a medium containing different concentrations of CTK and auxin to induce callus growth. The concentration of CTK 6-BA was 0.1, 0.5, 1.0 and 1.5mg/L, and the concentration of auxin NAA was 0.5, 1.0, 3.0 and 5.0 mg/L. A total of 11 treatments, 10 bottles per treatment, 3 explants per bottle. After 20 days, the number of explants initiated was counted and the initiation rate was calculated.
2.2.3 Screening of callus proliferation medium. The callus with ideal growth condition was selected, the browning and the dead part were cut out, and the tissue pieces of 0.5cm$^2$ were divided and inoculated into the proliferation medium. The concentration of CTK 6-BA was 1.0, 1.5 and 2.0mg/L, and the concentration of auxin NAA was 0.1, 0.2 and 0.5mg/L. There were 10 treatments in total, 5 bottles per treatment, 4 inoculations per bottle, repeated subculture three times; each culture period was 20d, record callus diameter, color, porosity, browning and pollution.

2.2.4 Screening of callus rooting medium. The callus in the proliferation medium is inoculated into the rooting medium, and the basic medium was selected as the MS medium, and 0.2g or 0.4g of auxin IBA were respectively added to it, simultaneously add 0.5g of activated carbon or 10g of banana puree per liter of medium respectively. A total of 4 treatments, 5 bottles per treatment, 2 pieces of callus per bottle, the culture period are 35-40d, record the number of roots, root length, and root growth.

2.2.5 Data processing. The data obtained in this experiment is processed by software such as Excel 2010. The calculation indicators involved are as follows:

1. Browning rate = $\frac{\sum\text{Browning}}{S} \times 100\%$
2. Dyeing rate = $\frac{\sum\text{Bacteria}}{S} \times 100\%$
3. Recovery rate = $\frac{\sum\text{Callus}}{S} \times 100\%$

Note: The browning area of the leaves exceeds 50% of the total area of the leaves, indicate browning of the leaves. $\sum\text{Browning}$ means the number of leaf browning; $\sum\text{Bacteria}$ was the number of bacteria in the leaves; $\sum\text{Callus}$ indicates the number of callus grown in the leaves; $S$ means the total number of inoculated leaves.

3. Results and analysis

3.1 Establishment of a sterile system
After 20 days of culture, it can be seen from observation and data recording that ethanol with a volume fraction of 75% and HgCl$_2$ with a mass fraction of 0.1% have a strong bactericidal effect on plant leaves, but also cause irreversible damage to plant leaves, leading to browning of the leaves, no longer have the ability to grow. When the HgCl$_2$ treatment time is 0min, the leaf infection rate is as high as 98.00%; as the HgCl$_2$ treatment time increases, the leaf bacteria rate decreases, the lowest is 10.00%, but at the same time, the leaf browning rate appears a tendency to increase-lower-rise; Under the condition of ensuring the treatment time of HgCl$_2$ is unchanged, as the ethanol treatment time increases, it can be seen that the browning rate of the leaves gradually decreases, and the browning rate is as low as 36.70%. Therefore, the comprehensive comparison is available: the best disinfection time of the R. rugosa tunb is 75% ethanol treatment for the 30s, and the mass fraction 0.1% HgCl$_2$ treatment 8min. The method is used to treat the leaves, and the browning rate and mortality are relatively low (in Figure 1-2).
3.2 Effects of different concentrations of hormone combinations on callus induction

Plant growth regulators assume the office of an important role in plant plasticity development. Newborn callus usually has two forms, light yellow transparent and white compact. The study found that after about 15 days of leaf inoculation, the edges of the leaves shrank and rolled, and green tumor-like protrusions appeared on the surface of the leaves, indicating that dedifferentiation began to enter the cell division stage; after 20 days, the callus began to grow in the leaf incision. According to the data, the recovery rate was 12.50% when no hormone was added; the average recovery rate gradually increased with the increase of the mass concentration of 6-BA; when the mass concentration of 6-BA reached 1.5 mg/L, the recovery rate is up to 75.00%, and the callus state is mostly yellow-green granular; and the higher the NAA mass concentration, the lower the recovery rate. When the 6-BA is 0.5 mg/L and the NAA is 3.0 mg/L, the recovery rate reaches a minimum of 7.14%, indicating that the plant growth regulator concentration ratio is too high, which has an inhibitory effect on plants: When the 6-BA mass concentration is 1.5 mg/L and NAA is 3.0 mg/L, the callus production rate reaches a maximum of 75.00%, and the growth state is a yellow-green granular shape with a large volume. In summary, the optimal medium for callus induction of R. rugosa tunb is: MS+1.5mg/L 6-BA+3.0mg/L NAA+3% sucrose+0.9% agar (in Table 1).

Table 1. Effect of different concentrations of plant growth regulators on leaf callus induction

| Handle | 6-BA(mg/L) | NAA(mg/L) | Recovery rate | Browning rate | Dying rate | Growth potential |
|--------|------------|-----------|---------------|---------------|------------|-----------------|
| A0     | 0.0        | 0.0       | 12.50%        | 37.50%        | 18.75%     | +               |
| A1     | 0.1        | 0.5       | 16.67%        | 70.83%        | 0.00%      | +               |
| A2     | 0.1        | 1.0       | 33.33%        | 66.66%        | 16.67%     | +               |
| A3     | 0.1        | 3.0       | 10.71%        | 16.67%        | 33.33%     | +               |
| A4     | 0.5        | 0.5       | 29.71%        | 45.83%        | 16.67%     | +               |
| A5     | 0.5        | 1.0       | 16.67%        | 83.33%        | 0.00%      | +               |
| A6     | 0.5        | 3.0       | 7.14%         | 85.75%        | 0.00%      | +               |
| A7     | 1.0        | 3.0       | 12.50%        | 37.50%        | 31.25%     | ++              |
| A8     | 1.0        | 5.0       | 70.83%        | 25.00%        | 29.17%     | ++              |
| A9     | 1.5        | 3.0       | 75.00%        | 25.00%        | 16.67%     | +++             |
| A10    | 1.5        | 5.0       | 70.83%        | 29.16%        | 16.67%     | ++              |

Note: + means growth; ++ means better growth; +++ indicates strong growth, and subsequent availability was strong

3.3 Effects of different concentrations of hormone combinations on callus proliferation

When the ratio of 6-BA and NAA hormones is changed, the callus has different effects and the callus can continue to grow. From the data, it can be seen that after changing the hormone ratio, the callus is obviously enlarged, and the growth state is mostly dark green, and the volume is large and granular, and only some of the growth state is poor, no obvious granular, no layering. Among them, when the concentration of 6-BA was 2.0 mg/L and the concentration of NAA was 0.2 mg/L, the callus had the best growth potential, and the average size was 2.493 cm. The callus after proliferation was dark green and the granular layer was obvious. There is a phenomenon of mild browning pollution. Therefore, the best proliferation medium for R. rugosa tunb callus is: MS+2.0 mg/L 6-BA+0.2 mg/L NAA+3% sucrose+0.9% agar (in Table 2).

Table 2. Effect of different concentrations of hormones on callus proliferation

| Group | 6-BA NAA (mg/L)(mg/L) | Uncontaminated number | The average diameter(cm) | Callus proliferation status | Growth potential |
|-------|-----------------------|-----------------------|--------------------------|----------------------------|-----------------|
| B1    | 1.0                   | 0.1                   | 1                        | Light green, small size, pollution, browning | +               |
| B2    | 1.0                   | 0.2                   | 5                        | Light green, dense, polluted, browned           | +               |
| B3    | 1.0                   | 0.5                   | 14                       | Light yellow, water stain, granular, pollution, browned | ++              |
| B4    | 1.5                   | 0.1                   | 2                        | Green, small size, pollution, browning          | ++              |
| B5    | 1.5                   | 0.2                   | 6                        | Green, loose, small, brown, pollution           | ++              |
| B6    | 1.5                   | 0.5                   | 8                        | Light green, small size, hard, granular         | +++             |
| B7    | 2.0                   | 0.1                   | 11                       | Dark green, small size, granular, pollution,    | +++             |
3.4 Effects of different media and different concentrations of hormones on adventitious bud rooting

The callus was proliferated for about 45 days and transferred to the rooting medium. It was found that the callus was inoculated in the medium without any hormone, and the callus could not take root due to the regulation of no plant growth regulator. It can be seen that as the mass concentration of IBA increases, the rooting rate increases gradually, and the root system is long and robust. The activated carbon has superior adsorption capacity and can adsorb toxic substances in the culture medium, which is beneficial to plant growth; Banana puree as an organic additive has obvious promoting effect in rooting culture of orchids. In this experiment, the average browning rate of C4 and C5 after using banana puree is 40.00%, and the average browning rate of C2 and C3 is 26.39%. Therefore, the adsorption effect of this plant activated carbon is stronger than that of banana puree. In summary, the optimal rooting medium is: MS+0.4 mg/L IBA+0.5 g/L AC+3% sucrose+0.9% agar (in Table 3).

### Table 3. Effects of different types of media and exogenous hormones on their rooting

| Handle | Medium | IBA(mg/L) | AC(g/L) | Banana puree(g) | Browning rate | Number of roots | Rooting rate | Growth state          |
|--------|--------|-----------|---------|-----------------|---------------|----------------|--------------|-----------------------|
| C1     | MS     | 0.0       | 0.5     | 0.0             | 52.94%        | 0              | 0.00%        | Rootless              |
| C2     | MS     | 0.2       | 0.5     | 0.0             | 27.78%        | 3              | 72.22%       | Root length           |
| C3     | MS     | 0.4       | 0.5     | 0.0             | 25.00%        | 3              | 75.00%       | Long roots and strong |
| C4     | MS     | 0.2       | 0.0     | 10.0            | 40.00%        | 2              | 60.00%       | Short roots and stout |
| C5     | MS     | 0.4       | 0.0     | 10.0            | 40.00%        | 2              | 60.00%       | Short root            |

4. Discussion and conclusion

4.1 Conclusion

After the study, the following conclusions were drawn: the best disinfection system for R. rugosa tunb leaves was 70% alcohol 30s+0.1%HgCl2 8min; the optimal medium for callus induction was MS+1.5mg/L 6-BA+3.0 mg/L NAA+3% sucrose+0.9% agar; suitable proliferation medium is MS+2.0mg/L 6-BA+0.2mg/L NAA+3% sucrose+0.9% agar; the best rooting medium was MS+0.4mg/L IBA+0.5 g/L AC+3% sucrose+0.9% agar.

4.2 Discussion

In recent years, with the continuous deepening of plant tissue culture research, any plant organ can be divided and differentiated to form a complete plant. In the process of tissue culture, browning, vitrification and pollution are inevitable. Non-standard aseptic operation and self-contamination of raw materials are the causes of excavation pollution death. The higher the pollution rate, the more serious the economic loss. The factors affecting browning are more complicated. Different types of explants, genotypes and culture conditions will cause different degrees of browning of plants. In this experiment, the low temperature storage and shading culture of explants were used to reduce the occurrence of browning, but HgCl2 was more toxic and caused damage to the leaves. The ratio of plant growth regulators is the main factor in the process of callus induction and differentiation. Commonly used 6-BA and NAA are mixed in a certain proportion, and low concentration of 6-BA is combined with high concentration of NAA to induce callus, and the highest rate, NAA is the dominant factor. CTK 6-BA was found to be dominant in callus proliferation. Both MS and 1/2MS can be used as the basic medium for rooting culture, and the addition of IBA can induce root formation. Among them, the
addition of banana mud and activated carbon can adsorb toxic substances and promote rooting. If too much is added, it will inhibit. Therefore, the dosage range can be further explored.

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