Tissue Culture of Regeneration Induced by Leaves of ‘Hongyang’

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Abstract. The test combined the concentration in 0.5mg·L⁻¹, 1.0mg·L⁻¹, 1.5mg·L⁻¹ of 1-naphthaleneacetic acid (NAA) and a concentration of 2.0mg·L⁻¹, 2.5mg·L⁻¹, 3.0mg·L⁻¹ of 6-benzylaminopurine (6-BA), from which we got a comprehensive evaluation of shoots survival ratio, growing point proliferated rate and shoots growth status together. From all the treatment we selected MS + 1.0mg·L⁻¹ NAA + 3.0mg·L⁻¹ 6-BA for the optimum combination, for its live ratio of budding leaves on 92.59%, growing point proliferation rate on 629.60%. In this experiment, each treatment adventitious buds were over 300.00%, that all the treatments are able to use as the media formulations for proliferation of adventitious buds.

1. Introduction

The fruit of ‘Hongyang’ (Actinidia chinensis cv.) is sweet, sour and delicious, and has a good flavor. It is widely used as an economically cultivated fruit tree that has become a commodity, and it has a large economic significance. Kiwifruit is a dioecious plant. Cultivation methods often use real-life rootstocks for grafting. Seed germination of seedlings has a long growth cycle, and it is difficult to ensure the stability of genetic traits, which makes the quality of kiwifruit products uneven and difficult to standardize production. The kiwifruit is a fruit tree with difficult rooting and cutting, slow growth and long term. Due to the advantages of using tissue culture techniques such as low demand for explants, high propagation coefficient, and low quality differentiation of offspring, it is possible to rapidly propagate large numbers of seedlings with consistent genetic traits. At the same time, tissue culture technology has been widely used in the research of breeding new varieties, preserving high-quality germplasm resources and genetic transformation [1].

Among the various genetic transformation methods of various horticultural plant germplasm resources that have been applied, the high frequency and good reproducibility of obtaining stable genetic transformants by Agrobacterium-mediated method is the clearest one in the research. Agrobacterium tumefaciens was used to transform woody fruit trees mainly by leaf disc method that is to cut small leaves and inoculate them on a special medium in a petri dish for culture. The leaf disc method occupies a small space and each dish can be inoculated with a large number of explants leaves, and the high regeneration efficiency it is. It can also avoid the cumbersome operation of the protoplasts, and directly use the regenerative explants such as leaf cells. In the research, the transformed plants were obtained by the process of forming leaf callus and then promoting the adventitious buds from callus. Explanted leaves undergo a process of dedifferentiation to re-
differentiation, which takes a long time and the expression of genetic transformation is not stable enough. The ratio of growth regulators in plant tissue culture is more critical, especially in terms of the ratio of auxin to cytokinin. Some studies have found that in the process from the beginning of dedifferentiation to the formation of part of the meristematic tissue, the stimulating and inducing effects of cytokinin and auxin from the interior of the plant are generally required, and higher levels of cytokinin are maintained. It is essential for the development of tissue, and high levels of auxin can induce plant callus production and maintain the proliferation of the tissue.

At present, researches on the tissue culture of Chinese kiwifruit have been carried out in the culture system of various explants such as leaf blade, stem segment, stem tip, and endosperm [2]. Since Y.L. Gui 1979 [3] study on tissue culture of kiwifruit stem segments, more in vitro culture systems were studied in the 1990s [4-6].

However, in the genetic transformation of leaf disc culture, X.P. Zhao et al [7] found that only BA but no NAA treated ‘Hongyang’ leaves did not form differentiated shoots. E. Rugini et al [8] used MS medium with 5 mg·L⁻¹ NAA and 0.1 mg·L⁻¹ BAP to induce the callus of kiwifruit leaves. X.H. Wu et al [9] found that for ‘Heywood’ kiwifruit leaves, when 6-BA concentration was less than 5 mg·L⁻¹ and the 6-BA/NAA concentration ratio was between 2.5:1 and 3:1, The effect of budding was the best; while 6-BA concentration was more than 5 mg·L⁻¹, the adventitious bud differentiation rate decreased. Although N. Tian [10] selected a suitable hormone ratio for the delicious kiwifruit, the petiole and veins still produced callus, and adventitious buds and callus grew together in the later culture.

2. Material and Method

2.1. Material
The material was ‘Hongyang’ kiwifruit tissue culture sterile seedling, provided by the Biotechnology Laboratory of Sichuan Agricultural University.

2.2. Method
The basic medium was MS medium containing 0.5 mg·L⁻¹, 1.0 mg·L⁻¹ and 1.5 mg·L⁻¹ of NAA, and 2.0 mg·L⁻¹, 2.5 mg·L⁻¹ and 3.0 mg·L⁻¹ 6-BA. A total of 9 combinations were processed. Under aseptic conditions, the young leaves of sterile seedlings were cut off without the leaf edge and tip, and cut into a disc with a diameter of 0.5cm with a puncher. It was inoculated in the adventitious bud induction medium containing 6-BA and NAA. Dark cultured for 30 days. Afterwards, they were transferred to light culture and cultured in an environment of 20°C with an interval of 2500 lx at 12 h intervals, repeat 3 times.

2.3. Statistics and Analysis
After 30 days of culture, the ratio of buds and discs and the growth rate of growth points were measured and the sensory evaluation of the seedlings formed by adventitious buds was evaluated to determine whether it was convenient for the post-cultivation.

3. Results and analysis

3.1. Effects of NAA and 6-BA on the survival of budding leaf disks
In this experiment, the highest percentage of sprouts in this experiment was treatment 6 (92.59%), while the lowest was treatment 7 (51.72%). With the same concentration of NAA, 9 treatments could be divided into 1 2 3, 4 5 6 and 7 8 9 three large groups, in the case of the same concentration of NAA, the survival rate of the leaves increased with the increase of 6-BA concentration, indicating that increasing the concentration of 6-BA in a certain range could increase the number of survival and bud number of the inoculated leaf disk. When 6-BA was grouped at the same concentration of 1 4 7, 2 5 8 and 3 6 9, the proportion of bladed discs changed little, it showed that the influence of 6-BA on the survival ratio of buds was greater than NAA.
3.2. Effects of NAA and 6-BA on Adventitious Shoot Propagation in Leaf Disk

The highest proliferation rate of adventitious buds was treatment 6 (629.60%), treatment 3 was the lowest. Grouping with the same NAA concentration showed a certain degree of intermediate low and higher two segments, indicating that NAA and BA had better effects when they were in the dominant position respectively. When compared with the same concentration of 6-BA, the proliferation effect of NAA at 1.0 mg L⁻¹ concentration was significantly higher than that of other groups, indicating that this concentration had a better effect on the growth of the adventitious buds of the leaves of kiwi fruit.

**Table 1.** Proportion of budding leaves and proliferation for each treatment

| Treatment number | NAA (mg L⁻¹) | 6-BA (mg L⁻¹) | Number of explants inoculated | Pollution rate (%) | Blasting rate (%) | Growth point number | Growth point proliferation rate (%) |
|------------------|--------------|--------------|-------------------------------|-------------------|------------------|---------------------|-----------------------------------|
| 1                | 0.5          | 2.0          | 28                            | 0                 | 71.43 b          | 129                 | 460.70 b                          |
| 2                | 0.5          | 2.5          | 30                            | 0                 | 90.00 a          | 129                 | 430.00 b                          |
| 3                | 0.5          | 3.0          | 27                            | 0                 | 85.19 a          | 105                 | 388.90 b                          |
| 4                | 1.0          | 2.0          | 26                            | 0                 | 73.08 b          | 151                 | 580.80 a                          |
| 5                | 1.0          | 2.5          | 30                            | 33.33             | 63.33 b          | 113                 | 565.00 a                          |
| 6                | 1.0          | 3.0          | 27                            | 0                 | 92.59 a          | 170                 | 629.60 a                          |
| 7                | 1.5          | 2.0          | 29                            | 34.48             | 51.72 c          | 100                 | 526.30 ab                         |
| 8                | 1.5          | 2.5          | 27                            | 0                 | 77.78 b          | 106                 | 392.60 b                          |
| 9                | 1.5          | 3.0          | 30                            | 33.33             | 56.67 b          | 122                 | 610.00 a                          |

3.3. Effects of NAA and 6-BA on the Growth of Seedlings Formed by Adventitious Buds

In the experiment, the root grew on the callus and the most root number was treatment 9, it was 4 with very good growth, and the more developed root hair could be observed. At the same NAA concentration, the growth of the original leaves appeared when the 6-BA concentration was higher. At the same 6-BA concentration showed that the rooting number increased with the increase of NAA concentration. The seedlings grew well in all treatments had treatment 1, treatment 2, treatment 6, and treatment 9.

**Table 2.** Evaluation of the growth of shoots

| Treatment number | Rooting number | Sprout description                                      | Is it easy to operate later |
|------------------|----------------|---------------------------------------------------------|----------------------------|
| 1                | 1              | obvious young plant, better growth                      | yes                        |
| 2                | 1              | 4 to 5 young plants, growing generally                   | no                         |
| 3                | 2              | the original leaves grow, the buds differentiate slowly, and the number is small | no                         |
| 4                | 0              | the buds are numerous and frail, with 2 young plants     | no                         |
| 5                | 2              | slow differentiation, few young plants                  | no                         |
| 6                | 0              | 11 young plants                                         | yes                        |
| 7                | 0              | 5 young plants, the buds are numerous and fragile, generally growing | no                         |
| 8                | 3              | slow differentiation, general growth, growth of the original leaves | no                         |
| 9                | 4              | numerous buds, growth of original leaves, only one young plant but vigorous growth | yes                        |
4. Discuss
In this experiment, the proliferation rate of adventitious buds in each treatment was more than 300.00%, indicating that the medium used in this study could be used for the proliferation of adventitious buds. Comprehensive assessment of the survival rate of buds, the proliferation rate of growth points, and the growth status of seedlings could know the best combination was treatment 6, MS + 1.0 mg·L⁻¹ NAA + 3.0 mg·L⁻¹ 6-BA. This was consistent with the conclusion that the kiwifruit leaf disk obtained by X.P. Zhao [7] had the best growth state at a concentration ratio of 1:3 between NAA and 6-BA.

The concentration ratio of NAA and 6-BA for treatment 4 and treatment 9 was 1:2, but the effect of growth regulator was obvious in treatment 9, such as the large rooting number and more exuberant, which also accorded with the effect of the growth regulator on the rooting of ‘Hongyang’ peach in hard branch cutting [11]. While they caused the growth of the original inoculation leaves and the vigorous growth of the seedlings, it showed that the use of the two growth regulators would still play an important role in the same concentration ratio. It was not enough to give only the applicable concentration ratio. Although the applicable concentration had its approximate range, more in-depth and accurate tests were needed.

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