Study on the effect of stress treatment on embryogenic callus of raspberry

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Abstract. In order to obtain the embryogenic callus of raspberry, the friable calluses of raspberry were used as materials to be treated by different time of starvation and desiccation, and were further cultivated and induced embryoid. By use of embryogenic observation, the embryogenic degree of callus could be determined. The results showed that the embryogenic degree of calluses which were treated by starvation for 6 days was the highest, and the desiccation treatment had no effect on embryogenic degree of calluses. Therefore, appropriate starvation treatment can facilitate the transformation of non-embryogenic callus into embryogenic callus. The results of this experiment could provide reference for callus embryogenesis induction of other plants.

1. Introduction

Raspberry, as a new functional fruit, is more and more popular among consumers due to its rich nutrition \cite{1}. With the expansion of the planting area of raspberry, the requirement for the provenance of raspberry also becomes diversified \cite{2-5}, so it is urgent to accelerate the breeding research of raspberry.

Raspberries are mostly wild varieties, and cultivated varieties obtained through artificial breeding are relatively stable plant varieties with heredity. Through conventional hybridization, it is difficult to obtain new excellent characters, so the development of conventional breeding meets a bottleneck. However, gene transformation technology is restricted by the market and cannot be industrialized. Therefore, mutagenesis technology is basically adopted in the breeding of raspberry \cite{6-10}. Conventional physical and chemical mutagenesis using seeds or plants has the lower mutagenesis efficiency. Mutation breeding with embryonic callus can greatly improve the mutagenesis efficiency \cite{11-13}.

Embryogenic callus is different from common callus and has strong advantages in somatic mutation breeding and gene transformation. Its main advantages are as follows: first, the embryonic callus is in the state of vigorous division, and the genetic material in the cell is very active, and it is easy to produce variation, which is more obvious under the condition of added mutagens; Second, the number of embryonic callus cells has an obvious advantage, and each cell can be regarded as a single mutagenesis object, thus increasing the mutagenesis efficiency. Third, embryogenic callus can be dispersed into multiple independent individuals, so that each mutant plant is formed by the division and differentiation of a single embryogenic mutant cell, thus avoiding the chimerism phenomenon under conventional mutagenesis. Fourthly, embryogenic callus can obtain regenerated plants at any
time. Because embryogenic callus can regenerate directly on the culture medium without hormone, without hormone induction, which saves time for research and improves the efficiency of research [14,15].

Although the regeneration efficiency of embryogenic callus is high, it is still not ideal to conduct tissue culture studies on some plants due to the difficulty in inducing embryogenic callus. How to improve the proportion of embryogenic cells is a difficult point for many scholars. Therefore, obtaining better callus induction conditions and in vitro regeneration techniques are the preconditions for the utilization of embryonic callus [16]. In this study, the callus of raspberry was treated by stress method in order to obtain embryonic callus, so as to provide good experimental materials for breeding research and lay a foundation for the cultivation and promotion of bramble.

2. Materials and methods

2.1. Test materials

The callus used in this study was the raspberry loose callus induced by the garden plant laboratory team of Tianjin agricultural university. The loose callus was easy to be separated and differentiated stably, which was a good material for loose embryo callus induction.

2.2. Test methods

2.2.1. Definition of embryonated callus. Generally, the morphological characteristics of loose embryogenic callus are loose texture, milky white or yellow color, and spherical particles on the surface. Nikon.P. s. XY64 somatoscope was used for observation. From the perspective of cytology, embryogenic callus is characterized by small cells, dense protoplasm, small vacuoles and large nuclei, while non-embryogenic callus is characterized by large cells, small nuclei and large vacuoles. The instrument used for observation was a LEICA DM 2000 microscope. The characteristics of the spherical embryo are the spherical structure composed of multiple cells. The spherical surface is smooth, the internal cell division is vigorous, and the cell individual is small, which will present the development and change process similar to the plant seed embryo by further cultivation.

2.2.2. Drying treatment of callus of raspberry. Dissected the cultivated raspberry loose callus into small pieces of 2 mm size with the scalpel, and paid attention not to crush the callus in the process of transfer and segmentation. Take the sterile petri dish and transfer the separated callus of raspberry to the empty petri dish in the ultra-clean table. The petri dish must be dry without the presence of steam. Distribute the callus evenly in the petri dish and seal the petri dish with paraffin sealing film. Twenty callus groups were placed in each culture dish, and three replicates were set for each treatment. The treated calluses were cultured under the following conditions: 2000 lux light intensity, 24 h light. Temperature is 23℃, dry processing time respectively 1 d, 2 d, 3 d 4 d.

2.2.3. Hunger management of raspberry callus. The starvation treatment adopted in this experiment was very simple. The cultivated loose callus of raspberry was taken and cut into small pieces of 2 mm size for later use. Take the sterile petri dish, put the sterile filter paper in the petri dish in the ultra-clean table, add a small amount of sterile water to the petri dish, and note that the amount of water is subject to all the soaked filter paper. Do not add more filter paper or do not completely soak. The calluses were placed in a prepared dish. 15 callus groups were placed in each culture dish, and 5 replicates were set for each treatment. The treated calluses were cultured under the following conditions: 2000 lux light intensity, 24 h light. Temperature of 23℃, hungry processing time respectively for 3 d, 6 d, 9 d, 12 d.

2.2.4. Recovery culture of callus of raspberry on MS medium. The dried callus and starved callus were transferred from the petri dish to MS medium for embryoid culture, and the changes of callus were
observed after 14 days.

3. Results and analysis

3.1. Experimental results of drying treatment of callus of raspberry

After drying for different days, the callus of raspberry changed significantly in appearance and under microscopic observation, and then transferred to MS medium. After about 14 days of culture, the callus basically did not show embryonic structure, as shown in Table 1.

| Treatment day | Number of callus inoculated | Changes of callus after treatment | Microscopic changes of callus after treatment | The change of culture was restored on MS medium after 14 days |
|---------------|-----------------------------|----------------------------------|-----------------------------------------------|-------------------------------------------------------------|
| 1             | 60                          | no change                        | The cells were parenchyma without obvious embryogenesis. | The callus grew normally, but no embryonic structure was produced. |
| 2             | 60                          | A small amount of callus were dehydrated and dried | The cells on the surface lost water and died. The internal cells are non-embryonic. | Callus could not grow and all died with culture time. |
| 3             | 60                          | Nearly half of callus lost water | Most cells lose water and die. | All callus died after Browning. |
| 4             | 60                          | All callus lost water and atrophied. | All cells lost water and died. | All callus died after Browning. |

As can be seen from Table 1, with the increase of drying treatment time, the surface of the callus loses water and the callus shrinks. When the treatment time reaches 4d, the callus is basically dry and has no living condition. In terms of the anatomical structure, the callus was still non-embryonated parenchyma cells even after 1 day of treatment. With the increase of treatment time, the cells outside the callus lost water, atrophied and died, forming a structure similar to the cork layer, while the internal cells were normal, but there was no change of embryogenesis. The callus of raspberry treated for 4d was completely dehydrated, and the cell structure could not be found under the microscope, so embryonic changes were more unlikely.

When these dried callus were transferred to MS medium, only the callus treated for 1d could grow, but there was no embryoid structure. The microscopic observation was non-embryonal callus. Other callus showed browning after 2-3 d on MS medium, and almost all of them turned black and died after 7d. Therefore, the drying treatment had no effect on the embryogenesis of the callus, and the drying treatment could not accelerate the embryogenesis of the callus, or even hinder the normal growth of the callus.

3.2. Effects of starvation treatment on embryogenesis of callus of raspberry

After starvation treatment for different days, the callus of raspberry changed significantly in appearance and under microscopic observation, and then was transferred to MS medium. After about 14 days of recovery culture, some callus were treated to spherical embryos, as shown figures 1-3. In figures 1-3, T3 represents the starvation treatment for 3 days, T6 represents the starvation treatment for 6 days, T9 represents the starvation treatment for 9 days, and T12 represents the starvation treatment for 12 days.
Figure 1. Effects of different starvation treatment days on callus morphology.

Figure 2. Effects of different starvation treatment days on granule structure formation of callus.

Figure 3. Spherical embryo structure at different starvation treatment time.

Table 2. Effects of starvation treatment on embryogenesis of callus of raspberry.

| Treatment day | Number of callus inoculated | Changes of callus after treatment | Microscopic changes of callus after treatment | The change of culture was restored on MS medium after 14 days |
|---------------|-----------------------------|----------------------------------|-----------------------------------------------|----------------------------------------------------------|
| 3             | 75                          | no change                        | Most of the cells were parenchyma, and 5% of them showed embryogenesis. | The callus grew normally and the smooth spherical embryo structure appeared on the callus with embryogenic characteristics. |
| 6             | 75                          | Callus are hard and granular     | More than 95% of the cells showed embryogenesis and several cells clustered together. | Almost all callus have smooth spherical embryo structure. |
| 9             | 75                          | Callus are hard and granular. Browning occurred in 15% of callus | All the living cells showed embryogenic characteristics, and multiple cells were clustered together. Individual cells die. | All the callus of the surviving cells had globular embryo structure with smooth surface. But soon 85% of callus died after Browning. |
| 12            | 75                          | Callus are hard and granular. Nearly 50% of callus showed Browning. | All the living cells showed embryogenesis, but half of them died. | Soon all callus died after Browning. |

As can be seen from table 2, the effect of starvation treatment on the number of spherical embryos generated after callus recovery culture was obvious. After 14 days of recovery culture, about 5% of the spherical embryos in the starved treated callus for 3 d grew normally, about 95% of the spherical
embryos in the starved treated callus for 6 d grew normally, about 13% of the spherical embryos in the starved treated callus for 9 d grew normally, and almost all the callus in the starved treated callus for 12 d died. It can be seen that the proportion of callus to embryogenesis is the highest when the starvation time of callus is 6 d, which is most conducive to the embryogenesis of callus.

4. Discussion

4.1. The theoretical basis that starvation treatment is beneficial to the embryogenic transformation of callus

The mechanism of the effect of starvation treatment on the embryogenesis of callus can be summarized as the effect of stress on cell differentiation. Specifically, under the premise of nutrient deficiency, some cells grow slower, some cells disintegrate and die, and some competitive cells will absorb the nutrients of these cells to maintain their basic needs. These cells to resist adversity, nutrition lack necessary absorbs the nutrition of surrounding cells, mainly is the nutrition of disintegration of some cells. These cells in order to continue to resist adversity stress gradually reduce their metabolism, leading to splitting speed drops, causing cell excessive nutrient accumulation, cytoplasm concentration increases, the intracellular nutrients increases, leading cell toward embryonic development and callus toward the embryonic callus. This is similar to the performance of many plant cells under adversity, such as the budding reproduction of some plants under adversity. This mechanism is the self-protection function acquired in the process of plant evolution, and it is the potential ability of plants [17].

4.2. In this experiment, the reason why drought treatment had little effect on callus embryogenesis and the measures to change

Drought stress can also promote the callus to embryonic callus transformation, but the effect is not obvious in this experiment. The main reason is that the callus was inoculated directly to the triangle in the bottle without any medium. The callus surface water loss fast and died, so as to no prepare for embryonic structure transformation. Therefore, to transform callus into embryonic by drying stress it is necessary to make a relatively dry environment, allows cells to respond to the environment gradually, to adjust, Rather than trying to induce cells to adapt in this extreme way. In the future, some humidity can be added to the environment to control the degree of drying, such as adding a few drops of water in a tripod bottle to create a relatively dry environment. Or control the relative humidity in the culture environment to prevent the rapid disappearance of water in the tripod. Another method is to add polyethylene glycol into the culture medium to cause physiological drought, so as to change the water absorption environment of the callus, which can also receive the same treatment effect as the drought. In a word, drought has some effect on embryogenesis of callus, but the treatment method must be selected correctly to get the desired effect.

4.3. Effects of the timing and mode of hunger treatment on the embryogenic effect

some preliminary experiments were done to find the best time to deal with hunger in this study. In the process of callus culture, the physiological state of cells is different, and with the change of medium composition and culture environment, the callus will also change correspondingly, and there will be various changes in physiology and structure. Generally speaking, the time should be chosen to transform the callus into embryogenic tissue as far as possible, that is, the closer the callus is to the embryogenic state, the easier it is to be induced to produce embryogenic transformation by stress. At this time, the embryogenic transformation of the callus can be realized once and for all by simple stress.

5. Conclusion

In this experiment, the callus of bramble was embryogenic induced by starvation and drought stress. The results showed that the embryogenic effect obtained by starvation treatment was more ideal, and
the embryogenic effect obtained by starvation treatment at 6d was the best. Although drought is not ideal for callus embryogenesis, it can be overcome by improving the experiment. In conclusion, adversity can promote the transformation of callus into embryogenic tissue, which is especially necessary for plant species whose embryogenic callus cannot be induced by common measures.

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