Effects of Sucrose and Browning Inhibitors on Callus Proliferation and Anti-Browning of Chinese Kale

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Abstract. The hypocotyl of Chinese kale ‘Cutiaoyusun’ was used as explants, the effects of the different concentrations of sucrose and different browning inhibitors (activated carbon, polyvinylpyrrolidone, and ascorbic acid) on the callus proliferation and anti-browning of Chinese kale were studied in this study. The results showed that the proliferation medium with 20 g·L⁻¹ sucrose had the best effects on the proliferation and anti-browning of Chinese kale callus, and its proliferation rate was the highest, reaching 213.5%, and the browning rate was as low as 27.77%. In addition, adding 0.2 g·L⁻¹ ascorbic acid to the culture medium can significantly promote callus proliferation and reduce browning rate. In this treatment, the callus had the lowest browning rate of 19.45% and the highest proliferation rate of 266.98%, and the morphology of the callus was friable. This study lays a foundation for future research in molecular biology and genetic improvement in Chinese kale.

1. Introduction

Chinese kale (Brassica oleracea var. alboglabra) is a cruciferous vegetable originated from southern China and Southeast Asia [1]. It has a long history of cultivation and is mainly served by bolting stems and young leaves. Its texture is crisp and tender, and the flavor is unique [2]. It is rich in nutrients and is one of the famous specialty vegetables in China. It has been reported that the plant tissues and buds of Chinese kale are rich in glucosinolates, which have medicinal healthy value [3-4] and play a role in anti-cancer.

Obtaining stable proliferation and healthy callus requires a suitable proliferation medium. As a key factor in the medium, sucrose provides organic nutrition and also plays an important role in regulating osmotic pressure. Therefore, sucrose can significantly affect the growth state of callus [5-6]. For example, Hua et al. found that the effect of adding different concentrations of sucrose in the medium to induce callus of Bracken was significantly different [6]. Browning is also a common problem in plant tissue culture, usually inhibiting the normal growth and differentiation of plant, and even plant death in severe cases [7-8]. There are plenty of research reports on the anti-browning of explants. The most common way is the addition of browning inhibitors [9-10], among which the main users are antioxidants such as ascorbic acid, polyvinylpyrrolidone (PVP) and adsorbents such as activated carbon. The results of Wen et al. showed that the addition of ascorbic acid and activated carbon to the
culture medium had the anti-browning effect on the callus, but the anti-browning effect was different [8].

In this experiment, in order to an improvement of proliferation and anti-browning of Chinese kale callus, different concentrations of sucrose and browning inhibitors were added into the proliferation medium, and then the optimal condition which can control browning and promote proliferation was finally selected to ensure the callus remains in good condition and proliferates steadily.

2. Materials and methods

2.1. Plant materials

The callus of Chinese kale ‘Cutiaoyusun’ was used as initial materials in this study, and it was obtained on MS solid medium supplemented with 0.1 mg L⁻¹ 2,4-D and 0.02 mg L⁻¹ 6-BA, and using Chinese kale hypocotyl as explant material.

2.2. Different concentrations of sucrose treatments

Based on the optimal hormone ratio proliferation MS solid medium with 0.4 mg L⁻¹ NAA and 2.5 mg L⁻¹ 6-BA, different concentrations of sucrose (10, 20, 30, 40 g L⁻¹) were added to the proliferation medium, respectively. All media were adjusted to pH 5.8 with 1 M NaOH, and the induced calli of substantially uniform size were transferred to the media above. Subsequently, they were incubated at (25±1) °C and 60-70% relative humidity in the dark, and subcultured every 7 d. After 30 d of culture, fresh weight, browning rate, and callus status were recorded respectively. The experiment was carried out with three replicates, with each replicate was containing six bottles.

2.3. Different browning inhibitors treatments

Based on the optimal hormone ratio proliferation medium with 0.4 mg L⁻¹ NAA and 2.5 mg L⁻¹ 6-BA, the induced calli were placed in medium supplemented with different concentrations of activated carbon (0, 1.0, 2.0, 3.0 g L⁻¹), PVP (1.0, 2.0, 3.0, 4.0 g L⁻¹) and ascorbic acid (0.2, 0.4, 0.6, 0.8 g L⁻¹). All media were adjusted to pH 5.8 with 1 M NaOH, and then 7 g L⁻¹ of agarose and 20 g L⁻¹ sucrose were added before autoclaving for 20 min at 121 °C. Other conditions and steps were the same as 2.2.

2.4. Statistical analysis

All experimental data were statistically analyzed by one-way analysis of variance (ANOVA) using the least-significant-difference (LSD) test (P<0.05), and data were evaluated using an analysis of variance from which mean ± standard error values were calculated for comparison between distinct treatments.

3. Results

3.1. Effects of different concentrations of sucrose on the proliferation and anti-browning of Chinese kale callus

On the whole, the browning rate of callus in each treatment was not significantly different on the medium supplemented with different concentrations of sucrose, but the proliferation effect was significantly different (Table 1). Calli are similar in status, are mostly light yellow and friable in structure (Figure 1). There was no significant difference in the browning rate of the four treatments, but it was found that when the sucrose concentration gradually increased, the degree of browning of the callus gradually increased. When the sucrose concentration is 10 g L⁻¹ and 20 g L⁻¹, the browning rate is at a low level. The proliferation rate was significantly different. It increased first and then decreased with the increase of sucrose concentration. When the sucrose concentration was 20 g L⁻¹, the proliferation rate was the highest and reaching 213.5%. Therefore, only when the sucrose concentration is appropriate, the callus can stably and rapidly proliferate. In this experiment, adding 20 g L⁻¹ sucrose is the best choice.
Table 1. Effects of different concentrations of sucrose on the proliferation and anti-browning of Chinese kale callus

| Sucrose concentration (g·L⁻¹) | Browning rate (%) | Fresh weight of pre-proliferation (g) | Fresh weight of post-proliferation (g) | Multiplication rate (%) | Callus status                      |
|-------------------------------|-------------------|--------------------------------------|---------------------------------------|-------------------------|-----------------------------------|
| 10                            | 16.67±16.65 a     | 1.10                                 | 2.79                                  | 154.12±2.18 c           | yellowish, friable and few compact|
| 20                            | 27.77±9.58 a      | 1.17                                 | 3.67                                  | 213.50±3.87 a           | yellowish, friable                |
| 30                            | 33.33±16.65 a     | 1.31                                 | 3.52                                  | 168.28±0.42 b           | yellowish, friable                |
| 40                            | 38.87±9.64 a      | 1.21                                 | 2.87                                  | 137.93±3.35 d           | yellowish, friable                |

Different letters indicate significant difference at the 0.05 probability level. The same was as below.

Figure 1. Callus status cultured on proliferation medium supplemented with different concentrations of sucrose. (a) Sucrose of 10 g L⁻¹; (b) sucrose of 20 g L⁻¹; (c) sucrose of 30 g L⁻¹; (d) sucrose of 40 g L⁻¹.

3.2. Effects of different browning inhibitors on the proliferation and anti-browning of Chinese kale callus

On the whole, the browning rate of callus in each treatment was not significantly different on the medium supplemented with different concentrations of sucrose, but the proliferation effect was significantly different (Table 2). Calli are similar in status, are mostly light yellow and friable in structure (Figure 2). There was no significant difference in the browning rate of the four treatments, but it was found that when the sucrose concentration gradually increased, the degree of browning of the callus gradually increased. When the sucrose concentration is 10 g L⁻¹ and 20 g L⁻¹, the browning rate is at a low level. The proliferation rate was significantly different. It increased first and then decreased with the increase of sucrose concentration. When the sucrose concentration was 20 g L⁻¹, the proliferation rate was the highest and reaching 213.5%. Therefore, only when the sucrose concentration is appropriate, the callus can stably and rapidly proliferate. In this experiment, adding 20 g L⁻¹ sucrose is the best choice. On the whole, the addition of different browning inhibitors to the culture medium has a great influence on the proliferation and anti-browning of callus. The browning of callus in each treatment was different, and the proliferation was significantly different (Table 2). In terms of callus status, most of the calli showed yellowish and friable, and the callus status was shown in Figure 2.
The proliferation rate of each treatment was compared and found to be significantly different. The treatment with 0.2 g·L⁻¹ ascorbic acid had the highest proliferation rate and was significantly higher than all other treatments, and the proliferation rate was as high as 266.98%. Analysis of the browning rate shows that the treatment with 0.8 g·L⁻¹ ascorbic acid has the highest browning rate, which is significantly higher than other treatments, and the browning rate is 83.33%. The addition of 0.2 g·L⁻¹ ascorbic acid had the lowest browning rate of 19.45%, which was not significantly different from the addition of the control, activated carbon of 2 g·L⁻¹ and 3 g·L⁻¹, and PVP of 2 g·L⁻¹ and 3 g·L⁻¹. The browning rate and proliferation rate of each treatment were comprehensively analyzed. The results showed that the addition of 0.2 g·L⁻¹ ascorbic acid had the ability to significantly promote callus proliferation. The callus had the lowest browning rate and the highest proliferation rate, and was yellowish and friable. This treatment is optimal for callus proliferation and browning resistance.

**Table 2.** Effects of different browning inhibitors on the proliferation and anti-browning of Chinese kale callus

| Browning inhibitors | Concentrations (g·L⁻¹) | Browning rate (%) | Fresh weight of pre-proliferation (g) | Fresh weight of post-proliferation (g) | Multiplication rate (%) | Callus status |
|---------------------|------------------------|-------------------|--------------------------------------|---------------------------------------|------------------------|--------------|
| Control             | 0                      | 36.10±12.54       | 1.23                                 | 3.86                                  | 214.25±5.89            | yellowish, friable |
| Activated carbon    | 1                      | 47.22±12.57       | 1.22                                 | 3.38                                  | 176.33±6.61            | yellowish, friable |
|                     | 2                      | 27.78±13.59       | 1.30                                 | 4.38                                  | 237.20±6.87            | yellowish, friable |
|                     | 3                      | 33.33±14.89       | 1.36                                 | 4.42                                  | 224.52±5.24            | yellowish, friable |
| PVP                 | 1                      | 50.00±10.56       | 1.31                                 | 3.35                                  | 155.22±7.68            | yellowish, friable and few compact |
|                     | 2                      | 22.22±13.59       | 1.35                                 | 4.66                                  | 246.12±9.83            | yellowish, friable |
|                     | 3                      | 30.55±12.53       | 1.32                                 | 4.31                                  | 226.12±8.12            | yellowish, friable |
|                     | 4                      | 38.87±8.62        | 1.32                                 | 4.15                                  | 215.28±6.17            | yellowish, friable |
| Ascorbic acid       | 0.2                    | 19.45±12.53       | 1.37                                 | 5.03                                  | 266.98±7.64            | yellowish, friable |
|                     | 0.4                    | 52.78±12.57       | 1.22                                 | 3.09                                  | 153.60±3.19            | yellowish, friable |
|                     | 0.6                    | 66.67±14.89       | 1.25                                 | 3.05                                  | 144.92±1.57            | yellowish, friable and few compact |
|                     | 0.8                    | 83.33±14.89       | 1.36                                 | 3.04                                  | 124.23±2.11            | yellowish, friable and few compact |
Figure 2. Callus status cultured on proliferation medium supplemented with different concentrations of browning inhibitors. (a) Activated carbon of 0 g L⁻¹; (b) activated carbon of 1 g L⁻¹; (c) activated carbon of 2 g L⁻¹; (d) activated carbon of 3 g L⁻¹; (e) PVP of 1 g L⁻¹; (f) PVP of 2 g L⁻¹; (g) PVP of 3 g L⁻¹; (h) PVP of 4 g L⁻¹; (i) ascorbic acid of 0.2 g L⁻¹; (j) ascorbic acid of 0.4 g L⁻¹; (k) ascorbic acid of 0.6 g L⁻¹; (l) ascorbic acid of 0.8 g L⁻¹.

4. Conclusion
At present, there are two main physiological mechanisms for browning of plant tissue culture, one is enzymatic browning, the other is stress browning caused by wound or culture conditions [7]. Therefore, for the cause of browning, it is concluded that the method of anti-browning is to add an antioxidant or an adsorbent to the medium, and to reduce the occurrence of stress browning by improving the culture conditions. A large number of reports have shown that sucrose [5-6] and browning inhibitors [8, 10] have a great influence on plant callus proliferation and anti-browning. In the present study, we found that when the sucrose concentration in the proliferation medium was 20 g·L⁻¹, the state of the callus was good. In addition, when 0.2 g·L⁻¹ ascorbic acid added to the proliferation medium, the browning rate of the callus was the lowest and the proliferation rate was the highest. This study provides a protocol for obtaining good quality and stable Chinese kale callus.
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References
[1] H.M. Qian, B. Sun, H.Y. Miao, C.X. Cai, C.J. Xu, Q.M. Wang, Variation of glucosinolates and quinone reductase activity among different varieties of Chinese kale and improvement of glucoraphanin by metabolic engineering, Food Chemistry. 168 (2015) 321-326.
[2] J.L. Ye, X.Y. Zhang, X.R. Chai, P.Y. Zhao, Y.Y. Kang, S.F. Li, X. Yang, Research on main nutritional components and active oxygen metabolism in Chinese kale (Brassica alboglabra Bailey), Guangdong Agricultural Sciences. 4 (2016) 57-62.
[3] B. Sun, L. Fang, N. Liu, H.Z. Yan, Y.J. Zhang, Q.Q. Shi, Q.M. Wang, Studies on main nutritional components of Chinese kale among different organs, Acta Horticulturae Sinica. 3 (2011) 541-548.
[4] H.Y. Miao, M.Y. Wang, J.Q. Chang, H. Tao, B. Sun, Q.M. Wang, Effects of glucose and gibberellic acid on glucosinolate content and antioxidant properties of Chinese kale sprouts, Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology). 12 (2017) 1093-1100.
[5] Rumiyati, Sisindari, E. Semiarti, A.F. Milasari, D.K. Sari, N. Fitriana and G. Sekar, Callus induction from various organs of dragon fruit, apple and tomato on some mediums, Pakistan Journal of Biological Sciences. 5 (2017) 244.
[6] Z.R. Hua, X.L. Li, Effects of hormones and sucrose concentration on callus inducement of Pteridium aquilinum, Acta Agriculturae Jiangxi. 2 (2015) 41-44.
[7] D.D. Feng, Y. Wang, J.P. Chen, Research progress of browning in the plant tissue culture, Acta Agriculturae Zhejiangensis. 6 (2015) 1108-1116.
[8] R.T. Wen, H.N. Wang, R.T. Yang, X.X. Jia, Z.Y. Zhuang, Primary study on reducing browning of callus induced from maize embryos, Molecular Plant Breeding. 3 (2010) 483-487.
[9] L.W. Ge, W. Guo, Z.K. Pan, S. Wang, The research literature analysis about tissue culture of genus camellia in China, Journal of Fujian Forestry Science and Technology. 1 (2015) 237-241.
[10] S.B. Fu, H. Li, J. Zhao, X.J. Fu, S.L. Zhu, J.F. Zhang, Investigation of browning factors in embryogenic callus culture of Pinus tabulaeformis and optimization of proliferation medium, Journal of South China Agricultural University. 5 (2017) 91-96