High Concentration of Benzyladenine Solution Stimulates Anthers for Inducing Callus in \textit{Ricinus Communis} L.

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\textbf{Abstract:} An high-frequency protocol for induction of callus from anther explants of \textit{Ricinus communis} was described. When anther explants of \textit{R. communis} was cultured directly onto medium containing 6-benzylaminopurine (BA) induced formation of only poor quality callus that had a low induction frequency of anther callus (10.67%). However, treating the anther explants with high concentrations (7.5-120 mg/L) of BA solution for short time periods (5-80 min) helped to improve the induction frequency and enhance the quality of the callus formation significantly. The best callus induction (41.25%) was observed when anther explants were treated with 15 mg/L BA solution for 10 min before being inoculated onto hormone-free Murashige and Skoog (MS) medium for 30 days. In order to further optimize the culture system, after treated with 15 mg/L BA for 10 min, anther explants were inoculated on the hormone-free MS medium contained concentrations of sodium nitroprusside (SNP). The results showed that SNP significantly promoted the response of callus induction, especially when 8 mg/L SNP was applied, the the highest percentage of callus induction (60.37%) were gained.

1. Introduction

Since anthers of \textit{Datura innoxia} have been used for tissue culture to obtain haploid plants successfully, study of anther culture has developed rapidly, and many countries, including China, have conducted research work in this area [1]. Up to now, about 300 haploid plants have obtained from all kinds of crops by in vitro culture of anthers [2].

Anther culture, combined with conventional hybrid breeding, distant crossbreeding, mutation breeding and transgenic technology, has become one of the more extensive and effective methods of biotechnology in crop breeding. So far, more than 40 kinds of intact plants have been gained via anther culture method in China [2].

Castor (\textit{Ricinus communis} L.) is an annual or perennial herb of \textit{Euphorbiaceae} and accounted as one of the top ten oil crops in the world [3]. Castor oil from seed is an important industrial oil with extensive applications [4]. However, the primary limitation for its large-scale cultivation as an oil crop is the low and inconstant seed yields due to nature heterozygous of \textit{R. communis} plants. In order to breed advanced \textit{R. communis} varieties for large scale cultivation and produce more seeds, haploid breeding of the species might be a possible solution. Haploid breeding has been considered as an advanced strategy for breeding new varieties with improved traits. The haploid plants can be obtained.
by using anther culture, and then the chromosomes can be doubled to obtain the purely diploid cultivars [2]. The characters of offspring of the excellent homozygous lines will not be separated. What’s more, the breeding period can be significantly shorten [2]. Therefore, establishment of tissue culture system for efficient callus induction of castor anther explants will accelerate the breeding progress in R. communis.

In this study, we have developed an efficient callus induction procedure for R. communis by utilizing high concentration of BA solution to deal with the anther explants. Details of the experiments are described in this paper.

2. Materials and Methods

2.1. Preparation of Anther Explants
Male flowers (The blossoms had not opened) of castor were surface-sterilized for 60 s with 75% (v/v) ethanol, immersed in 2% (v/v) sodium hypochlorite (NaClO) for 20 min and finally rinsed 5 times in sterile distilled water. The perianths were removed from the male flowers and anther explants could be gained by carefully separated from stamens with sterile dissecting needle.

2.2. Preparation of 6-benzylaminopurine (BA) Solution and Treating the Anther Explants with BA Solution
6-benzylaminopurine (BA) (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in 1 mol/L NaOH water solution, adjusted to final pH value of 5.8-6.0 and diluted to various concentrations of 0, 7.5, 15, 30, 60 and 120 mg/L. The prepared BA solution was sterilized by filtration using 0.22 μm water filters (Millipore, USA) prior to use.

Anther explants were soaked in glass bottles containing different concentrations (0, 7.5, 15, 30, 60 and 120 mg/L) of BA solution for various time periods (0, 5, 10, 20, 40 and 80 min). After treatment, the explants were briefly placed on sterile dry filter paper in petri dish to absorb excess moisture.

2.3. Direct Induction of Callus Using Anther Explants of R. communis L.
For inducing callus, anther explants were inoculated on hormone-free MS medium after treated with BA solution for various time periods. For comparison, anther explants were also treated using conventional methods and inoculated directly on MS medium containing different concentrations of BA (0, 0.5, 1, 2 and 4 mg/L) as reported previously [3,4]. The percentage of induction of callus was recorded after 30 days of culture.

2.4. Preparation of Culture Medium and culture Maintenance
Uniform culture conditions were applied in all experiments. Basal MS formula was used for all tissue culture experiments. Mediums used in our experiment contained 2.5% sucrose and 0.7% agar that were adjusted to pH 5.8-6.0 with 1 mol/L NaOH, and then being autoclaved at 1.4 kg cm-2 for 20 minutes. All culture treatments were kept at 25 ± 1°C under a 12 h photoperiod of 60-80 μmol m-2s-1 intensity (cool white fluorescent tubes).

2.5. Evaluation of the Results and Data Analysis
All experiments were based on a completely randomized factorial design and repeated three times with 25-30 replicates per treatment. Statistical analysis of the data was carried out using SPSS 17.0 software, and data in the same column followed by different letters were significantly different at p ≤ 5% level as determined by Duncan’s multiple range test. The results were expressed as means ± SD (standard deviation) of three independent experiments.
3. Results

3.1. Induction of Callus from Anther Explants with Conventional Culture Methods

Anther explants without BA solution treatment were inoculated onto MS medium containing different concentrations of BA as reported previously [3, 4]. The concentration of BA in the medium influenced the response of callus induction observably (Table 1). Of the different concentrations of BA (0, 0.5, 1, 2 and 4 mg/L) tested, when there was no BA added into callus-inducing medium, callus could not be induced from anther explants of R. communis (Table 1 and Fig. 1A); and the the highest percentage of callus induction (10.67%) were observed when 1 mg/L BA was applied (Table 1 and Fig. 1B). However, when BA was used at concentration higher than 1 mg/L, the induction rate of callus dropped sharply. What’s more, most of the induced callus were very tiny and underdeveloped (Table 1 and Fig. 1B).

| Number | BA concentration (mg/L) | Induction rate of callus (%) |
|--------|-------------------------|-----------------------------|
| 1      | 0                       | 0c                          |
| 2      | 0.5                     | 8.45±0.39b                  |
| 3      | 1                       | 10.67±0.45a                 |
| 4      | 2                       | 7.34±0.42b                  |
| 5      | 4                       | 4.21±0.35                   |

*Data in the same column followed by different letters are significantly different at $p \leq 5\%$ level as determined by Duncan’s multiple range test.

3.2. Induction of Callus from Anther Explants by Treated with BA Solution before Culture

To study the effect of time duration of BA treatment on callus induction, anther explants treated with 15 mg/L BA solution for various time periods before inoculation of explants on the hormone-free MS medium. The results showed that time duration of the treatment significantly influenced the response of callus induction (Table 2). Treatment with 15 mg/L BA solution for 10 min was the most suitable and it achieved the highest regeneration percentage (41.25%) (Table 2 and Fig. 1C). The percentage of induction of callus was directly proportional to time duration of BA treatment when the time periods of BA treatment was not longer than 10 min. However, when the time duration of BA treatment was longer than 10 min, i.e. 20 min, the callus induction frequencies decreased markedly, and then the percentage of callus induction was 35.42% (Table 2). Therefore, treatment with solution for 15 min was the optimum time periods for the induction of callus, and the callus seemed very healthy and vigorous.

| Number | Treating duration (min) | Induction rate of callus (%) |
|--------|-------------------------|-----------------------------|
| 1      | 0                       | 0e                          |
| 2      | 5                       | 19.31±3.45d                 |
| 3      | 10                      | 41.25±2.32a                 |
| 4      | 20                      | 35.42±1.96b                 |
| 5      | 40                      | 28.63±2.68c                 |
| 6      | 80                      | 17.72±2.51d                 |

* Data in the same column followed by different letters are significantly different at $p \leq 5\%$ level as determined by Duncan’s multiple range test.
**Figure 1.** Direct induction of callus from anther explants of *R. communis.*

(A) Anther explants were inoculated on hormone-free MS medium for 30 days; (B) anther explants were inoculated on MS medium supplemented with 1 mg/L 6-BA for 30 days; after being treated with 15 mg/L TDZ solution for 10 min, anthers explants were inoculated on (C) hormone-free MS medium; (D) MS medium contained 8 mg/L SNP for 30 days (bar = 1 cm).

Furthermore, anther explants were treated with various concentrations of BA solution for 10 min before being inoculated onto hormone-free MS medium. The concentrations of BA solution significantly influenced the response of callus induction (Table 3). The application of 15 mg/L BA resulted in the highest percentage of callus induction (41.25%) (Table 3 and Fig. 1C). The percentage of induction of callus was directly proportional to the concentration of BA solution when the concentration of TDZ was not higher than 15 mg/L (Table 3). However, when TDZ was used at concentrations higher than 15 mg/L, the percentage of callus induction was dramatically decreased (Table 3). Therefore, 15 mg/L BA solution was the optimum choice for the induction of callus, and the callus seemed healthy and lively (Table 3 and Fig. 1C). Finally, it’s clear that treatment with BA solution was more effective than conventional methods for induction of callus from anther explants (Table 1 and Table 3).

| Number | Concentrations of BA solution (mg/L) | Induction rate of callus (%) |
|--------|--------------------------------------|-----------------------------|
| 1      | 0                                    | 0e                          |
| 2      | 7.5                                  | 22.46±1.69c                 |
| 3      | 15                                   | 41.25±2.32a                 |
| 4      | 30                                   | 34.22±2.08b                 |
| 5      | 60                                   | 23.54±3.17c                 |
| 6      | 120                                  | 16.31±2.24d                 |

* Data in the same column followed by different letters are significantly different at p ≤ 5% level as determined by Duncan’s multiple range test.

3.3. **Influence of SNP in the MS Medium on the Culture Response of Anther Explants**

After being treated with 15 mg/L BA for 10 min, anther explants were inoculated onto MS medium containing different concentrations of SNP for tested of their culture response. Date summarized in Table 4 indicated that SNP supplemented to the MS medium had positive effects, facilitating the induction rate of callus. When 8 mg/L SNP was applied, the the highest percentage of callus induction (60.37%) were gained (Table 4 and Fig. 1D). However, when SNP was used at concentration higher than 8 mg/L, the callus induction was inhibited and the induction efficiency was gradually decreased.
Table 4. Effects of concentrations of SNP on callus induction of castor anthers*

| Number | Concentrations of SNP (mg/L) | Induction rate of callus (%)  |
|--------|-----------------------------|-------------------------------|
| 1      | 0                           | 41.25±2.32d                  |
| 2      | 2                           | 47.42±1.43c                  |
| 3      | 4                           | 54.26±2.64b                  |
| 4      | 8                           | 60.37±2.78a                  |
| 5      | 16                          | 50.61±3.49bc                 |
| 6      | 32                          | 37.19±2.57d                  |

* Data in the same column followed by different letters are significantly different at p ≤ 5% level as determined by Duncan’s multiple range test.

4. Results

Haploid breeding helps to modify traits of the species and cultivate new varieties. Whilst tissue culture methods for inducing callus from anther explants are very important and a prerequisite for haploid breeding. Conventional culture methods for inducing callus from anther explants of R. communis included direct inoculation of the explants on a medium containing cytokinin such as BA at low concentrations (usually 0.5-4 mg/L) [3,4]. These methods showed low efficiency of inducing callus and the callus were hardly to further enlargement and growth. However, treatment of anther explants with BA solution at high concentrations (7.5-120 mg/L) in this study for a short time periods (5-80 min) before inoculation on hormone-free MS medium increased the frequency of callus induction and caused the formation of bigger callus as compared with the conventional methods.

BA pertains to the cytokinin family of hormones. cytokinin is an essential factor for the induction of callus formation in most cases of plant tissue culture [5,6]. The success of experimental results given in this paper implied that the induction of callus formation might not requires cytokinin during the whole culture period; when the process of cell division for the formation of callus was activated, the presence of cytokinin was no longer necessary. On the contrary, long presence of cytokinin in the medium might have only negative effects. What’s more, it has been well documented in many academic journals and text books that cytokinin has the effects of inhibition growth and development of plant organs and tissues [7,8].

In our previous study, high concentrations (30 mg/L) of BA treatment for 20 min before inoculating the explants on hormone-free MS medium promoted the buds regeneration frequency from hypocotyl explants in soybean [7]. These results suggested that the new culture method might be applicable to other kinds of plant species and different types of explant.

SNP is usually used as NO donor in plant tissue culture studies [9]. NO might interact with auxins for the regulation of plant cell division, dedifferentiation and redifferentiation [10]. Furthermore, NO was involved in the auxin response during adventitious root formation and the development of buds and shoots might also be promoted [11]. Results in this study confirmed the effect of SNP on the promotion of callus induction, but meanwhile the results demonstrated that the concentration of SNP was vitally important. Such as 16-32 mg/L SNP causes negative effects in the present study.

In conclusion, an efficient callus formation protocol was established for R. communis using anther explants. These methods have the potential to facilitate the haploid breeding.

5. Summary

An efficient callus formation protocol was developed for R. communis by using anther explants. The best effect of callus induction was gained from anther explants treated with 15 mg/L BA solution for 10 min, followed by 30 days of culture on MS medium contained 8 mg/L SNP.

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7. References

[1] Hu D F. Advances in plant flower breeding. Beijing: China Agricultural Science and Technology Press, 1996, 22-42.

[2] Sa R N, Chen Y S, Huang F L, et al. Research progress of plant anther culture technology. Journal of Inner Mongolia University for Nationalities, 2008, 23(6): 650-653.

[3] Shao Z M, Chen Y S, Huang F L, et al. Effects of low temperature pretreatment and light conditions on callus induction of castor anthers. Journal of Inner Mongolia University for Nationalities, 2012, 27 (2): 189-193.

[4] Li G R, Huang F L, Wang W Y, et al. Optimization of callus induct conditions of Ricinus communis anthers. Journal of Inner Mongolia University for Nationalities. 2012, 27(6): 670-673.

[5] Khemkladngoen N, Cartagena J, Shibagaki N, et al. Adventitious shoot regeneration from juvenile cotyledons of a biodiesel producing plant, Jatropha curcas L. Journal of bioscience and bioengineering, 2011, 111(1): 67-70.

[6] Kumar N, Reddy M P. Thidiazuron (TDZ) induced plant regeneration from cotyledonary petiole explants of elite genotypes of Jatropha curcas: a candidate biodiesel plant. Industrial Crops and Products, 2012, 39: 62-68.

[7] Liu Y, Yu L, Zhang Q, et al. High concentration short duration treatment of benzyladenine stimulates adventitious bud regeneration from hypocotyl explants in soybean. Advanced Materials Research, 2013, 647: 331-337.

[8] Liu Y, Tong X, Hui W, et al. Efficient culture protocol for plant regeneration from petiole explants of physiologically mature trees of Jatropha curcas L. Biotechnology & Biotechnological Equipment, 2015, 29(3): 479-488.

[9] Petřivalský M, Vaníčková P, Ryzí M, et al. The effects of reactive nitrogen and oxygen species on the regeneration and growth of cucumber cells from isolated protoplasts. Plant Cell, Tissue and Organ Culture, 2012, 108(2): 237-249.

[10] Ötvös K, Pasternak T P, Miskolczi P, et al. Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. The Plant Journal, 2005, 43(6): 849-860.

[11] Pagnussat G C, Simontacchi M, Puntarulo S, et al. Nitric oxide is required for root organogenesis. Plant Physiology, 2002, 129(3): 954-956.