CALLUS INDUCTION AND PLANT REGENERATION IN ELEVEN PERENNIAL RYEGRASS CULTIVARS

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ABSTRACT

Perennial ryegrass (Lolium perenne L.) is an important grass species as turf and forage. The optimal combination of 2,4-D and BAP in the medium for callus induction and the optimal kinetin concentration in the medium for plant regeneration were investigated. Cultivar variation in the callus induction and plant regeneration of eleven perennial ryegrass was studied using the optimized culture medium. The results showed that the highest callus induction rate with the best callus quality assurance was obtained on MS medium containing 5 mg l⁻¹ 2,4-D and 0.05 mg l⁻¹ BAP. MS medium containing 3 mg l⁻¹ BAP, 1 mg l⁻¹ NAA and 1 mg l⁻¹ kinetin was most suitable for plant regeneration. Callus induction and plant regeneration of the eleven cultivars of perennial ryegrass were varied greatly, and higher overall plant regeneration rates were observed in cultivars Barlennium, Pace, Emerald and Premier.

Introduction

Perennial ryegrass (Lolium perenne L.) is an important grass as forage and turf in temperate zone throughout the world (23), and is suitable for growing in the northern area of China. Its general characteristics of rapid germination, strong seedling vigor, quick establishment, and excellent wear tolerance are highly valued. Currently, drought and salinity of soil are serious limiting factors affecting plant growth, also are global problem restricting agriculture production. In order to reduce water demand for plant maintenance growth and grow in dry and salty areas, breeding salt-tolerant, drought-tolerant, high temperature-tolerant new cultivars of perennial ryegrass is an urgent need.

High self-infertility of perennial ryegrass results in slow progress in breeding success through conventional selection procedures (23). Biotechnological approaches are a significant way to obtain new cultivars of perennial ryegrass. Transgenic perennial ryegrass has been obtained by various methods, such as microprojectile bombardment (1, 14, 15, 21, 23, 24) and silicon carbide fiber-mediated transformation (13). Although Agrobacterium-mediated transformation of perennial ryegrass was recently reported (27), but relevant information is limited. However, it is necessary to optimize tissue culture conditions which involve genotypes, explant tissues and culture media and supplements (3) in genetic transformation of perennial ryegrass.

In regard to plant regeneration of perennial ryegrass, successful regenerations from embryogenic callus (10, 26, 28), cell suspensions (11, 12, 19, 26, 28), protoplasts (12, 26, 28) were reported. Low plant re-
TABLE 1

<table>
<thead>
<tr>
<th>Combinations of 2,4-D and BAP</th>
<th>D0B0.05</th>
<th>D2B0.05</th>
<th>D5B0.05</th>
<th>D8B0.05</th>
<th>D12B0.05</th>
<th>D5B0</th>
<th>D5B0.05</th>
<th>D5B0.1</th>
<th>D5B0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D (mg l⁻¹)</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>8</td>
<td>12</td>
<td>5</td>
<td>5</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>BAP (mg l⁻¹)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0</td>
<td>0.05</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

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Culturing method

The sterilized seeds were placed on the seed germination medium and were kept at 25 °C in the dark. When shoots elongated 2 to 3 cm, they were cut off with scissors from their bases and stayed on the original medium. Surplus parts scissored shoots were treated as explants, and were transferred to induction medium and were kept at 25 °C in darkness.

Calli normally appeared within 5-6 weeks on the induction medium, and then primary calli (Fig. A) were transferred to the subculture medium for culture under 25 °C in the dark. Callus was subcultured once every 25 days.

After 6 times subcultures, the compact, friable embryogenic callus (Fig. B) was selected and transferred to the regeneration medium. Green spot (Fig. C) firstly ap-
TABLE 2

Effects of concentrations of 2,4-D and BAP on callus induction rates of cultivar Barlennium and Pirouette of perennial ryegrass

<table>
<thead>
<tr>
<th>Medium</th>
<th>cv. Barlennium</th>
<th>cv. Pirouette</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Callus induction rate (%)</td>
<td>Overall plant regeneration rate (%)</td>
</tr>
<tr>
<td>D0B0.05</td>
<td>1.3c</td>
<td>0.9c</td>
</tr>
<tr>
<td>D2B0.05</td>
<td>18.7ab</td>
<td>6.2a</td>
</tr>
<tr>
<td>D8B0.05</td>
<td>18.1ab</td>
<td>4.9a</td>
</tr>
<tr>
<td>D12B0.05</td>
<td>24.9a</td>
<td>2.6ab</td>
</tr>
<tr>
<td>D5B0</td>
<td>17.0ab</td>
<td>2.1b</td>
</tr>
<tr>
<td>D5B0.05</td>
<td>13.4ab</td>
<td>3.6b</td>
</tr>
<tr>
<td>D5B0.1</td>
<td>13.4ab</td>
<td>3.6b</td>
</tr>
<tr>
<td>D5B0.2</td>
<td>11.1b</td>
<td>3.3b</td>
</tr>
</tbody>
</table>

Note: Data with different small letters within the same column indicate significant difference (p<0.05) according ANOVA and LSD test.

Effects of 2,4-D and BAP on callus induction rates of cultivar Barlennium and Pirouette of perennial ryegrass

1. Materials and methods
   A. Callus induction
      Auxin 2,4-D, by itself or in combination with cytokinins, has been widely used to enhance callus induction and maintenance (30), and it was considered to be especially important for cereal and grass tissue culture (4). The explants of cultivars Barlennium and Pirouette were used for callus induction in the tested induction medium, and the results are shown in Table 2. Under certain BAP concentration (0.05 mg l⁻¹), callus induction rates of cultivar Barlennium gradually increased from 1.3% to 24.9 with increased 2,4-D. Callus induction rates were no significant difference (p>0.05) on the induction medium with 40-50 calli per repetition.

   B. Overall plant regeneration
      The callus induction rate was defined as the number of seeds with induced callus over the total number of seeds germinated ×100. The callus regeneration rate was calculated as the percentage of callus that had regeneration shoots.

   C. Evaluation of tissue culture responses for eleven cultivars
      Eleven cultivars were evaluated on the optimized culture media according to the investigations for induction medium and regeneration medium. The experimental methods were the same as above-stated.

   D. Statistical analysis
      For the callus induction experiment, each treatment included three repetitions with 300 seeds per repetition. Fifty seeds were inoculated per 9-cm culture dish. The regeneration studies were repeated three times with 40-50 calli per repetition.

2. Results and Discussion
   A. Effects of 2,4-D and BAP on callus induction
      The callus induction rate was defined as the number of seeds with induced callus over the total number of seeds germinated ×100. The callus regeneration rate was calculated as the percentage of callus that had regeneration shoots.

   B. One-way ANOVA and LSD test
      One-way ANOVA and LSD test were carried out for the data gained in this study, using software of SPSS (version 11.5).
2,4-D from 5 mg l\(^{-1}\) to 12 mg l\(^{-1}\) for cultivar Barlennium, but the significant difference (p<0.05) occurred between 2 and 12 mg l\(^{-1}\) or with no 2,4-D added. Callus induction rates of cultivar Pirouette significantly increased when the 2,4-D concentration increased from 0 to 8 mg l\(^{-1}\), no obvious differences were observed between 5 mg l\(^{-1}\) and 8 mg l\(^{-1}\) 2,4-D. In comparison with other concentration of 2,4-D, 5 mg l\(^{-1}\) 2,4-D increased overall plant regeneration rate for cultivars Barlennium and Pirouette tested. Creemers-Molenaar et al. (10) indicated that the increasing concentration of 2,4-D in the callus initiation medium resulted in a lower regeneration frequency of green shoots for perennial ryegrass, which was coincident with the results obtained in our study. The result showed that no optimal 2,4-D concentration could be determined for the induction of compact and embryogenic callus from immature inflorescence explants under a wide range of 2,4-D concentration (2.5-15.0 mg l\(^{-1}\)), but they adopted a concentration of 5 mg l\(^{-1}\) 2,4-D for their further study (10). Bradley et al. (5) supplemented with 2 mg l\(^{-1}\) 2,4-D on the induction medium for studying callus induction of 13 cultivars of perennial ryegrass, and the callus induction rate obtained ranged from 2.3% to 21.0%. In the present study, the callus induction rate ranged from 10.7% to 28.8% under 5 mg l\(^{-1}\) 2,4-D. Because higher concentrations of 2,4-D might result in a greater possibility of somatic mutation (8, 17), 5.0 mg l\(^{-1}\) 2,4-D may be the optimal concentration.

Cytokinins, such as BAP and kinetin, at low concentrations, in combination auxins were often used in turfgrass species to promote callus initiation (7). When concentration of 2,4-D was specified at level of 5 mg l\(^{-1}\), callus induction rates of the cultivar Barlennium and Pirouette responded to changes of BAP concentration (Table 2). Although there were no significant differences (p<0.05) for callus induction of the cultivar Barlennium among the induction medium with BAP from 0 mg l\(^{-1}\) to 0.10 mg l\(^{-1}\), positive effect was found in 0.05 mg l\(^{-1}\). For cultivar Pirouette, explants from medium with no BAP added had the highest induction rate (25.4%). No obvious difference was observed between 0 mg l\(^{-1}\) BAP and 0.05 mg l\(^{-1}\) BAP for callus induction of the cultivar Pirouette, but the medium with no BAP added was significantly higher medium containing 0.1 mg l\(^{-1}\) BAP and 0.2 mg l\(^{-1}\) BAP. 0.05 mg l\(^{-1}\) BAP significantly increased plant regeneration for cultivar Barlennium, but not significantly increased regeneration for cultivar Pirouette. Optimal concentration of BAP for callus induction of turfgrass varied widely. For example, for bermudagrass (Cynodon dactylon), the optimal concentration of BAP was rather low, only 0.01 mg l\(^{-1}\) (9), while for Kentucky bluegrass (Poa pratensis L.) and tall fescue, it was 0.1-0.3 mg l\(^{-1}\) (2, 25) and rather high concentration of BAP (0.5-2 mg l\(^{-1}\)) was needed for creeping bentgrass (Agrostis stolonifera L.) (29). In our study, it was found that concentrations of BAP more than 0.05 mg l\(^{-1}\) on the induction medium would not increase the callus induction of cultivar Barlennium and Pirouette, and it seemed to be general for perennial ryegrass because of the same results observed in all of other tested cultivars in our later experiment (data not be shown). Bradley et al. (5) also reported that callus induction rate on the medium containing 0.02 mg l\(^{-1}\) BAP (medium supplemented with 2 mg l\(^{-1}\) 2,4-D) was significantly higher than on the medium containing BAP higher than 0.1 mg l\(^{-1}\) for cultivar Majesty of perennial ryegrass. Accordingly, the optimal BAP concentration in the induction medium should be 0.02-0.05 mg l\(^{-1}\) for perennial ryegrass.

Accordingly, the culture medium containing 5 mg l\(^{-1}\) 2,4-D and 0.05 mg l\(^{-1}\) BAP was chosen as callus induction medium and the modified induction medium which containing lower 2,4-D concentration (3 mg l\(^{-1}\)
TABLE 3

Effects of concentration of kinetin on plant regeneration of cultivar Premier and Barlennium of perennial ryegrass

<table>
<thead>
<tr>
<th>Concentration of kinetin (mg l⁻¹)</th>
<th>Cv.Premier (%)</th>
<th>Cv.Barlennium (%)</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>15.6 c</td>
<td>35.6 b</td>
<td>25.6bc</td>
</tr>
<tr>
<td>1</td>
<td>27.8 ab</td>
<td>52.2 a</td>
<td>40.0 a</td>
</tr>
<tr>
<td>1.5</td>
<td>30.6 a</td>
<td>34.1 b</td>
<td>32.4ab</td>
</tr>
<tr>
<td>2</td>
<td>16.7 bc</td>
<td>13.4 c</td>
<td>15.0c</td>
</tr>
</tbody>
</table>

Note: Data with different small letters within the same column indicate significant difference (p<0.05) level according ANOVA and LSD test.

was used for callus subculture to avoid the inhibition of the auxin to plant regeneration in this study.

Effect of kinetin on plant regeneration
Friable calli of cultivars Premier and Barlennium subcultured 6 times on the subculture medium were used for testing plant regeneration with different kinetin concentration (Table 3). The highest regeneration rate was obtained on the regeneration medium with 1.0 mg l⁻¹ kinetin for Barlennium. For cultivars Premier, callus from medium containing 1.5 mg l⁻¹ kinetin had the highest regeneration rate. The regeneration rate on the medium with 1.5 mg l⁻¹ kinetin was not significantly higher than the regeneration medium containing 1.0 mg l⁻¹ kinetin. Therefore, the regeneration medium containing 1.0 mg l⁻¹ kinetin was chosen for plant regeneration in this study.

Variations of tissue culture among the eleven cultivars of perennial ryegrass
Results of callus induction and plant regeneration of the eleven cultivars of perennial ryegrass on the selected medium are shown in Table 4. Callus induction rates varied evidently among the tested cultivars and ranged from 10.7% to 28.8%. The highest callus induction rate occurred in the cultivar Premier, which was 28.8%, and significantly higher (p<0.05) than the other tested cultivars excepting cultivar Barclay. Cultivars APM and Fairway had the lowest callus induction rates which were significantly lower (p<0.05) than the highest ones (cultivars Premier and Barclay). The callus induction rates of the other cultivars ranged from 15.5% to 20.6%, and were without significant differences (p>0.05) each other.

Variation of plant regeneration rates among the tested cultivars was also extremely significant (p<0.05) according to ANOVA, and ranged from 2.8% to 38.8%, the maximum difference reached to near 14 times. The cultivar Barlennium had the highest plant regeneration rate (38.8%), and it was no significant differences (p<0.05) with those of cultivars Emerald (31.5%) and Pace (31.0%). Plant regeneration rate of cultivar Fairway was similar to those of the cultivars Emerald and Pace, but significantly lower than the cultivar Barlennium (p<0.05).

Overall plant regeneration rates varied also significantly among the tested cultivars (p<0.05) based on ANOVA. The plant regeneration ability of cultivars Barlennium, Emerald, Pace and Premier were higher than most of the other tested cultivars significantly (p<0.05) excepting Fairway. Bradley et al. (5) reported that callus induction and plant regeneration were significantly varied among 13 cultivars of turf-type perennial ryegrass, and cultivar Roadrunner had the highest callus induction rate and the highest plant regeneration rate. The overall plant regeneration rates of the 13 cultivars varied from 0.0 to 12.2% and averaged 2.7% (5). By testing 21 genotypes of perennial ryegrass, Olesen et al. (20) estimated that differences between genotypes accounted for approximately 40% and 59% of the total variation for callus induction and plant regeneration, respectively. In our study, the maximum callus induction rate was gotten in cultivar Premier, but for plant regeneration rate, cultivar Barlennium ranked above other cultivars, and the overall plant regeneration rates of the eleven cultivars ranged from 0.7 to 6.9 % with the maximum 11.7 folds...
Callus induction and plant regeneration of the eleven perennial ryegrass cultivars

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Callus induction (%) (I)</th>
<th>Plant regeneration (%) (R)</th>
<th>Overall plant regeneration (%) (I×R)</th>
<th>albino(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accent</td>
<td>15.5 bc</td>
<td>8.2 def</td>
<td>1.3 c</td>
<td>0.0</td>
</tr>
<tr>
<td>APM</td>
<td>11.7 c</td>
<td>12.5 cde</td>
<td>1.4 c</td>
<td>3.1</td>
</tr>
<tr>
<td>Barball</td>
<td>18.4 bc</td>
<td>16.3 cd</td>
<td>3.0 c</td>
<td>0.0</td>
</tr>
<tr>
<td>Barclay</td>
<td>22.9 ab</td>
<td>2.8 f</td>
<td>0.7 c</td>
<td>0.0</td>
</tr>
<tr>
<td>Barlennium</td>
<td>18.1 bc</td>
<td>38.8 a</td>
<td>6.9 a</td>
<td>6.0</td>
</tr>
<tr>
<td>Emerald</td>
<td>17.3 bc</td>
<td>31.5 ab</td>
<td>5.4 ab</td>
<td>9.9</td>
</tr>
<tr>
<td>Fairway</td>
<td>10.7 c</td>
<td>28.4 b</td>
<td>3.2 bc</td>
<td>0.0</td>
</tr>
<tr>
<td>Matilda</td>
<td>15.6 bc</td>
<td>18.9 e</td>
<td>2.9 c</td>
<td>0.8</td>
</tr>
<tr>
<td>Pace</td>
<td>18.1 bc</td>
<td>30.9 ab</td>
<td>5.5 ab</td>
<td>0.0</td>
</tr>
<tr>
<td>Pirouette</td>
<td>20.6 b</td>
<td>6.2 ef</td>
<td>1.3 c</td>
<td>0.0</td>
</tr>
<tr>
<td>Premier</td>
<td>28.8 a</td>
<td>19.4 e</td>
<td>5.6 ab</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Notes: Different small letters within the same column indicate significant difference at p<0.05 level according ANOVA and LSD test.

difference, and averaged 3.4%. These results suggested that although the tested cultivars and the used methods for tissue culture of perennial ryegrass were different, genotype played the most important role which might be as decisive as the concentration of supplements in culture media in tissue culture responses. Some other studies for cereal or grass plants, such as tall fescue (3), barley (6), etc. proved also that genotype difference is a primary factor affecting plant tissue culture responses.

There were some albino plants (Fig. J) observed in the cultivars APM, Barlennium, Emerald, Matilda and Premier, and the albino rates were 3.1%, 6.0%, 9.9%, 0.8% and 11.5%, respectively. While there was no albino found in the cultivars Accent, Barball, Barclay, Fairway, Pace and Pirouette. Therefore, albino was easier to be formed in cultivars Premier and Emerald than the other tested cultivars.

The formation of albino is frequently occurred in tissue culture of grass species including perennial ryegrass (5, 28) and tall fescue (3). The occurrence of the albino varied also among cultivars in perennial ryegrass. Because the cultivars with higher plant regeneration rates, such as Barlennium, Emerald and Premier, had higher albino rates (6.0-11.5%) simultaneously, it lead to the loss of plant regeneration efficiency in the cultivars being useful for tissue culture.

As a conclusion, the system established in the present study for tissue culture of perennial ryegrass can get enough callus plant regeneration efficiency to perform transgenic operation, but choice of cultivar is necessary. Among the tested cultivars, Barlennium, Pace, Emerald and Premier are recommended.

REFERENCES