REGENERATION OF PLANTS FROM IN VITRO CULTURE OF PETIOLES IN PRUNUS DOMESTICA LINDL. (EUROPEAN PLUM)

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ABSTRACT
Adventitious shoot regeneration from the petioles of European plum ‘Tardicots’ (Prunus domestica) was studied in this study. The influences of different concentrations of thidiazuron (TDZ) and 2,4-Dichlorophenoxyacetic acid (2,4-D), dark culture period, and different basal salts on shoot regeneration were evaluated. Woody plant medium (WPM) was the best basal medium for the European plum initial culture, supplemented with 2.0 mg L⁻¹ thidiazuron (TDZ) and 0.2 mg L⁻¹ 2,4-D. The explants, which were maintained in darkness for 14 d, as a result had the highest regeneration rate (83.33%). The regenerated shoots were subcultured at 4 weeks intervals. Addition of IBA (1.0 mg L⁻¹) in the rooting medium produced the highest roots (93.3%) when maintained in dark for 7 d.


Keywords: Prunus domestica, regeneration, petiole, adventitious shoot
Abbreviations: TDZ: thidiazuron; BA: 6-Benzyladenine; IBA: Indole-3-butyric acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid; MS: Murashige and Skoog medium; WPM: woody plant medium; PGR: plant growth regulator

Introduction
Plum (Prunus domestica L.), belonging to the Rosaceae family, is one of the most important stone fruit trees in the world. Plum trees exhibit strong adaptability with the climatic conditions of China, and thus it is planted in most parts of China. Stone fruits, as important fruit crops, are very popular in our life, such as peach (P. persica), European plum (P. domestica), Japanese plum (P. salicina), apricot (P. armeniaca), sweet cherry (P. avium), sour cherry (P. cerasus), and almond (P. dulcis). They all belong to the genus Prunus (29). Plums have a lot of important purposes, in addition to being used as fresh fruits. They are also being used for food processing, pharmaceuticals, and export. Therefore, it is very necessary to cultivate and grow superior plum cultivars. Most of stone fruit trees have the characteristics of long breeding cycles, high levels of heterozygosity, and high degrees of incompatibility, which make them difficult to improve via sexual hybridization (25). Modern biological technologies, especially tissue culture and gene engineering for fruit tree germplasm creation, are opening a new route to shorten the breeding cycle and produce seedlings free of viruses. Adventitious shoot regeneration is a prerequisite for any plant improvement program that aims at improving plants by in vitro techniques and genetic engineering (7). So, high efficiency and stability of regeneration is necessary for transformation studies.

In Rosaceae family, regeneration using leaves has been reported for apple (8, 33), pear (5, 14, 15, 26), peach (10, 34), almond (1), and cherry (4, 18, 21). Additionally, regeneration using cotyledons has been reported for almond (2) cherry (6). For plum, adventitious shoot regeneration from many explants had also been reported in European plum, such as cotyledons (7, 20), hypocotyls (19, 27), leaves (3, 22, 23). Some of these explants had been used in transformation, such as hypocotyls (19, 24, 27). In recent years, successful regeneration from different explants of Japanese plum has been reported including hypocotyls (29, 30) and cotyledons (7).

Medium composition was a decisive factor for plant regeneration. In previous reports, MS or WPM was used frequently as basal medium in regeneration of Prunus species, and the most used cytokinins were TDZ or BA. 2,4-D was used as auxin in this study, because IBA easily induces the differentiation of root of plum.

As a new kind of explant of plum, petiole was used to construct a new regeneration system for plum in this study. To attain a high efficient regeneration system, some factors, such as basal medium, hormone and photoperiod were also studied.

Materials and Methods
Plant material
Plum petiole explants were obtained from in vitro ‘Tardicots’ seedlings, which were regenerated from stem segment explants on MS medium supplemented with IBA 0.05 mg L⁻¹ and BA 0.5 mg L⁻¹. Fully developed leaves were excised from the seedlings after subculture for 30 days. Leaf blades were removed, and the excised petiole explants were placed on the shoot induction medium (SIM). The pH of the medium was...
adjusted to 6.0 with NaOH before the addition of agar and autoclaved at 1.1 kg cm\(^{-2}\) and 121°C for 15 min. A total of 20 petioles were cultured for each 9 cm-diameter petri dish, and each treatment was replicated three times. Regeneration data were noted and analyzed after 30 days of culture.

**Medium composition**

To identify the optimal medium for shoot induction of petiole explants, the experiment was carried out using an orthogonal design (orthogonal array, L\(_{16}(4^3)\)) consisting of: 1. basal medium ((1) WPM (Lloyd and McCown, 1980), (2) MS (Murashige and Skoog 1962), (3) 1/2MS (Macro elements halved), (4) 1/2WPM (Macro elements halved)); 2. TDZ (1.0, 2.0, 3.0 and 4.0 mg L\(^{-1}\)); and 3. 2,4-D (0.1, 0.2, 0.3 and 0.4 mg L\(^{-1}\)) (Table 1). Each medium was supplemented with 3% sucrose, 0.7% agar, and the pH of the medium was adjusted to 6.0 with NaOH.

**Effect of different dark period**

To examine the effect of dark period on shoot induction, the explants were maintained in darkness for 0~4 weeks on the optimal medium that was identified in shoot induction experiment, and then transferred to the 16-h photoperiod (Light intensity: 2000-3000 Lux) provided by fluorescent lamps at 25(±1)°C. Differentiation rate of petiole explants and growth status were analyzed after 30 days.

**Rooting and acclimatization**

Up 1.5 cm subcultured shoots containing 3~4 leaves were inoculated on rooting medium containing MS medium supplemented with different concentrations of IBA (0, 0.25, 0.5, 0.75, 1.0 mg L\(^{-1}\)), 3% sucrose, pH 6.0, and 7 g agar.

<table>
<thead>
<tr>
<th>Orthogonal design</th>
<th>A Basal medium</th>
<th>B TDZ (mg L(^{-1}))</th>
<th>C 2,4-D (mg L(^{-1}))</th>
<th>Regeneration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (WPM)</td>
<td>1(1)</td>
<td>1(0.1)</td>
<td>3.33±5.77</td>
</tr>
<tr>
<td>2</td>
<td>1 (WPM)</td>
<td>2(2)</td>
<td>2(0.2)</td>
<td>83.33±12.58</td>
</tr>
<tr>
<td>3</td>
<td>1 (WPM)</td>
<td>3(3)</td>
<td>3(0.3)</td>
<td>46.67±7.64</td>
</tr>
<tr>
<td>4</td>
<td>1 (WPM)</td>
<td>4(4)</td>
<td>4(0.4)</td>
<td>15.00±0.00</td>
</tr>
<tr>
<td>5</td>
<td>2 (MS)</td>
<td>1</td>
<td>2</td>
<td>1.67±2.89</td>
</tr>
<tr>
<td>6</td>
<td>2 (MS)</td>
<td>2</td>
<td>3</td>
<td>26.67±5.77</td>
</tr>
<tr>
<td>7</td>
<td>2 (MS)</td>
<td>3</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>2 (MS)</td>
<td>4</td>
<td>1</td>
<td>11.67±2.89</td>
</tr>
<tr>
<td>9</td>
<td>3 (1/2MS)</td>
<td>1</td>
<td>3</td>
<td>11.67±2.89</td>
</tr>
<tr>
<td>10</td>
<td>3 (1/2MS)</td>
<td>2</td>
<td>4</td>
<td>43.33±7.64</td>
</tr>
<tr>
<td>11</td>
<td>3 (1/2MS)</td>
<td>3</td>
<td>1</td>
<td>3.33±2.89</td>
</tr>
<tr>
<td>12</td>
<td>3 (1/2MS)</td>
<td>4</td>
<td>2</td>
<td>3.33±2.89</td>
</tr>
<tr>
<td>13</td>
<td>4 (1/2WPM)</td>
<td>1</td>
<td>4</td>
<td>0.00</td>
</tr>
<tr>
<td>14</td>
<td>4 (1/2WPM)</td>
<td>2</td>
<td>1</td>
<td>21.67±2.89</td>
</tr>
<tr>
<td>15</td>
<td>4 (1/2WPM)</td>
<td>3</td>
<td>2</td>
<td>15.00±5.00</td>
</tr>
<tr>
<td>16</td>
<td>4 (1/2WPM)</td>
<td>4</td>
<td>3</td>
<td>10.00±0.00</td>
</tr>
</tbody>
</table>

\(^a\) each treatment was repeated 3 times

\(^b\) regeneration data were noted and analyzed after 30 days of culture

<table>
<thead>
<tr>
<th>IBA (mg L(^{-1}))</th>
<th>No. of inoculation</th>
<th>Rooting rate (%)</th>
<th>Rooting times</th>
<th>Height of plants (cm)</th>
<th>Length of roots (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>0.00 c</td>
<td>0.00 d</td>
<td>0.00 c</td>
<td>0.00 d</td>
</tr>
<tr>
<td>0.25</td>
<td>15</td>
<td>60.00±20.00 b</td>
<td>2.20±0.40 c</td>
<td>1.78±0.11 d</td>
<td>2.82±0.10 c</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
<td>66.67±11.55 b</td>
<td>2.13±0.50 c</td>
<td>2.49±0.30 c</td>
<td>3.91±0.08 a</td>
</tr>
<tr>
<td>0.75</td>
<td>15</td>
<td>80.00±0.00 b</td>
<td>3.07±0.31 b</td>
<td>3.10±0.29 b</td>
<td>3.98±0.06 a</td>
</tr>
<tr>
<td>1.0</td>
<td>15</td>
<td>93.33±11.55 a</td>
<td>4.00±0.20 a</td>
<td>3.63±0.10 a</td>
<td>3.69±0.17 b</td>
</tr>
</tbody>
</table>

Effects of IBA on rooting of ‘Tardicots’

Values in each column followed by the different letters are significantly different (\(P < 0.05\)) according to LSD test

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**TABLE 1**

**TABLE 2**
The cultures were maintained in dark for 7 days, and then transferred to 16 h photoperiod. Every 5 subcultured shoots were cultivated in a culture bottle. Rooting, rooting coefficient, height of plants and length of roots were analyzed after 30 days.

The acclimatization of rooted shoots was tested by the methods described by Padilla et al., 2003 (24) with slight alteration. Plantlets were cultured in tap water for 3 days after being rinsed and then were transferred to pots containing autoclaved soilless potting mixture, which consisted of both peat and vermiculite in the ratio of 2:1 (v/v). This medium was moistened with liquid MS salts, pH 6.0. These pots were placed inside re-sealable plastic bags. The bags were sealed and returned to the culture room. After 1 week, the bags were partially opened, and 1 week later they were fully opened and transferred to the greenhouse. Fifty plants were used in acclimatization experiment, and the plants survival rate, average height and leaf number of plant were recorded after 30 days.

**Statistical analysis**
The data in this study were calculated by the methods as follow:

- **Shoot Regeneration (%)** = number of regenerating explants/total number of explants × 100
- **Rooting (%)** = number of rooted explants/cultivation number of explants × 100
- **Rooting coefficient** = number of roots/number of rooted plantlets

![Fig. 1.](image1.png)

**Fig. 1.** (A) 1-day-old petiole explants; (B) Callus on explants that were incubated in dark on the 15th day; (C and D) Close ups of the adventitious shoots from petiole explants after 30 days of culture initiation
Survival (%)=number of survival plants/total number of transplanted plantlets × 100

The experimental data were analyzed by using the SAS software Version 9.1 (SAS Institute, 2003) and means were compared by Least Significant Difference (LSD) at the 0.05 level.

Results and Discussion

Effect of medium composition on shoot induction

Both ends of petioles were expanded and formed callus after being inoculated 1 to 2 weeks on the tested media. However, callus proliferated stronger at the end closer to stem. Adventitious shoots started regeneration after 2 to 3 weeks of culturing and from callus, formed at the ends of petioles closed to stem (Fig. 1).

Through the analysis of variance and multiple comparisons, the result revealed that WPM medium was significantly better than the other three media for the regeneration of shoots from petioles. A concentration of TDZ of 2.0 mg L\(^{-1}\) was determined to be optimal. The higher concentrations 3.0 mg L\(^{-1}\) and 4.0 mg L\(^{-1}\) were unfavorable for the regeneration. 2,4-D in concentrations of 0.2 mg L\(^{-1}\) or 0.3 mg L\(^{-1}\) produced more regeneration than in concentration of 0.1 mg L\(^{-1}\), and when 0.4 mg L\(^{-1}\) was used the results were intermediate between them. The highest regeneration frequency was noted on medium containing WPM supplemented with TDZ (2.0 mg L\(^{-1}\)) and 2,4-D (0.2 mg L\(^{-1}\)) (Table 1). The regeneration data indicated that adventitious buds could be regenerated on all the combinations of basal media augmented with TDZ and 2,4-D except MS medium supplemented with TDZ (3.0 mg L\(^{-1}\)), 2,4-D (0.3 mg L\(^{-1}\)) and 1/2WPM medium supplemented with TDZ (1.0 mg L\(^{-1}\)), 2,4-D (0.4 mg L\(^{-1}\)).

![Fig. 2. Effects of different dark periods on shoot regeneration of 'Tardicots' petioles](image)

The different letters mean significantly different (P<0.05) according to LSD test.

Explants were occasionally contaminated at very low level by endophytes (Fig. 3D). Cephamycin in the media was very effective to inhibit the contamination in the cultures at concentration of 10 mg L\(^{-1}\).

Effect of different dark periods on shoot induction

WPM medium supplemented with TDZ (2.0 mg L\(^{-1}\)) and 2,4-D (0.2 mg L\(^{-1}\)) was used in dark period experiment and the regeneration rate was significantly different under different dark period tests (Fig. 2). It was observed that explants cultured in dark for 2 weeks had highest regeneration capacity. At the dark culture period of 0 d or 7 d, the browning of explants was very intense, when explants at 0 d were all brown and most of explants were brown at 7 d (data not shown). If the dark culture period was 21 d or 28 d, the vitrification of explants was significant (data not shown), and with the prolongation of dark culture period the regeneration rate of explants dropped.

Rooting and acclimatization

Roots of the regenerated shoots were differentiated 2 weeks after inoculation on rooting medium. Rooting failed on medium that lacked IBA. However, rooting medium containing IBA (1.0 mg L\(^{-1}\)) resulted in highest (93.33%), rooting frequencies, vigor of plants and roots appeared comparatively earlier. In addition, IBA increased the rooting frequencies, and the effect of rooting coefficient and height of plants was most significant (Table 2). It was also noted that decrease in IBA concentrations (0.5~0.75 mg L\(^{-1}\)) produced longer roots of the inoculated shoot.

In acclimatization experiment, 33 plants survived and the survival rate reached 66.0% (Fig. 3C), average height and leaf number of plants were also recorded and respectively were 3.93 cm and 6.82.

Regeneration of adventitious shoots from petioles in European plum has not been reported until now. All the data here indicated that hormone and dark culture period were important factors for regeneration, whereas hormone was the most crucial factor in shoot differentiation. Similar results were achieved in regeneration of adult wild cherry (Prunus avium L.) (11). Auxin and/or cytokinin had been used in plant tissue culture for a long time and differentiation of shoot or root of explants depends on different concentrations and combinations of them. Adventitious shoots are differentiated at higher level of cytokinin and roots are differentiated conversely (31). This report testifies our findings. In this study, no matter which basal medium was used, the effect of regeneration was optimal when the concentration of TDZ was 2.0 mg L\(^{-1}\), and the effect did not depend on the concentrations of 2,4-D. This may be due to the high ratio of TDZ:2,4-D and strong cell division activity of TDZ.

MS and QL were often used as basal medium in previous reports about plum regeneration, but WPM was optimal basal medium in this study. This may be due to genotypic and biological characteristics of different explants. The nutrient requirement varies in plant tissue culture according to different genetic and biological characteristics, even with different tissues in a same plant or at different growth stages in a same tissue (9). Genotypic differences and types of explants are also known to influence regeneration efficiency (7). In addition, we found that adventitious shoots differentiated only from the end...
that is close to the stem, although callus were formed on the other end too. This may be due to the polarity of petioles.

Dark culture period is an important factor in regeneration of petiole explants. Regeneration rate was highest (83.3%) under 14 days of dark culture period, which was consistent with the previous reports about leaves regeneration of European plum (25), wild myrobalan plum (Prunus cerasifera) (17) and ‘Nubiana’ (Prunus salicina) studied previously in our laboratory. This result was different with 3-week of ‘Wegierka Zwykła’ plum (Prunus domestica) (23) and leaves of the peach rootstock ‘Nemaguard’ (Prunus persica × P. davidiana) (34). This may be due to the difference of types or species of the explants (7).

Contamination that is caused by endophytes can occasionally occur. An endophyte is a bacterial (including actinomycete) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intracellularly inside the healthy tissues of the host plant (28). The contamination that was caused by endophyte can be inhibited by adding some antibiotics to the medium (12, 16). The contamination of endophyte was very rare in this study and the effect of antibiotic (cephamycin) was pronounced.

Gene transformation of plum was carried out successfully by using hypocotyl segments as receptor of ectogenous genes (19, 24, 27, 30). However, their corresponding transformation efficiencies were not high.

Conclusions
In this report, we demonstrated that adventitious shoots could be induced from petioles of P. domestica and the regeneration rate was higher than most of those that had been reported by using other explants of plum. Furthermore, the calluses that were formed on explants from petioles were abundant. Thus, this protocol may be a good choice for establishing a high efficiency plum transformation by using petioles as the receptor of ectogenous genes.

In addition the petiole explants were obtained more easily than most other explants in plum, were very sufficient in number, and obtaining of petioles from in vitro seedlings was not restricted by the seasons. Therefore, this protocol might
be a potential system for improvement of the transformation efficiency in European plum.

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REFERENCES