Germlasm preservation in vitro of Polygonum multiflorum Thunb

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ABSTRACT

Background: The root of Polygonum multiflorum Thunb. is a common traditional Chinese medicine. In recent years, the wild resources of P. multiflorum have been seriously broken, and the cultivated varieties have been degrading. The germplasm resources of P. multiflorum need protection and preservation. So far, no in vitro germplasm preservation of P. multiflorum has been reported. Objective: To explore a method for the in vitro germplasm preservation of P. multiflorum. Materials and Methods: A large number of buds from seed explants were induced by tissue culture. The single buds were used as experimental materials to study the effects of plant growth regulator, temperature, and osmotic pressure on the preservation time, growth recovery, and genetic stability. Results: When the buds were inoculated onto Murashige and Skoog (MS) basal media containing 4% w/v sucrose, 2% w/v mannitol, and 1% w/v sorbitol, supplemented with paclobutrazol (PP333) 1.0 mg/l, abscisic acid (ABA) 5.0 mg/l, and daminozide (B9) 30.0 mg/l in an illuminated chamber under a 16 h photoperiod of 1500 lx light intensity at 15°C for 10 months, the survival rate was over 70% with good growth recovery and genetic stability. Conclusion: The results of this study can be used for medium-term in vitro germplasm preservation of P. multiflorum, and meeting actual needs of research and production.

Key words: Germplasm, Polygonum multiflorum Thunb., preservation in vitro

INTRODUCTION

Polygonum multiflorum Thunb., of the genus P. lapathifolium, is a perennial twisted herbal plant.[1] The root is a common traditional Chinese medicine. In addition, the stem and leaf of P. multiflorum can be also used as medicinal material.[2] In clinical practice, raw root was used for detoxification, elimination carbuncle, and catharsis.[3-5] After processing, it was used for nourishing liver and kidney, enriching blood, and blackening hair.[3-5] In the recent years, with the trend of returning to nature and the improvement of people’s living standards, the demand for natural herbs have surged. Owing to the increasing market demand, the wild resources of P. multiflorum are being seriously broken.[6,7] Besides, the cultivated varieties of P. multiflorum have been degrading in the process of long-term cultivation, which resulted in reducing the yield and quality of medicinal material.[8] Germplasm resources of P. multiflorum are being destroyed. Consequently, it is necessary to protect and preserve germplasm resources of P. multiflorum. However, there still has been no report on the germplasm preservation of P. multiflorum. To preserve germplasm preservation of P. multiflorum, we sought here a protocol for in vitro germplasm preservation of P. multiflorum.

Germplasm resources are the material basis of breeding.[9] Preservation of plant germplasm is significant for the conservation of biodiversity and preventing the loss of good gene. Research into germplasm preservation on crops was frequently reported.[10-13] Nevertheless, studies on germplasm preservation of medicinal plants were focused on preservation in vitro.[14-16]

With the seeds or roots used as propagation materials, P. multiflorum can only maintain genetic stability in short time. Because of frequently natural hybridization, the genetic basis presents high heterogeneity, which leads to the instability of good character in sexual reproduction. Asexual reproduction is susceptible to the virus with germplasm degradation.[8] Preservation in vitro based on

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tissue culture, not only can effectively preserve germplasm of *P. multiflorum* but also can rapidly propagate to meet production needs. To the best of our knowledge, no in vitro germplasm preservation of *P. multiflorum* has been reported. In this paper, an in vitro preservation protocol for *P. multiflorum* has been established and optimized. Buds from seed explants were induced by tissue culture. Buds in vitro were preserved for 10 months under room temperature (15°C), and the survival rate was over 70% with good growth recovery and genetic stability. The methods will provide a scientific basis for the germplasm preservation as well as further varieties breeding of *P. multiflorum*.

**MATERIALS AND METHODS**

**Plant material**

Seeds of *P. multiflorum* (2x = 22) were obtained from Qianshan County, Anhui Province, China. The Department of Genetics and Breeding of China Pharmaceutical University identified the original plant.

**Seed disinfection and germination and culture conditions**

Seeds of *P. multiflorum* were sterilized in 2% v/v sodium hypochlorite containing three to five drops/1 of Tween-20 for 12 min. The seeds were rinsed in sterile distilled water three to five times and then transferred to a petridish containing sterile filter paper to remove excess surface water. The sterilized seeds were placed onto Murashige and Skoog (MS) (Murashige and Skoog 1962) medium containing 3% w/v sucrose and 0.33% w/v agar powder (gel strength = 1,100 g/cm²) at pH 5.8. The inoculated seeds were incubated in an illuminated chamber under a 16 h photoperiod of 1,200 lx light intensity at 25 ± 1°C to initiate germination.

**Experiment on effects of plant growth regulators by orthogonal test**

MS medium was the basal medium used throughout these studies. To extend in vitro preservation time of plant material, three phytohormones, namely, paclobutrazol (PP333; 0.5, 1.0, and 2.0 mg/l), abscisic acid (ABA; 1.0, 5.0, and 10.0 mg/l), and daminozide (B9; 10.0, 20.0, and 30.0 mg/l) were used at three concentrations each for the orthogonal test. In this experiment, the survival materials, preserved for 300 days by the optimal preservation method, were subcultured on the breeding media supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA under a 16 h photoperiod of 1,200 lx light intensity at 25 ± 1°C. After they have been subcultured for three 25-day subculture cycle, the propagation coefficients, leaf length, and leaf width of survival materials were observed. Furthermore, after three 25-day subculture cycle, survival materials were transferred to rooting media consisting of semi-solid MS media at 1/2 macronutrient concentration and supplemented with 0.3 mg/l NAA to induce roots for observing rooting rate, as well as subsequent chromosome determination. In our experiment, the control materials routinely subcultured with 25-day subculture cycle on breeding media supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA, were also incubated in an illuminated chamber under a 16 h photoperiod of 1,500 lx light intensity at 25 ± 1°C.

**Growth recovery of materials preserved in vitro**

The survival materials, preserved for 300 days by the optimal preservation method, were subcultured on the breeding media supplemented with 0.8 mg/l BAP and 0.2 mg/l NAA under a 16 h photoperiod of 1,200 lx light intensity at 25 ± 1°C. After they have been subcultured for three 25-day subculture cycle, the propagation coefficients, leaf length, and leaf width of survival materials were observed. Furthermore, after three 25-day subculture cycle, survival materials were transferred to rooting media consisting of semi-solid MS media at 1/2 macronutrient concentration and supplemented with 0.3 mg/l NAA to induce roots for observing rooting rate, as well as subsequent chromosome determination. In our experiment, the control materials routinely subcultured with 25-day subculture cycle on breeding media supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA, were also incubated in an illuminated chamber under a 16 h photoperiod of 1,500 lx light intensity at 25 ± 1°C.

**Genetic stability determination**

Root tips approximately 0.5 cm in length were excised and pretreated in a 0.2% w/v colchicine solution for 2 h. The root tips were fixed in Carnoy’s fluid (containing 3:1 ethanol and glacial acetic acid) at 3–5°C for 2-24 h, rinsed with 95% (w/v) alcohol, 70% (w/v) alcohol, and distilled water three times, respectively, and then macerated for 16 min with 0.2 M HCl at 60°C. After being soaked in distilled water for 40 min, the fixed root tips were stained with improved Carbol Fuchsin (1.8 g sorbitol dissolved in 10 ml Carbol Fuchsin, then mixed with 45% v/v acetic acid 90 ml). A photomicroscope (Olympus BX 40, Japan) was used for chromosome determination.
RESULTS AND DISCUSSION

Effects of plant growth regulators on preservation in vitro

For many plant species, growth retardants applied to the medium of preservation in vitro are necessary to inhibit growth and delay aging. Pablounat (PP 333), a plant growth inhibitor, works by blocking the oxidation of kaurene to kaurane, different acids inhibit the biosynthesis of plant gibberellin (GA) to delay the growth of plant.[17,18] Studies have demonstrated that PP 333 played an important role in preservation in vitro.[19] From the 1960s, the discovery and identification of ABA,[20] the physiological functions of ABA, such as inhibition of plant cell elongation and division, induction of seed dormancy, and prevention of premature germination, have been constantly revealed.[21] Daminozide (B9), a plant growth retardant, can inhibit the biosynthesis of the plant endogenous hormones to play an important role in regulating plant growth, and promoting strong seedling.[22,23]

In our research, the orthogonal test revealed that the variation of the PP 333 concentration (2,379.00), and the variation of the ABA concentration (900.33) had significant effects on the preservation time [Table 1]. Further analysis showed that the longest preservation time was 133.33 days found at PP 333 concentration of 1.0 mg/l, and 121.33 days found at ABA concentration of 5.0 mg/l, respectively [Table 2]. The effect of B9 on the preservation time was not significant. According to these results, we may draw a conclusion that the best preservation media for buds in vitro of P. multiflorum were the MS media supplemented 1.0 mg/l PP 333, 5.0 mg/l ABA, and 30.0 mg/l B9.

Furthermore, in our experiment, preservation time of three treatments with survival rate of over 70% was over four months (data not presented). Moreover, the survival materials can normally grow in breeding medium supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA in an illuminated chamber under a 16 h photoperiod of 1500 lx light intensity at 25°C.

The K-value is the sum of the preservation time of all tests with the same factor at the same level and the R-value is the difference between the maximum and minimum value of K with the same factor. The K-values and the effects of each level with the same factor are positive correlation. R-values and the effects of each factor are positive correlation.

Effects of temperature on preservation in vitro

Room-temperature preservation and cryopreservation are two methods of germplasm preservation in vitro. Cryopreservation is suitable for long-term preservation,[24] while room-temperature is often used for medium-short-term preservation. Compared with cryopreservation, room-temperature preservation without special equipment is more convenient and cheaper.

In preservation in vitro, appropriate temperature is key to extending the preservation time. Different plants and different genotypes of the same plant have different sensitivity of temperature.[25] It was believed that 0-6°C was suitable for temperate plants to preserve, while 15-25°C was suitable for tropical plants.[26] P. multiflorum is a widely distributed species in China.[1] In our experiment, when buds were preserved in 0°C, 5°C, 15°C, and 25°C, respectively, the preservation time were 211, 192, 173, and 62 days, and the start time of wilt were 188, 169, 154, and 47 days [Table 3]. With the temperature dropping, the growth of materials slowed down until it stopped. Compared with 15°C, when materials were preserved in 0 and 5°C, the prolongation of preservation time was limited, but the equipment conditions and energy consumption correspondingly increased. Because the materials rapidly grew in 25°C, preservation time (62 days) could not meet the objective of medium-short-term preservation in vitro. Therefore, we believed 15°C was the most appropriate temperature to medium-short-term preservation in vitro in our experiment.

When survival materials kept for six months at 15°C were subcultured into breeding media supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA in an illuminated chamber under a 16 h photoperiod of 1500 lx light intensity at 25°C,

| Source of variance | Sum of variance squares | Degree of freedom | Variance | F value | P value |
|--------------------|------------------------|------------------|----------|---------|---------|
| PP333              | 4,758.00               | 2                | 2,379.00 | 63.73*  | 0.01< P< 0.05          |
| ABA                | 1,800.67               | 2                | 900.33   | 24.12*  | 0.01< P< 0.05          |
| B9                 | 98.67                  | 2                | 49.33    | 1.32    | P>0.1                |
| E (error)          | 74.67                  | 2                | 37.33    | 1.00    |         |
| Sum                | 6,732.01               | 8                |          |         |         |

F1-0.01 (2,2)=99.0, F1-0.05 (2,2)=19.0, F1-0.1 (2,2)=9.0, *Significant at P<0.05

| Concentration of phytohormone (mg/l) | Factor | A (PP333) | B (ABA) | C (B9) |
|--------------------------------------|--------|-----------|---------|--------|
| 0.5                                  | 1.0    | 10.0      | K^13=90.33 | K^13=91.00 | K^13=103.33 |
| 1.0                                  | 5.0    | 20.0      | K^23=133.33 | K^23=121.33 | K^23=96.67 |
| 2.0                                  | 10.0   | 30.0      | K^33=80.33  | K^33=91.67  | K^33=104.00 |
| R (range)                            | 53.00  | 30.33     | 7.33     |        |
materials were able to restore growth in 12 days. Then, room temperature (15°C) in vitro preservation of buds of *P. multiflorum* could meet the needs of production with a large number of propagations in short time.

Preservation time was the period with material survival rate of not less than 70%. Start time of wilt was the period from inoculation to the start of browning and death. Growth state was observed with naked eye.

**Effects of osmotic pressure on preservation in vitro**

Increase of medium osmotic pressure can inhibit the materials' in vitro growth to extend the preservation time. When the negative osmotic potential of the medium was increased to result in water stress, cell would difficulty absorb water to diminish metabolism, slow down growth and simultaneously, enzyme activity inhibition retard the cell wall growth to reduced nutrient consumption.[19,20] That medium was supplemented with carbohydrate, such as sucrose, mannitol, and sorbitol, and was a common method to improve the osmotic pressure.[27-30] Sucrose, mannitol, and sorbitol are of hypertonic compounds, which can increase the osmotic pressure of the medium to prevent water absorption and decrease metabolic activity of cell. The orthogonal test revealed that the variation of the sucrose concentration (525.78), and the variation of the mannitol concentration (360.44) had significant effects on the preservation time [Table 4]. Further analysis showed that the longest preservation time was 82.00 days found at sucrose concentration of 40 g/l, and 75.33 days found at mannitol concentration of 20 g/l, respectively [Table 5]. The effect of sorbitol on the preservation time was not significant. According to these results, we may draw a conclusion that the MS medium containing 4% w/v sucrose, 2% w/v mannitol, and 1% w/v sorbitol was most suitable in vitro preservation medium for buds of *P. multiflorum*.

The results of our experiment were in accord with the conclusion that hypertonic compounds can delay the growth of plant reported in many literatures.[29-31] Moreover, The carbohydrate not only adjust the medium osmotic pressure, but also was the main carbon source for the plantlets growth in vitro.

The *K*-value is the sum of the preservation of all tests with the same factor at the same level and the *R*-value is the difference between the maximum and minimum value of *K* with the same factor. The *K*-values and the effects of each level with the same factor are positive correlation. *R*-values and the effects of each factor are positive correlation.

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**Growth recovery and chromosome determination**

Preservation in vitro is an important means for the preservation of plant germplasm resources.[32] The preservation time was extended while the genetic stability of materials was maintained, these were the purpose of germplasm preservation in vitro.[33] According to our experimental results, in vitro buds were inoculated onto MS basal media containing 4% w/v sucrose, 2% w/v mannitol, and 1% w/v sorbitol supplemented with PP 3,33, 1.0 mg/l, ABA 5.0 mg/l, and B9 30.0 mg/l in an illuminated chamber under a 16 h photoperiod of 1500 lx light intensity at 15°C. After 10 months, buds survival rate was over 70%. When the survival materials were inoculated onto the breeding media supplemented with 0.8 mg/l BAP, 0.2 mg/l NAA at 25°C, after three 25-day subcultures, multiplication time of buds, leaf length, and leaf width, rooting rate of survival materials and control materials were not significantly different. The multiplication time of buds of survival materials was 14.21, while that of the control materials was 19.21.

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**Table 3: The results of the experiment in different preservation temperature**

| Temperature/°C | Preservation time/d | Start time of wilt/d | Growth state          |
|---------------|---------------------|----------------------|-----------------------|
| 0             | 211                 | 188                  | Buds stopped growing  |
| 5             | 192                 | 164                  | Buds grew very slow   |
| 15            | 173                 | 154                  | Buds grew slowly      |
| 25            | 62                  | 47                   | Buds grew rapidly     |

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**Table 4: Variance analysis of the preservation time of *P. multiflorum* in vitro buds by an orthogonal test**

| Source of variance | Sum of variance squares | Degree of freedom | Variance | F value | P value |
|--------------------|-------------------------|-------------------|----------|---------|---------|
| Sucrose            | 1,051.56                | 2                 | 525.78 | 45.96*  | <0.05   |
| Mannitol           | 720.89                  | 2                 | 360.44 | 31.51*  | <0.05   |
| Sorbitol           | 37.56                   | 2                 | 18.78  | 1.64    | P>0.1   |
| E (error)          | 22.89                   | 2                 | 11.44  | 1.00    |         |
| Sum                | 1,832.90                | 8                 |         |         |         |

**Table 5: Visual analysis of the preservation time of *P. multiflorum* in vitro buds by an orthogonal test**

| Concentration of phytohormone (g/l) | Factor |
|-------------------------------------|--------|
| **A** (Sucrose)                    | **B** (Mannitol) | **C** (Sorbitol) |
| 5                                   | 5       | 5           |
| 10                                  | 10      | 10          |
| 20                                  | 20      | 20          |

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was 15.67. The leaf length and leave width of survival materials were about 9.2 mm and 8.3 mm, respectively, while the control materials were about 9.6 mm and 8.2 mm. The rooting rate of the survival materials was 93%, while the control material was 91% [Table 6].

In our experiment, the morphological appearance mutation of survival materials in vitro did not occur as compared with the control materials. Microscopic studies confirmed the chromosome number of survival materials to be 22 which was consistent with the literatures [Figure 1].

Compared with the control materials, the chromosome morphology of survival materials appeared normal. According to the growth recovery, the number and morphology of chromosome of the survival materials, buds in vitro of P. multiflorum had a good genetic stability in our experiment.

Leaves were chosen from each of 10 survival materials and each of 10 control materials. Ten stomata were measured for each leaf. Number of samples used for rooting is both 100.

In summary, under a 16 h photoperiod of 1500 lx light intensity, when in vitro single-buds of P. multiflorum inoculated onto MS media containing 4% w/v sucrose, 2% w/v mannitol, and 1% w/v sorbitol supplemented with PP 1.0 mg/l, ABA 5.0 mg/l, and B9 30.0 mg/l were preserved for 10 months in an illuminated chamber at 15°C, survival rate was not less than 70%. Moreover, the survivals had a good growth recovery and genetic stability.

### Table 6: The growth recovery of the survival materials in vitro

| Material                  | Times of buds multiplication | Leaf length (mm) | Leaf width (mm) | Rooting rate (%) |
|---------------------------|-----------------------------|-----------------|-----------------|------------------|
| Control materials         | 15.67                       | 8.2             | 9.6             | 93               |
| Preservation materials    | 14.21                       | 8.3             | 9.2             | 91               |

**Figure 1:** The chromosome of preservation materials in vitro (2n=2x=22; bar: 5.18 x 10-4 cm)

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