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•Research Articles•

Morphological and chemical studies of artificial *Andrographis paniculata* polyploids

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[ABSTRACT] *Andrographis paniculata* (Burm. f.) Nees (AP) is commonly used for the treatment of many infectious diseases and has been cultivated widely in Asian countries, and has been included in United States Pharmacopoeia as a dietary supplement, but the cultivars of *A. paniculata* are not abundant due to its self-pollinated. With the aims to enrich AP resources and provide materials for after breeding we explored the polyploidy induction. Different explants, colchicine concentration, and treatment time were tested. After identification by flow cytometry, eleven polyploid plants with different morphologic traits were obtained. The agronomic traits and andrographolide concentration of the polyploids were improved greatly. One of the polyploids (serial 3-7) was chosen for further study. The traits of the second and third generation polyploids (serial 3-7) were stable. Compared with the normal plants, the seeds (2nd generation) weight increased by 31%, and the andrographolide concentration of the leaves increased by 14% (2nd) and 28% (3rd). In conclusion, AP autopolyploids with different morphologic traits were established successfully for the first time, and the polyploids induction might be effective for crop improvement of AP.

[KEY WORDS] Herba Andrographis; Crop improvement; Autopolyploid; Andrographolide; Cultivar; Colchicine

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Introduction

Artificial induction of polyploids has been long developed and has become a common breeding method to improve the yield and quality of crops. Polyploidy provides increased allelic diversity and heterozygosity and permits generation of novel phenotypic variation. And compared with diploids, polyploids have stronger organs and higher content of some useful metabolites [1-3]. In China, some herbs, such as *Lonicera japonica* and *Isatis indigotica*, their polyploids have been induced for quality and yield improvements [4-5]. Among the reagents used for polyploid induction, colchicine is often used with good results. In the process of induction, using the cold treatment first can promote the agent to seep into the embryo and then transferring explants to heat treatment can promote the chemical reaction of agent [6].

Herba Andrographis (*Andrographis paniculata, AP*) has been widely used in China and other Asian countries for the treatment of many ailments, including fever, cough, tuberculosis, snake bites, active ulcerative colitis, and some animal diseases [7-9]. The therapeutic activities of this plant are mainly attributed to andrographolide, which has been found to have anti-cancer, anti-bacterial, anti-inflammatory, and anti-oxidative effects in target organs such as liver, lung, and bladder [10-13]. And there is a need to improve the yield and the content of effective components to enhance the therapeutic effect and meet the wide demand.

However, the cultivars of AP are not abundant because the plant is self-pollinated. The genetic diversity researches by RAPD have shown that the collected samples from various locations in Thailand or India are likely to belong to the same clusters [14-15], and AP germplasm from various regions in China have poor genetic diversity [16]. Focusing on these issues, many efforts have been put on the optimization of the

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planting technology and application of chemical or biological agents to improve the AP yield and andrographolide content [17]. But no research has been reported about the induction of polyploid AP.

The aims of the work described here were to induce AP polyploids by colchicine and to test the value of the new autopolyploids preliminary through the determination of the agronomic characteristics and chemical ingredients. The traits of second and third generation polyploids were also determined. Our study would provide valuable materials for selection of plants with higher production potential of important diterpene compounds.

Materials and Methods

Plant materials and tissue culture

The seeds of AP used in the present study were purchased from market (Qiaoxu, Guigang, Guangxi, China) and authenticated by Prof. YU Bo-Yang, the corresponding author of this work. The AP seeds were kept underrunning tap water for 30 min, rinsed with distilled water, and then sterilized with 0.1% (*W/V*) solution of mercuric chloride (SCR, Sinopharm Chemical Reagent, Shanghai city, China) for 5 min, followed by five rinses in autoclaved distilled water. The disinfected seeds were inoculated on autoclaved tissue towel moistened with autoclaved tap water in Petri dishes sealed with parafilm.

Seven day-old axenic seedlings served as the explants. After removal of the radicle, the cotyledonary nodes were inoculated in 200-mL glass jars (3 explants/jar) containing Murashige and Skoog [18] medium (0.6% agar, 3% sugar) augmented with 0.2 mg·L^{-1} of BAP (6-benzyl amino purine) and 0.1 mg·L^{-1} of IBA (indolebutyric acid). The pH of the medium was adjusted to 5.8 prior to autoclave at 121 °C for 20 min. The reagents used for tissue culture were purchased from N.S (Nanjing Chemical Reagent, Nanjing, Jiangsu, China) and SCR (Shanghai city, China). All cultures were maintained at 25 ± 1 °C with 60% relative humidity under 2 000 lux with 16 h/8 h light/dark cycles.

Polyploidy induction by colchicine

There were four kinds of treatments, respectively, for the internode, seedlings and seeds.

Test 1: The seven day-old axenic seedlings were treated with colchicine (Xi'an Shanchuan Biotechnology, Xi'an, Shanxi, China) solution for 24 h at 25 ± 1 °C. They were washed thrice with sterilized distilled water, cultured individually on MS medium for seven days, and then transferred to the proliferation medium for subculture.

Test 2: The single nodes were excised from tissue cultures and subsequently incubated in filter-sterilized colchicine solutions at 25 ± 1 °C, either for 2 or 12 h. The treated nodes were then cultured on proliferation medium, allowing the axillary buds to grow out and elongate.

Test 3: The seeds were immersed in filter-sterilized colchicine solution for 24 h at 55 ± 1 °C, and distilled water was used as a control. Soaked seeds were washed three times with sterilized distilled water and cultured on MS medium. The survival rate was determined after two months, and then the cotyledonary nodes were excised and inoculated into proliferation medium.

Test 4: The seeds were pre-soaked at 25 ± 1 °C for 12 h. For four treated groups, the seeds were soaked in colchicine solution at 4 ± 1 °C for several minutes, and then subjected to the fresh colchicine solution at 40 ± 1 °C for several minutes. And the control group was without any treatment. Finally, the seeds were washed with tap water for four h. All the seeds were planted in trays containing a 1 : 3 (vermiculite : peat, *V/V*) mixture substrate and then maintained in culture room (August 2012). The survival rate was determined after two months of culture.

Plant cultivation

For the first generation, when the seventh pair of true leaves had grown, 10 plants from each group were transplanted into 11-cm pots containing substrate (greenorchids, Foshan, Guangzhou, China) and then kept in culture room with natural light (25 ± 2 °C, September 2012 to February 2013) until analysis. The plants that remained in the trays were used in other experiments.

The second generation was planted in greenhouse with garden soil (natural light, natural temperature, April to October, 2013; and then natural light, 20 ± 2 °C, November 2013 to January 2014). The sowed seeds were half of the total harvested seeds. We recorded the seedlings time and the seedlings number, and transplanted them when the plants had have five pairs of true leaves.

The third generation was planted in test field, garden soil, with natural temperature and light from April to November, 2014.

Pesticides and fertilizers were not used in our study.

Flow cytometry analysis

For flow cytometry assays, 200 mg of fresh leaf from each test plant was cut with a scalpel and mixed with 2 mL of Otto's buffer-1 (100 mmol·L^{-1} of citric acid and 0.5% Tween 20; Nanshi, Nanjing, China) [19-20]. The homogenate was passed through a nylon filter (40 μm) and then centrifuged at 1 500 × *g* for 5 min at 4 °C. The supernatant was discarded, and the pellet was re-suspended with 1.5 mL of Otto's buffer-1 and then centrifuged at 1 500 × *g* for 5 min at 4 °C. After the supernatant was discarded, the pellet was re-suspended with 2 mL of Otto's buffer-2 (400 mmol·L^{-1} of $Na_2HPO_4 \cdot 12H_2O$; Nanshi, Nanjing, Jiangsu, China), to which 20 μL of 1 mg·mL^{-1} RNase (Aladdin, Shanghai, China) and 20 μL of 1 mg·mL^{-1} propidium iodide (Aladdin,) were added, and the mixtures were incubated for 30 min at 4 °C, protected from light. For each sample, 20 000 nuclei were analyzed on a flow cytometer (FACSCalibur, BD, Franklin Lakes, NJ, USA). And the data were analyzed using ModFit LT (Verity Software, Topsham, ME, USA). The leaves from plants without treatment were used as control.