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Effects of exogenous salicylic acid on polysaccharides production of *Dendrobium officinale*

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ABSTRACT

Polysaccharides from *Dendrobium officinale* possess unparalleled medicinal value. In order to produce the active polysaccharides from *D. officinale* through tissue culture, the effects of salicylic acid on the accumulation of polysaccharides were investigated. Salicylic acid (SA) was beneficial to increasing the contents of polysaccharides. The highest polysaccharide production occurred on the medium supplied with 100 $\mu mol \cdot L^{-1}$ salicylic acid. After 30 days of culture the production of polysaccharides reached 10.09% and 15.81% by phenol-sulfuric acid method and 3, 5-dinitrosalicylic (DNS) colorimetric method respectively. The major constituent of polysaccharides, glucose and mannose, was determined by pre-column derivatization-HPLC. The results showed that the glucose contents were reduced and mannose contents were increased with different salicylic acid concentration. The ANOVA of the mannose contents demonstrated that among the different SAs concentration was significantly different. The SA application influenced polysaccharide production rather than degradation because the sucrose metabolic enzyme activities were modified by SA. The experiments suggest that salicylic acid could be an effective compound to enhance the production of active polysaccharides from *D. officinale*.

1. Introduction

The genus Dendrobium is one of the largest genera in Orchidaceae (Wood, 2006). Some Dendrobium species are used as traditional Chinese medicine. But the number of plants in the world is steadily declining due to their lower rate of propagation in nature and overexploitation. As a rare and endangered perennial herb endemic to China, *Dendrobium officinale* Kimura et Migo (Orchidaceae) is famous for its unparalleled medicinal value. For its clinical use, *D. officinale* has been applied to nourish the body, clear away heat-evil, benefit the stomach, moisten the lung, relieve cough, promote the production of body fluid and prolong life (Ding et al., 2008; Xiao et al., 2011). Polysaccharides, as the major active constituent and rich in Dendrobium species (Liu et al., 2005), are recently reported to be positive for immunomodulation (Zhao et al., 2007), antioxidant (Luo et al., 2009), antitumor (Wang et al., 2010), inhibiting apoptosis (Lin et al., 2011), and prevention of liver injury and fibrosis (Pan et al., 2012). Therefore, for effective conservation and utilization of *D. officinale* resource, it is necessary to develop a method for the rapid, mass production of polysaccharides. Cell and organ culture possess potential to produce secondary metabolites than to produce a field-grown plant owing to active growth and secondary metabolite biosynthesis in culture within a shorter period.

As an important endogenous signal molecule, salicylic acid (SA) has been proven to be a major component in signal transduction systems which can induce particular enzymes catalyzing biosynthetic reactions in plants to biotic or abiotic stresses and is essential for the development of systemic acquired resistance (Klessig and Malamy, 1994). In the recent years, application of exogenous SA at non-toxic concentrations to plants has been shown to be effective in the regulation number of processes, such as improving productivity and quality (Elwan and El-Hamahmy, 2009), seed germination (Loïc et al., 2006), nutrient uptake and vegetative growth (Shakirova and Sakhabutdinova, 2003), protein synthesis (Hao et al., 2012), chlorophyll synthesis and photosynthesis (Zhao et al., 1995). In addition, SA has been involved in inducing the formation of secondary metabolites in plants, such as polyphenols, alkaloids, quinines, terpenoids, and polypeptides (Nadeem et al., 2012; Masidur et al., 2012). There is no report on the effects of salicylic acid and on the production of polysaccharides in *D. officinale* tissue culture seedlings. Therefore, the objective of this work was to investigate the impact of exogenous salicylic acid on the yield of polysaccharides.

2. Materials and methods

2.1. Plant material, tissue culture seedlings and salicylic acid treatments

The capsules of *D. officinale* were procured from Yandang Mountains, ZheJiang province, China. Healthy seeds of uniform size were selected and surface was sterilized with 5% solution of NaClO for 10 min followed

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by repeated washing with distilled water in ultra clean cabinet. Sterilized seeds were sown into each culture flask containing MS basal medium with 30 $g \cdot L^{-1}$ sucrose and germinated in vitro. After pre-cultivated for 2 months, uniform size seedlings with completely expanded cotyledons were selected and transplanted into new glass containers with 60 seedlings per container containing MS medium. The experiment was carried out under a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1} with a time period of 12/12 (day/night), temperature of 25/25 °C (day/night) and relative humidity of 65–67%.

After 60 days of culture, seedlings with the height of 3–5 cm were transferred to new glass containers with 30 seedlings per container containing MS medium respectively supplemented by a final concentration with 0 (control), 50, 100, 150, 200, and 300 $\mu mol \cdot L^{-1}$ of SA under above conditions. The treatments were arranged with three replicates. Every 6 days, 5 g of fresh seedlings is sampled from every treatment and the seedlings were stored in −70 °C for use.

2.2. Polysaccharide extraction

Polysaccharide extraction was performed followed by the cellulase protocol reported by Zhang et al. (2011) that follows with some modifications. The seedlings were ground to a powder after being dried in an oven (60 °C, 3 days). The crushed powder (40 meshes) of *D. officinale* was extracted with petroleum ether at 70 °C for 1 h thrice and filtered. The residue was hydrolyzed with 0.5% cellulase solution at 50 °C for 2 h, and then further extracted with 90% ethanol at 90 °C for 0.5 h. After each extraction, the solutions were separated from residues through filtration, and then extracted with double-distilled water at 25 °C for 2 h thrice. After combined and concentrated using a rotary evaporator at 55 °C, the above extracts were precipitated by adding fivefold volume of ethanol at 4 °C for 24 h, followed by centrifugation at 4000 rpm for 20 min. The precipitate was lyophilized to yield the crude *D. officinale* polysaccharides (DOP). The DOP was washed successively with ethyl acetate and acetone, and then dissolved in water, and then lyophilized to yield the purified polysaccharides.

2.3. Determination of polysaccharides

The polysaccharide contents of the samples were determined by the phenol-sulfuric acid method (Dubois et al., 1956). The DOP was added with 100 mL of water. The color reaction was initiated by mixing 2 mL of polysaccharide solution with 1 mL of a 10% phenol solution and 5 mL of concentrated sulfuric acid. The reaction mixture was incubated in a boiling water bath for 15 min. After cooling at room temperature, the optical density (O.D.) of the mixture was determined at 490 nm and the total carbohydrate content was calculated with D-glucose as a standard. The contents of polysaccharides were quantified by calibration factor with D-glucose as a standard. The results were expressed as percentage composition with polysaccharides is equivalent to dry powders of seedlings.

Contents of polysaccharides $(\%) = \frac{L \times D \times f \times V}{W} \times 100\%$ where L is the glucose concentration in the sample solution ($mg \cdot mL^{-1}$), D is the dilution factor of sample solution, f is the conversion factor, V is the sample volume (mL), and W is the weight of Dendrobium powder (mg).

2.4. Determination of reducing sugar

Reducing sugar content and total sugar were analyzed by 3, 5-dinitrosalicylic (DNS) colorimetric method (Miller, 1959). For each of the 2 mL of the polysaccharide solution, 6 mL of DNS reagent was added. The mixture was then heated in boiling water for 15 min until the red brown color was developed. Then, the mixture was cooled to room temperature in a water bath. The absorbance was then measured at 520 nm and the concentration of reducing sugars was calculated based on a standard curve obtained with D-glucose. For each of the

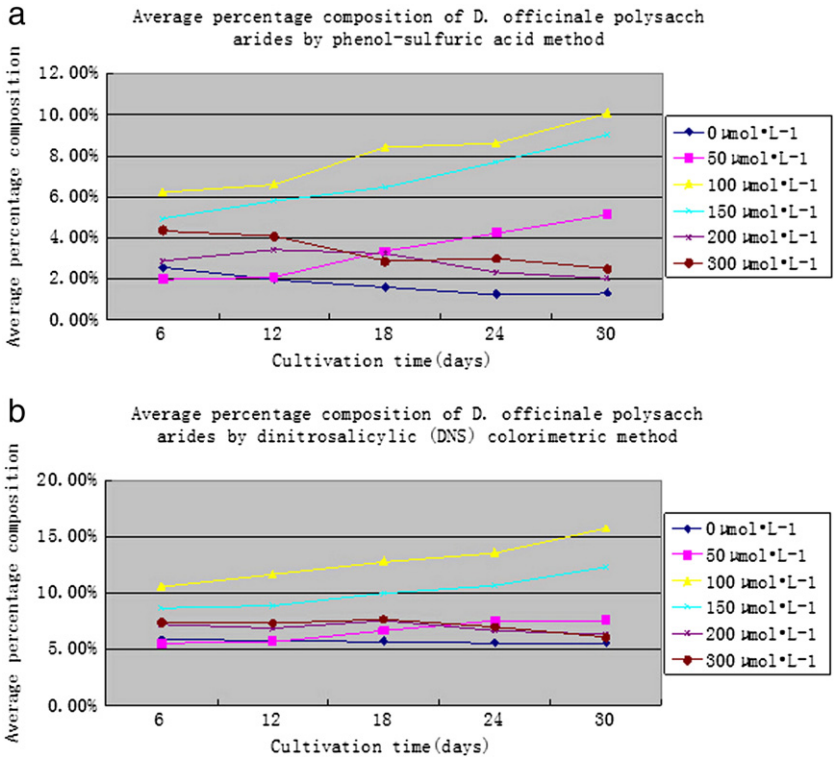

Fig. 1. Effect of different concentrations of salicylic acid on polysaccharide contents by phenol-sulfuric acid method (a) and polysaccharide contents by dinitrosalicylic (DNS) colorimetric method (b).