African Journal of Biotechnology Vol. 9(23), pp. 3437-3442, 7 June, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Stimulation of artemisinin biosynthesis in *Artemisia annua* hairy roots by oligogalacturonides

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Accepted 23 March, 2010

The different fractions of oligogalacturonides (OGA) from polygalacturonic acid by pectinase hydrolysate have been partially purified using column chromatography of Sephadex G-10. The isolated fraction OGA2 (degree of polymerization, DP = 4.57) was found to stimulate the accumulation of artemisinin in *Artemisia annua* hairy roots. When hairy roots of 16-day old cultures were exposed to the OGA elicitor (60 µg/mL) for 4 days, the maximum production of artemisinin reached 11.3 mg/L, a 55.2% increase over the control. OGA could induce H_2O_2 production in hairy root culture as one of early defense events. Moreover, the OGA-induced reactive oxygen species (ROS) were involved in stimulating the artemisinin biosynthesis in the hairy roots. This is the first report on the stimulation of artemisinin production in hairy roots by an oligogalacturonide elicitor.

Keywords: *Artemisia annua*, oligogalacturonide, artemisinin, reactive oxygen species.

INTRODUCTION

Artemisinin, a sesquiterpene lactone with an endoperoxide bridge isolated from *Artemisia annua* L. (Asteraceae), is a potential drug effective against multidrug resistant strains of malarial parasite, *Plasmodium* (Klayman, 1985). Since its chemical synthesis is difficult and expensive, the plant source has been considered an attractive alternative in the production of this secondary metabolite. Artemisinin can be produced in acceptable quantities by differentiated shoot cultures of *A. annua* (Woerdenbag et al., 1993). Weathers et al. (1994) reported artemisinin at 0.4% dry wt in hairy root cultures of *A. annua* that had been transformed with *Agrobacterium rhizogenes*. But the commercial application of large-scale cultures of *A. annua* hairy root is still be hampered by low artemisinin production.

In recent years, several elicitors have been used for stimulation on artemisinin biosynthesis, such as miconazole, chlorocholine chloride (Li et al., 1999), homobrassinolide (Wang et al., 2002) and crude extracts of fungal mycelia of *Verticillium dahliae*, *Rhizopus stolonife and Colletotrichum dematium* (Wang et al., 2000). Oligogalacturonides (OGAs), the pectic fragments released from the plant cell walls, are among the well-known oligosaccharides triggering a variety of defense responses in plants (Reymond et al., 1995; Lu et al., 2008). OGA elicitor also exhibits highly specific activity of inducing secondary metabolite production. Shikonin accumulation in *Lithospermum erythrorhizon* cell cultures was improved via induced synthetic intermediates (geranylhydroquinone and dihydroechinofuran) by OGA (Tani et al., 1993). We reported that accumulations of paclitaxel and 10-deacetyl baccatin III (10-DAB III) in suspension cultures of *Taxus yunnanensis* could be stimulated by the application of OGA (Guo and Wang, 2008). However, presently, there have been no reports regarding OGA elicitation on artemisinin production. In continuation of our characterization of artemisinin elicitation (Wang et al., 2001), we therefore wish to report the preparation of active OGA elicitor and the effect of OGA on artemisinin production in hairy root cultures of *A. annua.*

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Abbreviations: **OGA,** Oligogalacturonides; ROS, reactive oxygen species; MS, Murashige and Skoog medium; **PGA,** polygalacturonic acid; **OGAC,** oligogalacturonic acid crude; **DP,** degree of polymerization; **DPI,** diphenylene iodonium; **HPLC,** high performance liquid chromatography; **FTIR,** fourier transform infrared; **IR,** infrared spectroscopy; **CAT,** catalase.

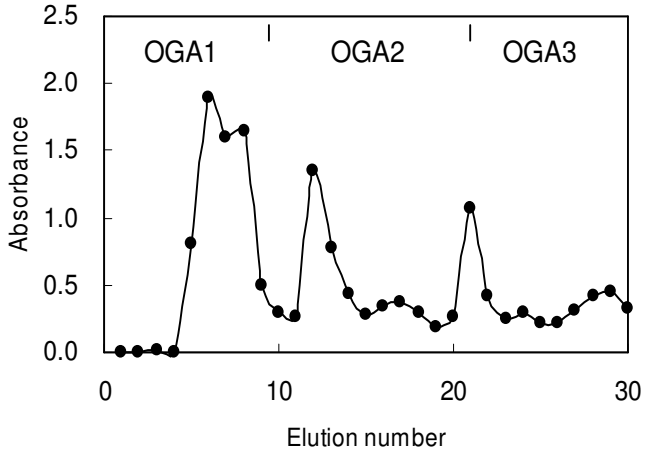

Figure 1. Chromatogram of oligogalacturonides from polygalacturonic acid by enzymatic hydrolysate of pectinase in the column of Sephadex G-10.

MATERIALS AND METHODS

Hairy root culture

Hairy root (HR3) of *A. annua* was used in this study, which was routinely maintained on liquid Murashige and Skoog (1962) medium (MS) with 0.01 mg/l gibberellic acid, 0.5 g/l casein hydrolysate and 3% (w/v) sucrose and grown at 25 - 27°C on a rotary shaker (120 - 130 rpm) under 16 h light (100 µmol photons/m^2 s) per day. The roots were sub-cultured once every two weeks. For experiments in flasks, 2 cm of root apices was taken from 10-days old roots and 0.1 g (fresh weight) hairy roots were incubated in 50 ml liquid MS medium in 250 ml Erlenmeyer flasks.

Preparation of OGA

OGA was prepared from polygalacturonic acid (PGA, Cat. 81325, Fluka) according to the procedure of Hu et al. (2003) with modification. PGA at 50 µg/ml was incubated with 30 µg/ml pectinase (Cat. 17389, Fluka) from *Aspergillus niger* for 20 min at room temperature. Then the solution was boiled for 5 min and filtered. The filtrate was concentrated to give oligogalacturonic acid crude (OGAC). The OGAC was freeze-dried and adjusted to a final concentration of 1 mg/ml. The OGAC was sub-sequently subjected to a Sephadex G-10 (MW ≥ 700, Pharmacia) column. The OGAC sample (10 mL) was placed on the top of the column, eluted with distilled water with a flow rate of 0.25 - 0.35 ml/min and collected for OGA analysis by sulfate-carbazole method (Wang et al., 2006). Then, the effluent was rotary concentrated and sterilized by filtration. The average degree of poly-merization (DP) for OGA as the ratio between the direct reducing sugar and total reducing sugar was determined according to Somogyi method (Sun et al., 2007).

Elicitation and inhibition experiments

For the investigation on the effects of OGA on artemisinin biosynthesis in *A. annua* hairy root cultures, the elicitor at 50 µg/ml was added to 20-days old hairy root cultures for 3 days. Control received the same volume of water only. Then the artemisinin content and biomass of hairy roots were quantified. To evaluate the effects of OGA concen-tration, OGA at 0 – 100 µg/ml was added to 16-days old hairy root cultures for 3 days. All OGA elicitors were filter sterilized using separate 0.22 µm sterile syringe filters before addition into liquid MS medium.

For tests on the effect of reactive oxygen species (ROS), exogenous hydrogen peroxide (H_2O_2) and super-oxide anion ($O_2^{\bullet-}$) generator (xanthine + xanthine oxidase, X + XO) were used as ROS donors. NADPH oxidase inhibitor, diphenylene iodonium (DPI) and a scavenger of H_2O_2, catalase (CAT), were used to pretreat hairy root cultures before the elicitation. They were all prepared at 50 - 100 times of the final concentrations in the culture and sterilized by filtration. The scavenger CAT (1 unit/mL) and NADPH oxidase inhibitor, DPI, (50 µM) were applied to the culture at 30 min prior to the OGA treatment. H_2O_2 at 10 mM and $O_2^{\bullet-}$ generator (0.5 mM X + 1 unit XO) were added at the same time with the OGA application, respectively. Their dosages used in the experiments were chosen based on previous reports (de Pinto et al., 2006).

H_2O_2 generation

H_2O_2 production from elicited hairy roots was measured by chemiluminescence method (Hu et al., 2003). The luminescence counts were read in a luminometer (BPCL Ultra-weak, Institute of Biophysics, Chinese Academy of Sciences, China). The concentration of H_2O_2 in the sample was determined with a calibration curve constructed with standard H_2O_2 solutions.

Artemisinin extraction and analysis

Artemisinin was extracted and quantified according to the modified method of Zhao and Zeng (1985). The *A. annua* hairy roots were harvested, dried in 50°C for 24 h and weighed. Dry tissue (0.5 g) was ground into a fine powder and extracted with petroleum ether (30 ml) by 30 min ultrasonication. The extract was evaporated and dissolved in 2 ml ethanol. After 10 min centrifugation at 3000 g, the supernatant was decanted in 10 ml volumetric flask and dissolved by ethanol. This supernatant (200 µl) with 800 µl ethanol was treated with 0.2% (w/v) NaOH 4 ml at 45°C for 30 min and cooled to room temperature. The solution (500 µl) was acidified with 100 µl ethanol and 0.5 ml acetic acid (0.16 M) and then centrifuged at 3000 g for 10 min before high performance liquid chromatography (HPLC) analysis. The artemisinin reference substances (Sigma) in different concentrations were prepared in the same manner. HPLC analysis conditions were: Waters 600E HPLC system equipped with 150 × 4.6 mm Hypersil BDS C8 column; samples were eluted with methanol/0.01 M Na_2HPO_4-NaH_2PO_4 buffer (pH = 7) (40: 60, v/v) at 1 mL/min and monitored at 260 nm.

RESULTS

Effect of the OGA preparation on artemisinin concentration

After the digestion of PGA solution by pectinase, the obtained OGAC was partially purified by column chroma-tography of Sephadex G-10 into OGA1-3 fractions (Figure 1). The isolated fraction OGA2 (DP 4.57) was revealed to promote artemisinin concentration of *A. annua* hairy roots significantly (Figure 2). Figure 3 gave the fourier transform infrared (FTIR) spectra for the comparison among PGA, OGAC and OGA2. In the infrared spectroscopy (IR) spectrum of OGA2, there was no obvious band change; indicating that the carbohydrate chain structure and the characteristic functional groups of

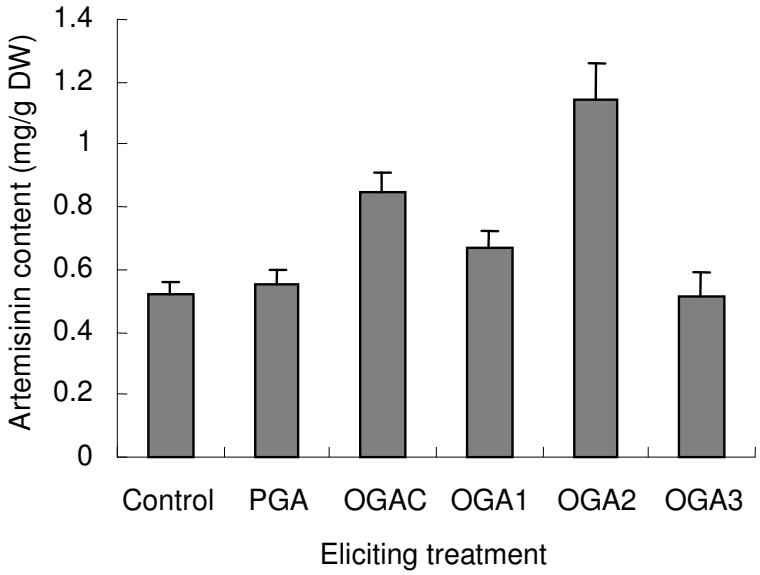

Figure 2. Effects of partially purified oligogalacturonide fractions on artemisinin content in *A. annua* hairy root cultures. The elicitors at 50 μg/mL were added to 20-day-old hairy root cultures for 3 days treatment. Control received the same volume of water only. Values are means of triplicate results and error bars show standard deviations.

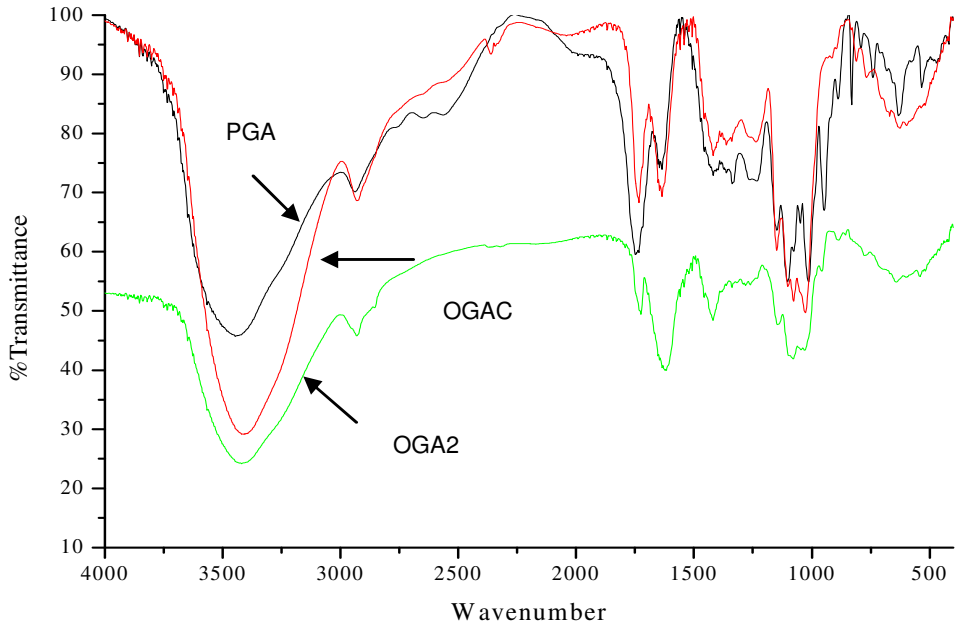

Figure 3. FTIR spectra of polygalacturonic acid (PGA), oligogalacturonide crudes (OGAC) and purified fraction (OGA2).

PGA was not changed after hydrolysis. On the other hand, the intermolecular ester bonds were broken and more and more carboxyl groups exposed during PGA hydrolysis, leading to the increase of the water solubility of OGA2. The fraction of OGA2 was then used as the OGA elicitor for subsequent study.

The effect of OGA dosage and time course of elicitation

When OGA2 at different concentrations were added to 16-days old hairy root cultures for 3 days treatment, artemisinin accumulation in hairy roots was dose-dependent

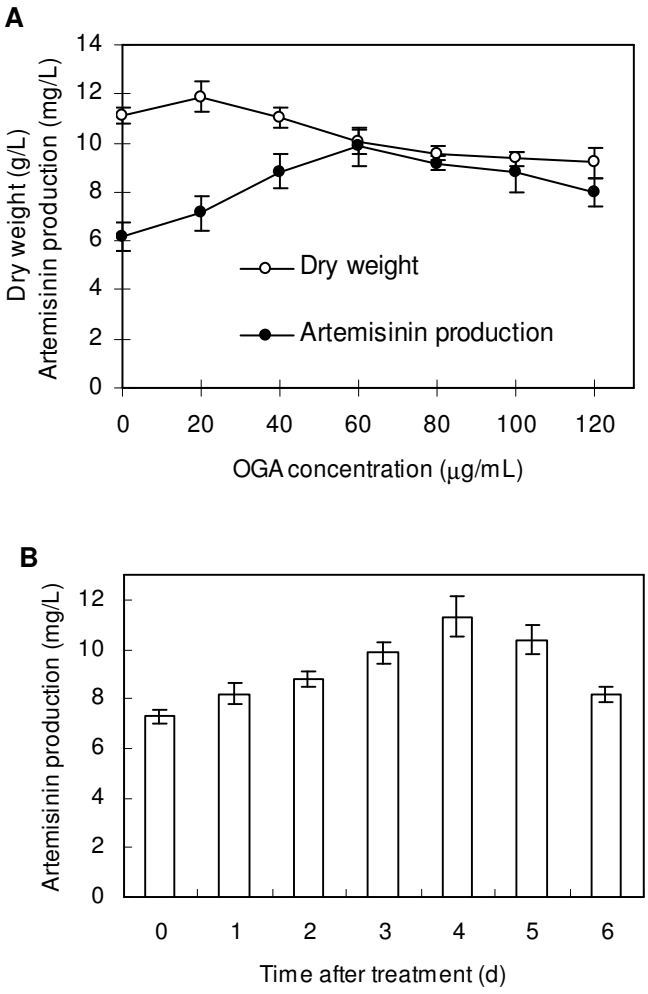

Figure 4. Effects of different concentrations of OGA elicitor (A) and treatment time (B) on artemisinin production in hairy root cultures of *A. annua*. OGA at different concentrations was added to 16-day-old hairy root cultures for 3 days treatment (A). OGA at 60 μg/mL was added to 16-day-old hairy root cultures (B). Control received the same volume of water only. Values are means of triplicate results and error bars show standard deviations.

as shown in Figure 4A. The highest yield of artemisinin (9.8 mg/l) was observed after elicitation with OGA2 at 60 μg/ml which was then used for subsequent study. The time course of the effect of OGA2 on artemisinin accumulation for 6 days is shown in Figure 4B. The increase of artemisinin production in hairy roots was initiated at day 1 and peaked at day 4, reaching a maximum value (11.3 mg/l), 55.2% higher than the non-elicited control.

OGA2-induced oxidative burst and its effect on artemisinin production

The OGA2 treatment induced rapid production of H_2O_2 in

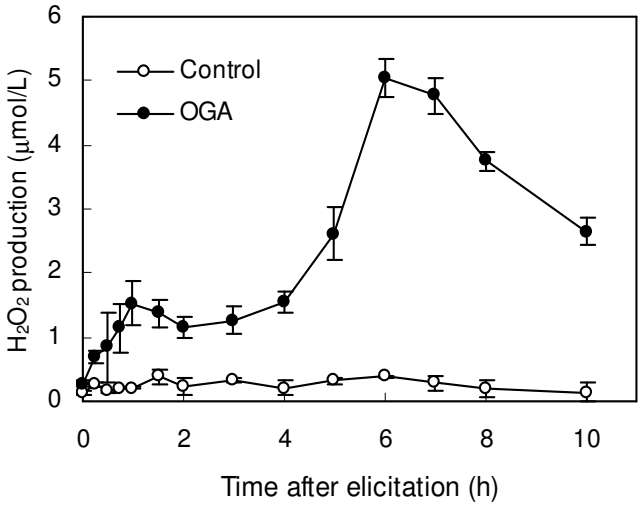

Figure 5. OGA2-induced H_2O_2 generation in *A. annua* hairy root cultures. The same procedure and dosage as specified in Figure 4B. Values are means of triplicate results and error bars show standard deviations.

A. annua hairy root cultures, reaching a peak of 1.5 μM around 1 h and another higher and broad peak (5.1 μM) around 6 h (Figure 5). To test whether OGA2-induced ROS production is required for artemisinin biosynthesis, NADPH oxidase inhibitor DPI and the scavenger of H_2O_2 CAT were used to pretreat hairy root cultures before the elicitation. Exogenous H_2O_2 and $O_2^{\bullet-}$ generator (xanthine + xanthine oxidase, X + XO) were used for investigation on the effect of ROS. As shown in Figure 6, The OGA2 treatment stimulated artemisinin concentration in hairy root, while exogenous H_2O_2 treatment alone could not improve artemisinin production. The low dose of $O_2^{\bullet-}$ (0.5 mM X + 1 unit XO) increased artemisinin content. When OGA2 was applied to root cultures, DPI and CAT treatments all reduced elicitor-induced artemisinin accumulation, suggesting that the OGA-induced artemisinin biosynthesis requires endogenous $O_2^{\bullet-}$.

DISCUSSION

In our previous study, OGA was found to induce taxol and 10-deacetyl baccatin III biosynthesis in *T. yunnanensis* (Guo and Wang, 2008). Our present results showed that OGA can also induce artemisinin biosynthesis in *A. annua* hairy root cultures. Artemisinin is not only an antimalarial drug for human health, it is also known to be very effective against a wide spectrum of microorganisms including blood flukes, protozoa, bacteria, fungi and viruses (Utzinger et al., 2001; Allen and Fetterer, 2002; Brisibe et al., 2008; Efferth et al., 2008) as well as serve as a selective phytotoxin and phytoalexin (DiTomaso and Duke, 1991). The artemisinin synthesis can be induced by crude extracts from pathogenic and endophyte fungal

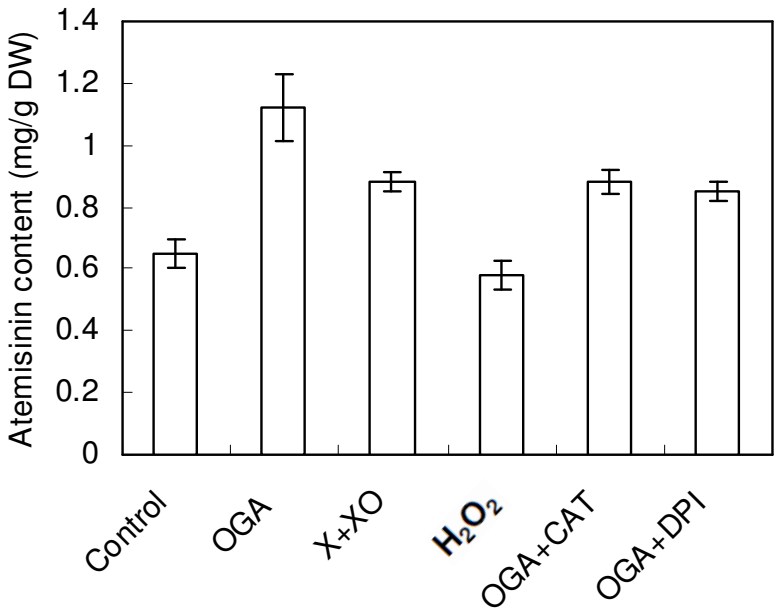

Figure 6. Effect of reactive oxygen species (ROS) on OGA2-induced artemisinin in *A. annua* hairy roots. CAT (1 unit/mL), DPI (50 μM) were applied to the culture at 30 min prior to the OGA treatment. The $O_2^{\bullet-}$ generation system (0.5 mM xanthine + 1 unit xanthine oxidase, X + XO) and H_2O_2 (10 mM) were added simultaneously with the OGA2 application. The same procedure and dosage as specified in Figure 4. Values are means of triplicate results and error bars show standard deviations.

mycelia (Liu et al., 1997; Wang et al., 2001). To our knowledge, this is the first report on the stimulation of the OGA elicitor on artemisinin production. With strain improvement of hairy roots and optimisation of elicitation treatment, the greatly enhanced production of artemisinin could be achieved in hairy root cultures of *A. annua.*

The ROS generation is reported as one of the early OGA-induced defense reactions (RouetMayer et al., 1997; Romani et al., 2004). Hydrogen peroxide and oxygen free radicals can be the second messenger in accelerating the production of salicylic acid, jasmonate acid and ethylene, directly activating the expression of defense response genes (Orozco-Cárdenas et al., 2001). On the other hand, ROS is also a signal for the synthesis of plant defensive secondary metabolites, including glyceollin (Guo et al., 1998), catharanthus roseus (Zhao et al., 2001), indole alkaloids and ginsenoside (Hu et al., 2003). Our present study has also shown that elicitation of artemisinin biosynthesis by OGA is strongly dependent on the OGA-induced ROS production (Figure 6). El-Feraly et al. (1986) converted artemisinic acid to another artemisinin precursor artemisinin B by single oxygen ($^1O_2$) generated through sensitized photo-oxygenation. It has been demonstrated that the oxidation reaction was involved in the biological transformation of dihydro-artemisinic acid to artemisinin (Brown and Sy, 2004). Although the detailed mechanisms for ROS-induced artemisinin by OGA are still unclear and need to be further explored, our study provides new effective strategies such as OGA elicitation for the production of this pharmaceutically important compound by *A. annua* hairy root.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (30772731), the fifth Jiangsu Leading Discipline Program (BU 132802) and Open Funding Project of the State Key Laboratory of Bioreactor Engineering.

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