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Phytosterol of *Potamogeton maackianus* and Its Change under the Stress of *Microcystis aeruginosa*

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Abstract: The phytosterol in *Potamogeton maackianus* was identified and quantified. From the hexane extracts analysis of unsaponifiable fraction of *P. maackianus* by gas chromatography - mass spectrometry(GC-MS), campesterol, stigmasterol and *β*-sitosterol were the main phytosterols in *P. maackianus* with the contents of 0.368, 1.17 and 0.824 mg·g^{-1}, respectively. After 7 days' exposure under *M. aeruginosa*, the contents of campesterol, stigmasterol and *β*-sitosterol in *P.maackianus* increased by 23.1%, 40.4% and 40.8%, respectively. Both the contents of 24-ethyl sterols (sitosterol and stigmasterol) and 24-methyl sterol (campesterol) in macrophytes increased, but the percentage of total sterol composition did not change significantly. This result indicated that the content of 4-demethyl sterols and the second alkylation of the phytosterol side-chain at C-24 in *P. maackianus* were affected by *M. aeruginosa*.

Key words: *Potamogeton maackianus*; *Microcystis aeruginosa*; phytosterol; enviromental stress
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0 Introduction

Plant sterols (phytosterols) are bioactive components of almost all plant species, which resemble cholesterol in vertebrates in terms of both function (to stabilize phospholipid bilayers in plant cell membranes) and structure (steroid nucleus, 3*β*-hydroxyl group, 5, 6 double bond). In plants, more than 200 different types of phytosterols have been reported and the principal plant sterols are campesterol, stigmasterol and *β*-sitosterol[1-3]. The physiological functions of phytosterol on human being are concluded[3] and phytosterols have been used to serving as supplements and as drugs since 1950s[4]. Many bio-activities of phytosterols have already been demonstrated, such as anti-cholesterol[3], anti-cancer properties[5], anti-atherosclerotic[6], anti-inflammatory[7] and anti-oxidative effects[2].

Macrophytes play important roles in the shallow aquatic ecosystem, by not only keeping the water ecosystem stable and clean, but also providing botanic production. Unlike their terrestrial counterparts, all submersed aquatic angiosperms are secondarily adapted for life in water and are used to coexisting with algae in natural water system, especially in eutrophic lakes. Algae would affect the growth and production of submerged plant[8,9]. Many experiments have been performed to evaluate the effects of water bloom on submerged macrophytes and aquatic ecosystem[10-12].

Although some researchers have investigated the effects of algae on the growth and development of submerged macrophytes[8,9,13], there are little information on

the effects of algae on the metabolism and composition of sterols in macrophytes.

Potamogeton maackianus is a kind of submerged macrophytes widely distributing in East Asia[14]. In this study, the phytosterols were identified and quantified in the dry materials of *P. maackianus*. The effects of *Microcystis aeruginosa* on the dynamics of free phytosterols in *P. maackianus* were also studied to understand the reaction of macrophytes to stress of toxic algae on the sterol metabolism.

1 Materials and Methods

1.1 Macrophyte and Phytoplankton Cultures

P. maackianus was collected from the pond of Wuhan Botanical Garden, Chinese Academy of Sciences at Wuhan, China. Plant material was washed free of debris and microorganisms with deionized water. The macrophytes were grown in 30 L aquarium with a quarter of Hoagland medium and illuminated by fluorescent lamps for 16 h (light)∶8 h (dark) cyclically (3 000 lx) at 20 ºC. 1/4 volume of the culture medium in each aquarium was replaced with fresh a quarter of Hoagland medium every two days. Toxic *M. aeruginosa* (FACHB 942) was selected as the experimental algae and was kept in BG-11 medium[15,16].

Prior to the experiment, phytoplankton was transferred into 500 mL Erlenmeyer flasks containing 200 mL a quarter of Hoagland medium, exposed to 16 h(light)∶8 h(dark) illumination cyclically (3 000 lx) at 20 ºC and shaken at 100 r/min untill a stationary state was reached. The growing cells were used as inoculums for coexisting culture experiment.

1.2 Co-culture Experiment

After incubation for 10 days in the laboratory, the macrophytes were transferred into a cubic jar with capacity of 6 liters, in which aquaria (containing 4 L a quarter of Hoagland medium) was at natural biomass concentration of 10 g / L (fresh weight). The algae was also placed in the aquaria at three cell densities (1.0×10^3, 1.0×10^4, 1.0×10^6 mL^{-1}) and plants without algae were used as control. To avoid the competition between optical irradiance and carbon dioxides, the coexistence culture system were cultivated under 3 000 lx with 16 h (light)∶ 8 h (dark) cyclically at 20 ℃ and bacteria-free air was aerated to make up CO_2.

Concentrations of both media (control and treatments) were equal, i.e. a quarter of Hoagland medium. Each treatment was replicated 3 times in a randomized block design. The experiments lasted for 7 days.

1.3 Phytosterols Extraction and Preparation of Unsaponifiable Sample

Reference phytosterols, stigmasterol, β-sitosterol and campesterol were bought from Sigma–Supelco USA Ltd. Methods and procedures applied were essentially those employed by Ham earlier[17].

The plants of each aquarium were taken out, washed by deionized water, frozen-dried, pulverized and weighed after the 7 days coexistence culture was completed. 5 g dry powder was continuously extracted in circumfluence for 6 hours with 300 mL *n*-hexane in Soxhlet extarction apparatus. The crude extracts, after evaporated by means of rotary, were added with 40 mL methanol solution containing 10% KOH, shaken for 15 min, and then heated at 60 ℃ by water bath for reaction. After 1 h reaction, 40 mL water of super purity was added to the medium, which was then extracted with 80 mL ethyl ether for 3 times, the ether layer was dried by anhydrate $NaSO_4$, evaporated, and diluted with *n*-hexane to a constant volume at 25 mL, from which 1 mL solution was taken out and was further diluted with *n*-hexane into 10 mL brown vials subsequently for analysis. The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (GC6890-MS5973, Agilent, Santa Clara, CA, USA). Mass fragments of components were compared with the mass fragmentation data contained in the NIST 02 Database.

GC-MS equipped with HP 7673 autosampler and an Agilent 5973 mass spectrogram detector was employed to analyze the phytosterols. Data acquisition, processing, and instrumental control were performed by Agilent WS software. Sterol samples were separated with a HP-5 MS capillary column (19091S-433, 30 m long, 0.25 mm i.d., with 0.25 μm film, Agilent, Santa Clara, CA, USA). The oven temperature was isothermal at 280 ℃. The injector operated at conditions: injection volume 1 μL, temperature 250 ℃ with the splitless mode. The column temperature was 270 ℃ during the entire run and the transfer line temperature was 280 ℃. Helium (purity of 99.999 5 %) was used as a carrier gas with a flow rate of 1mL/min at an inlet pressure of 4.93×10^4 Pa. Electronic impact (EI) ionization mode mass spectra were obtained at 70 eV and monitored on the full-scan range (*m*/*z* 50-550). Quantitative analysis was carried out using selected ion monitoring (SIM) analysis mode.

1.4 Data Analysis

All analysis was performed three times repeatedly and the results were displayed with the mean value and

standard deviation. Statistical differences between control and treatments were tested using One-way analysis of variance (ANOVA) with SPSS software (13.0) (SPSS Inc., Chicago, IL, USA) at a 95% confidence level.

2 Results

2.1 Identification and Content Analysis of Phytosterols in *Potamogeton maackianus*

Chromatographic analysis of the unsaponifiable fraction of *P. maackianus n*-hexane extracts showed that there were 3 identifiable phytosterol peaks labeled 1 to 3 (Fig.1).

Peak assignments were carried out by comparing their MS fragments with fragmentation data contained in the NIST 02 database and their structures were determined simultaneously by comparing the mass fragmentation patterns and relative retention times with that of reference compounds and literature sources. The peak 1 (retention time 26.77 min) was equivalent to molecule ion value of 400, so that it was determined to be campesterol with spectrum retrieving matching ratio of 99%. The peak 2 (retention time 27.44 min) and 3 (retention time 28.76 min) were identified as stigmasterol and sitosterol, respectively. The structures were shown in Fig.2. The contents of the main three types of phytosterols in *P. maackianus* were showed in Fig.3. The contents of campesterol, stigmasterol and *β*-sitosterol were 0.368, 1.17 and 0.824 $mg \cdot g^{-1}$, and the proportions of individual sterols to the total sterols (2.36 $mg \cdot g^{-1}$) were 15.6%, 49.5% and 34.9%, respectively, in the controlled *P. maackianus*.

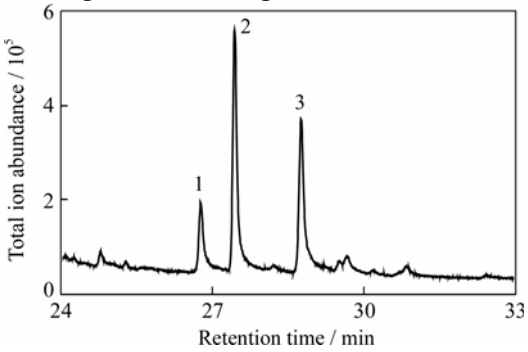

Fig.1 Gas chromatogram of the mass spectrometry detector response of hexane extracts unsaponifiables of *Potamogeton maackianus*

2.2 The Effects of *Microcystis aeruginosa* on Contents of Individual and Total Phytosterols in *Potamogeton maackianus*

As shown in Fig.3, the contents of individual and total sterols both increased under the stress of *M. aeruginosa*. Comparing with the control, the contents of campesterol, stigmasterol, *β*-sitosterol and the total phytosterol in *P. maackianus* treated with algae increased 23.1%, 40.4%, 40.8% and 37.8%, up to 0.453, 1.648, 1.16 and 3.25 $mg \cdot g^{-1}$, respectively. The contents of sterols in the higher density of algae treatment (1.0×10^4 and 1.0×10^6 mL^{-1}) were significantly different to that in control samples ($p<0.05$), which indicated that the contents of 24-ethyl sterols (stigmasterol and sitosterol), 24- methyl sterol (campesterol), and the second alkylation of the phytosterol side-chain at C-24 in *P. maackianus* were affected by *M. aeruginosa*.

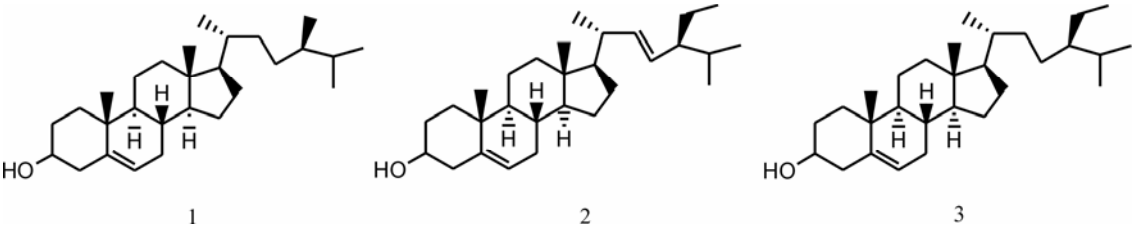

Fig.2 The structures of the main sterols in *Potamogeton maackianus*

1.campesterol; 2.stigmasterol; 3.*β*-sitosterol

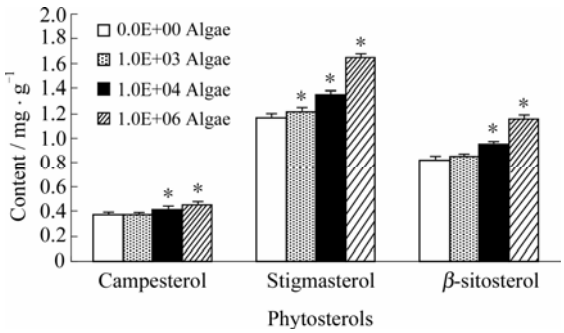

Fig.3 Contents' changes of the individual and total phytosterols in *P. maackianus* under the stress of *M. aeruginosa*

The column is the mean, and the bar is the standard deviation, and * means the significant difference to the control

3 Discussion

This experiment showed that campesterol, stigmas-

terol and *β*-sitosterol were the main forms of phytosterols in *P. maackianus*, which is a kind of typical macrophyte. This result was in accordance with that of terraneous plants[1]. The qualitative and quantitative analysis indicated that stigmasterol was the most abundant sterol component in *P. maackianus*, which had ever been found in *Potamogeton nodosus*[18]. Few quantitative determination of sterol in macrophyte was reported, although there are several reports on phytosterol in macrophytes, such as 10 kinds of sterols from *Zantedeschia aethiopica*[19], a kind of allelopathic sterol from *Typha latifolia*[20] and two kinds of polyhydroxylated sterols from *Ruppia maritime*[21].

The functional foods enriched with plant sterols were developed rapidly since 1950s because phytosterol has ability to decrease the levels of low-density lipoprotein (LDL) cholesterol in serum[4]. Compared with the sterols' contents of crops, which are commonly considered as phytosterol-abundant plant[22,23], *P. maackianus* belongs to the phytosterol-abundant plant species since the content of phytosterol in *P. maackianus* reaches 2.36 $mg \cdot g^{-1}$.

In this paper, there were no significant changes in sterol composition of the macrophyte when it was co-cultured with algae. Stigmasterol, *β*-sitosterol, and campersterol were the main forms of sterols in both control and the treated plants. Compared with the control, the compounds released by the toxic stems of *M. aeruginosa* affected the content of 4-demythel sterol in *P. maackianus*. Along with the algal concentration going up, 24-ethyl sterol (sitosterol and stigmasterol) content in plants increased more than that of 24-methyl sterol (campesterol), moreover, the ratio between campesterol and stigmasterol plus sitosterol decreased from 18.5% to 16.2%, which showed that *M. aeruginosa* affected the second alkyl side chain at C-24 position of plant sterol in the biosynthesis process of sterol *in vivo P. maackianus*[24-27]. The first deteriorative change during stress injury is an alteration in the structure and function of cell membranes, which results in an impaired ability to retain solutes[28]. The structure and function of cell membranes would be influenced by sterols[26]. Many forms of sterols are localized in membranes, but only free sterols play an important role in the permeability of plant membranes. Qualitative and/or quantitative differences in composition of sterol, which was caused by environmental stress, would reflect the relative abilities of plants to regulate membrane permeability to ions, to tolerate or to adapt to environmental stress[25,26,29]. So in this experiment, the increase of sterol content probably revealed that *P. maackianus* could resist algal stress by improving the capacities to regulate the permeability of its cell membranes. There were reports that content of sterol could be increased when treated by toxic compounds at low concentration and could be decreased when treated by high concentration toxic ones[26,30]. It means that the content of free sterol in *P. maackianus* would be affected by the chemicals released from toxic *M. aeruginosa*. Furthermore, the sterols in *P. maackianus* increased under algal stress, but the ratio of sitosterol to stigmasterol kept constant and the percentage composition of the three types of free sterols changed very little in total sterol. Macrophytes could maintain the membrane structure 'more planar' by keeping the percentage composition of sterols[26]. The constant ratio of sitosterol to stigmasterol would contribute to the control of membrane permeability and reducing stress effects.

4 Conclusion

In summary, three sterols have been identified and quantified in *P. maackianus*, which had never been reported before. These three main forms of sterols are stigmasterol, *β*-sitosterol and campersterol. Sterol in *P. maackianus* is abundant with the content reaching 2.36 $mg \cdot g^{-1}$. There were no obvious changes in sterol composition when plant was co-cultured with toxic *M. aeruginosa*, but the contents of the three individual and total sterols of *P. maackianus* increased significantly ($p < 0.05$). It proved that the phytosterol biosynthesis of *P. maackianus* was affected by toxic *M. aeruginosa*. And the constant percentage composition of free sterols in the macrophyte would be one of the resistant mechanisms for macrophyte to keep the membrane structure planar from the stress of toxic algae.

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References

[1] Moreau R A, Whitaker B D, Hicks K B. Phytosterols, Phytostanols, and Their Conjugates in Foods: Structural Diversity, Quantitative Analysis, and Health-Promoting Uses. [J]. *Progress in Lipid Research*, 2002, **41**:457-500.

[2] Berger A, Jones, P J H, Abumweis S S. Plant Sterols: Factors

Affecting Their Efficacy and Safety as Functional Food Ingredients [J]. *Lipids in Health and Disease*, 2004, **3**:5-23.

[3] Kritchevsky D, Chen S C. Phytosterols—Health Benefits and Potential Concerns: A Review [J]. *Nutrition Researc*h, 2005, **25**:413-428.

[4] Pollak O J. Reduction of Blood Cholesterol in Man [J]. *Circulation*, 1953, **7**:702-706.

[5] Awad A B, Fink C S. Phytosterols as Anticancer Dietary Components: Evidence and Mechanism of Action [J]. *Journal of Nutrition*, 2000, **130**:2127-2130.

[6] Nashed B, Yeganeh B, Hayglass K T, *et al*. Antiatherogenic Effects of Dietary Plant Sterols are Associated with Inhibition of Proinflammatory Cytokine Production in Apo E-KO Mice [J]. *Journal of Nutrition*, 2005, **135**:2438-2444.

[7] Okoli C O, Akah P A. Mechanisms of the Anti-Inflammatory Activity of the Leaf Extracts of *Culcasia scandens* P. Beauv (Araceae) [J]. *Pharmacology Biochemistry Behavior,* 2004, **79**: 473-481.

[8] Ozimek T, Pieczyńska E, Hankiewicz A. Effects of Filamentous Algae on Submerged Macrophyte Growth: A Laboratory Experiment [J]. *Aquatic Botany*, 1991, **41**:309-315.

[9] Asaeda T, Sultana M, Manatunge J, *et al*. The Effect of Epiphytic Algae on the Growth and Production of *Potamogeton perfoliatus* L. in Two Light Conditions [J]. *Environmental and Experimental Botany*, 2004, **52**:225-238.

[10] Jupp B P, Spence D H N. Limitations of Macrophytes in a Eutrophic Lake, Loch Leven: I. Effects of Phytoplankton [J]. *Journal of Ecology*, 1977, **65**:175-186.

[11] Bayley S, Stotts V D, Springer P F, *et al*. Changes in Submerged Aquatic Macrophyte Populations at the Head of Chesapeake Bay, 1958-1975 [J]. *Estuaries*, 1978, **1**:73-84.

[12] Carter V, Rybicki N B, Turtor M. Effect of Increasing Photon Irradiance on the Growth of *Vallisneria americana* in the Tidal Potomac River [J]. *Aquatic Botany*, 1996, **54**:337-345.

[13] Chen Kaining, Li Wenchao, Wu Qinglong, *et al*. Impacts of Cycanobacteria on the Growth of Submerged Macrophytes, Dianchi Lake [J]. *Journal of Lake Sciences*, 2003, **15**:364-368 (Ch).

[14] Sun Xiangzhong. *Flora Sinica*, Vol.8[M]. Beijing: Science Press, 1992, 8.

[15] Stanier R N, Kuniswa R, Mandel M, *et al*. Purification and Properties of Unicellar Blue-Green Algae (Order Chroococcales) [J]. *Bacteriology Reviews*, 1971, **35**: 171-201.

[16] Boualcha N, Rlvasseau C, Henniona M C, *et al*. Detection of Cyanobacterial Toxins (microcystins) in Cell Extracts by Micellar Electrokinetic Chromatography [J]. *Journal of Chromatography B*, 1996, **685**:53-57.

[17] Ham B, Butler B, Thionville P. Evaluating the Isolation of Quantification of Sterols in Seed Oils by Solid-Phase Extraction and Capillary Gas-liquid Chromatography [J]. *LC-GC North America*, 2000, **18**: 1174-1181.

[18] Qais N, Mandal M R, Rasgud M A, *et al*. A Furanoid Labdane Diterpene from *Potamogeton nodosus* [J]. *Journal of Natural Products*, 1998, **61**:156-157.

[19] Dellagreca M, Ferrara M, Fiorentina A, *et al*. Antialgal Compounds from *Zantedeschia aethiopica* [J]. *Phytochemistry*, 1998, **49**:1299-1304.

[20] Dellagreca M, Mangoni L, Molinaro A. (20s)-4-α-Methyl-24-Methylenecholest-7-en-3β-ol, An Allelopathic Sterol from *Typha latifolia* [J]. *Phytochemistry*, 1990, **29**:1797-1798.

[21] Dellagreca M , Fiorentino A, Monaco P , *et al*. Two New Polyhydroxylated Sterols from *Ruppia maritima* [J]. *Natural Product Letters*, 2001, **15**:111-118.

[22] Normén L, Bryngelsson S, Johnsson M, *et al*. The Phytosterol Content of Some Cereal Foods Commonly Consumed in Sweden and in the Netherlands [J]. *Journal of Food Composition and Analysis*, 2002, **15**: 693-704.

[23] Lagarda M J, Garéia-Llatas G, Farré R. Analysis of Phytosterols in Foods [J]. *Journal of Pharmaceutical and Biomedical Analysis*, 2006, **41**:1486-1496.

[24] Guo D, Jia Z, Nes W D. Phytosterol Biosynthesis: Isotope Effects Associated with Biomethylation Formation to 24-Alkene Sterol Isomers [J]. *Tetrahedron Letters*, 1996, **37**: 6823-6826.

[25] Khalil I A, Rahman H. Effect of Paclobutrazol on Growth, Chloroplast Pigments and Sterol Biosynthesis of Maize (*Zea mays* L.) [J]. *Plant Science*, 1995, **105**:15-21.

[26] Navari-Izzo F, Izzo R, Bottazzi F. Effects of Water Stress and Salinity on Sterols in *Zea mays* Shoots [J]. *Phytochemistry*, 1988, **27**:3109-3115.

[27] Nes W D, Nichols S D. Phytosterol Biosynthesis Pathway in *Mortierella alpine* [J]. *Phytochemistry*, 2006, **67**: 1716-1721.

[28] Sanchez-Diaz M, Aparicio-Tejo P, Gonzales -Murua C, *et al*. The Effect of NaCl Salinity and Water Stress with Polyethylene Glycol on Nitrogen Fixation, Stomatal Response and Transpiration of *Medicago sativa*, *Trifolium repens* and *Trifolium brachycalycinum* (Subclover) [J]. *Physiologia Plantarum*, 1982, **54**: 361-366.

[29] Cerdon C, Rahier A, Taton M, *et al*. Effect of Diniconazole on Sterol Composition of Roots and Cell Suspension Cultures of Fenugreek [J]. *Phytochemistry*, 1995, **39**: 883-893.

[30] Muchembled J, Sahraoui A L H, Grandmougin -Ferjani A, *et al*. Changes in Lipid Composition of *Blumeria graminis* f.sp. Tritici Conidia Produced on Wheat Leaves Treated with Heptanoyl Salicylic Acid [J]. *Phytochemistry*, 2006, **67**:1104-1109.

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