Plant Biochemistry

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2009

www.studiumpress.com

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***Series Editor*: Dr. S.S. Narwal**

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ISBN: 1-933699-43-4

Published by:

STUDIUM PRESS, LLC
P.O. Box-722200, Houston, Texas-77072, USA
Tel. 713-541-9400; Fax:713-541-9401
E-mail: studiumpress@studiumpress.com

Printed at:

Thomson Press (India) Ltd.

SECTION III

METABOLIC PROCESSES

CHAPTER 19

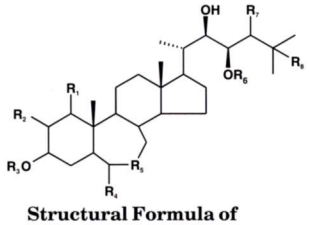
BRASSINOSTEROIDS

JANA SWACZYNOVÁ[1*], ONDREJ NOVAK[1], MIROSLAV STRNAD[1] AND ANNA JANECZKO[2]

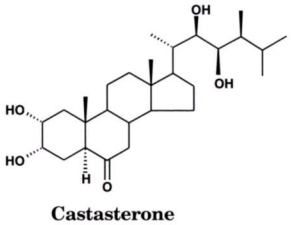
1. INTRODUCTION

Steroid hormones are well-known regulators in mammal organisms, where several classes of these compounds are involved in regulation of carbohydrate metabolism, mineral and water balance, and reproduction processes. The search for plant regulators among groups of steroids resulted in the discovery of brassinosteroids (BR). The first brassinosteroid (brassinolide) was discovered in *Brassica napus* L. pollen in 1979. Until now, more than 60 related compounds have been identified (Fig 1). Similar to steroid hormones in mammals, the structures of BR consist of a sterane skeleton with various attached functional groups which determine their physiological activity (Zullo and Adam, 2002). There are few bioassays to estimate brassinosteroid activity but the most popular is rice leaf lamina inclination (Wada *et al.*, 1984; Kim *et al.*, 1990; Fujioka *et al.*, 1998).

Brassinosteroids stimulate plant growth, influence the effectiveness of photosynthesis and increase protein and carbohydrate content (Khripach *et al.*, 2000). Application of BR can protect plants against stress conditions and increase the quality and yield of different crops (Khripach *et al.*, 2000). Brassinosteroids are natural plant constituents which make them environmental friendly regulators that can be used in agricultural and horticultural production.



Structural Formula of Brassinosteroids



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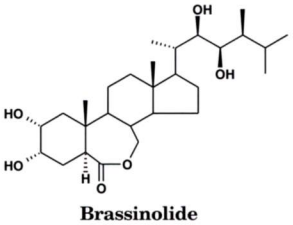

Brassinolide

Fig 1. Chemical structure of brassinosteroids (adapted from Zullo and Adam, 2002)

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2. BIOASSAYS

Experiment 1. Rice lamina inclination bioassay for brassinosteroids

Principle

Rice lamina inclination test is a simple and specific bioassay to evaluate the brassinosteroids activity (Wada *et al.*, 1984; Kim *et al.*, 1990; Fujioka *et al.*, 1998). In this bioassay, explants comprising lamina, lamina joint and leaf sheath are excised from rice seedlings and floated on test solution with BRs. Cells in adaxial side of lamina joint between leaf sheath and lamina are swollen by BRs, causing bending (Fujioka *et al.*, 1998). The external angle between the lamina and its leaf sheath is measured (Fig 2). The angle differ according to chemical structure, concentration and time of exposure to the brassinosteroid assayed (Wada *et al.*, 1984; Kim *et al.*, 1990; Fujioka *et al.*, 1998). Kim *et al.* (1990) modified rice lamina inclination test by using intact seedlings of dwarf rice.

Materials and equipments

Seeds of dwarf rice (*Oryza sativa* L., *i.e.* cv. Tan-ginbozu); glass jar (*i.e.* 26 mm i.d. x 60 mm); microsyringe; microwave oven.

Reagents

Brassinolide; **castasterone**; **96% ethanol**; **1% (w/v) agar medium:** Dissolve 1 g of agar in 100 ml of distilled water, heating it in a microwave oven.

Procedure

(i) Soak seeds of dwarf rice in water at 28 °C for 2 days. Select seeds with a ca. 1-2 cm coleoptile length. Put them on 30 ml of 1% agar medium in a glass jar and incubate under the above conditions for three days.

(ii) Dissolve samples in ethanol 96% and apply 0.5 ml of each sample solution to the top portion of leaf lamina using a microsyringe. Amounts of BR applied should be 0 (control), 0.01, 0.1, 1, 10 and 100 ng/plant.

(iii) After application, plants need to growth 2 days in the same conditions.

(iv) Measure the external angle between the lamina and its leaf sheath using a circular protractor as shown in Fig 2.

Observations

Avoid boiling during heating of agar solution.

Calculations

Collect results of measured angles and calculate means for both tested brassinosteroids and all treatments. Prepare a plot according to example of Fig 2.

Statistical analysis

Calculate standard deviation for each mean.

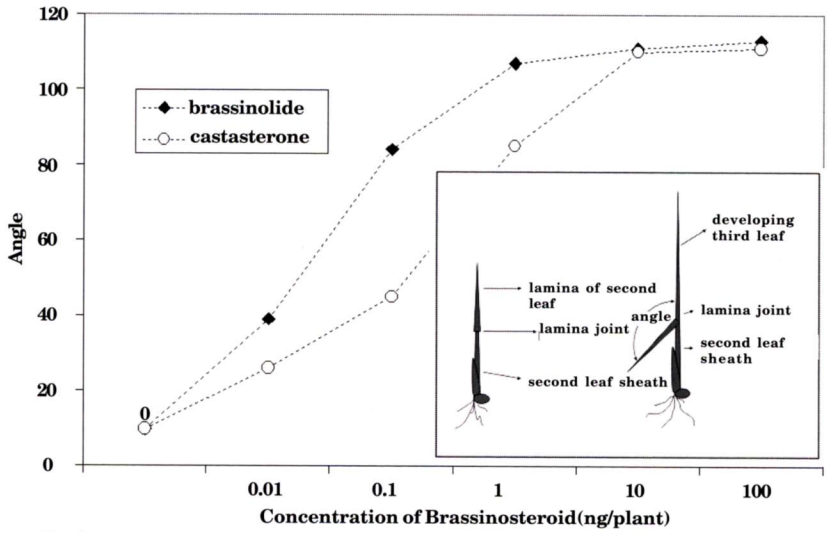

Fig 2. Different biological activity of brassinosteroids in the rice lamina inclination bioassay. Inserted figure shows diagram for the dwarf rice lamina inclination bioassay before and two days after BR treatment (adapted from Kim *et al.*, 1990)

Observations

(i) Germinated seeds selected for the experiment need to be as uniform as possible.

(ii) Use about 30 plants (N=30) per treatment.

(iii) Dwarf rice variety Tan-ginbozu is recommended for this experiment.

3. SAMPLE PREPARATION AND EXTRACTION FROM PLANT MATERIAL

Experiment 2. Extraction and purification of brassinosteroids from plant material

Principle

Brassinosteroids are found in few micro- or nanograms per kilogram of fresh plant tissue, so that purification techniques are quite important before qualitative and quantitative analysis of these hormones. The procedure below combines a method previously described for extraction and purification of endogenous BRs (Yokota *et al.*, 1997) with solid phase extraction (SPE). SPE allows rapid sample preparation. It consists of a solid stationary phase typically packed into a syringe barrel and used to selectively extract, concentrate and purify target analytes prior to analysis by chromatography techniques.

Materials and equipments

Plant material (*i.e.* pollen of oilseed rape, *Brassica napus* L.); 10-d-old seedlings of *Phaseolus vulgaris* L.; or 10-d-old seedlings of *Arabidopsis thaliana* L.; Strata X reversed phase column (33 µm, Phenomenex, Torrance, CA, USA); mortar; glass tubes; evaporator; centrifuge capable 7,100 x g.

Reagents

Cold 80% (v/v) methanol: Add 166 ml of distilled water to 834 ml of 96% methanol. Mix vigorously, then keep in -20 °C; **liquid nitrogen**; **[^{3}H]24-epicastasterone** (1400 Bq, for HPLC-ELISA); **100% acetonitrile**; **100% methanol**; **distilled water**.

Procedure

(I) BR Extraction

(i) Grind freeze-dried plant tissues (ca. 1-2 g) to a fine powder under liquid nitrogen and extract twice in ice-cold 80% (v/v) methanol for 2 hours (10 ml/g FW).

(ii) Add about 1400 Bq (80 000 dpm) of [^{3}H]24-epicastasterone to the extracts, for measure recovery during purification, to facilitate the location of natural BR on the basis of their co-elution with authentic internal standards, and to estimate exact levels of brassinosteroids in plant samples.

(II) BR purification

(i) After centrifugation for 10 minutes at 7,100 g, recover supernatants and purify using a Strata X reversed phase column. Before use, the Strata X sorbent has to be activated with 3 ml 100% methanol and 3 ml of distilled water.

(ii) Elute BRs retained on Strata X with 3 ml of 100% acetonitrile. Dry eluates under vacuum and store at – 20 °C until further analysis.

Observations

Plant material must be frozen in liquid nitrogen after harvest and stored at – 80 °C or freeze-dried.

Experiment 3. Brassinosteroid estimation

Principle

Many different techniques have been developed to estimate BRs in plant tissues. Bioassays are the most widely used as well as different variations of chromatography coupled to mass-spectrometry (GC-MS and HPLC-MS).

The best LC-MS methods allows detection of BRs at subfemtomolar levels (Svatoš *et al.*, 2004). Quantification of BRs by immunoassay is based

upon the competition of BRs found in a sample with a known quantity of labeled or immobilized BR bound to an antibrassinosteroid antibody. The degree of competition is measured using either radiolabeled BRs or enzyme-labeled probes. Although the immunoassay may take multiple forms, all are based upon the availability of high-affinity antibrassinosteroid antisera or monoclonal antibodies. There are also some reports describing the development of polyclonal or monoclonal antibodies against BRs (Yokota *et al.*, 1990; Swaczynová *et al.*, 2007; Khripach *et al.*, 2007). The following procedure describes sensitive and specific methods for the quantification of endogenous BRs combining high performance liquid chromatography (HPLC) with enzyme linked immunosorbent assay based on anti-brassinosteroid antibodies.

Experiment 3.1. Enzyme-linked immunosorbent assay (ELISA)

Materials and equipments

ELISA reader (Titertek Multiscan ® PLUS), microtiter plates (Nunc-Maxisorp), thermostat 37 °C.

Reagents

24-Epicastasterone; tracer solution: 24-epicastasterone-O-(carboxylmethyl) oxime-horseradish peroxidase conjugate; **Anti-24-epiCS-CMO polyclonal antibody (0.66 µg/ml of 50 mM $NaHCO_3$:** Dissolve 1.5 g of Na_2CO_3 and 2.94 g of $NaHCO_3$ in 1 litre of distilled water, adjust pH to 9.6 with HCl or NaOH); **2 M H_2SO_4**; mix 108.8 ml of H_2SO_4 with 891.2 ml of H_2O. **PBS solution:** Dissolve 2.7 g $Na_2HPO_4.12\ H_2O$, 0.3 g of $NaH_2PO_4.10\ H_2O$ and 8.5 g of NaCl in 1 litre of distilled water, adjust pH to 7.2 with HCl or NaOH. **Bovine serum albumin (BSA):** Prepare 0.1% (w/v) BSA in PBS buffer. **PBS-Tween 20:** Prepare 1% (w/v) Tween 20 in PBS buffer. **TMB solution:** Dissolve 0.1 mg 3,3′,5,5′-tetramethylbenzidine (TMB) in 949 µl of distilled water and mix with 50 µl CH_3COONa and with 1 µl 16% (v/v) H_2O_2/distilled water.

Procedure

(i) Coat the wells of microtiter plates with 150 µl of anti-24-epiCS-CMO polyclonal antibody (Swaczynová *et al.* 2006). Incubate overnight at 4 °C for binding and then wash and block twice with 200 µl of 1% PBS-TWEEN 20 buffer per well for 15 minutes.

(ii) After decanting, fill the wells with the following reagents, in the sequence provided: 100 µl of PBS, 50 µl of 24-epicastasterone in PBS and 50 µl of tracer solution (in BSA in PBS buffer). Incubate the plates at 37 °C for 2 hours.

(iii) Remove unbound conjugate by rinsing the plates three times with PBS containing 1% Tween 20.

(iv) Fill the plates with 150 µl of a TMB solution which acts as a substrate.

(v) Stop the reaction, after incubation for 15 minutes at 25 °C, by adding 50 µl of 2 M H_2SO_4. Measure the final absorbance at 450 nm on an ELISA reader.

Observations

(i) All ELISA buffers should be stored at 4 °C in the dark. Do not store the buffers more than 1 month. Use BRs standards, anti-BRs antibodies and BR-HRP conjugates solutions as fresh as possible and store stock solutions of standards, antibodies and conjugates at -20 °C.

(ii) Preparations of BR-BSA and BR-HRP conjugates can be found in Swaczynová *et al.* (2006, 2007).

Calculations

Step 1. Calculate the levels of B/B_0 needed to build calibrations curves:

$$B/B_0 = \{(A_{(B)} - A_{(UB)})/(A_{(B0)} - A_{(UB)})\} * 100\%.$$

where, $A_{(B)}$ is absorbance of standard/sample, $A_{(B0)}$ is absorbance without standard, $A_{(UB)}$ is absorbance with abundance (50 pmol per well) of standard, B and B_0 represent binding of peroxidase tracer in the presence or absence of standard respectively.

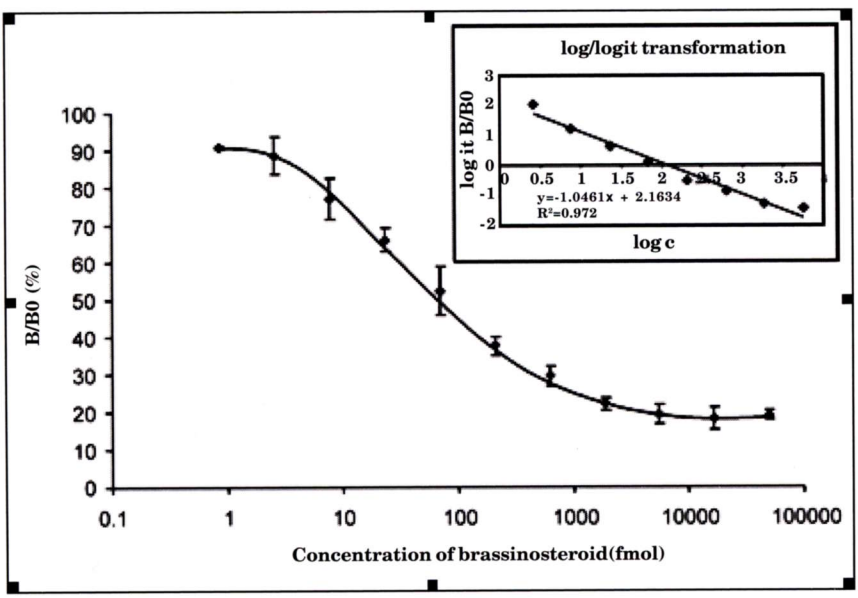

Fig 3. An example of calibration curve obtained for brassinosteroid ELISAs and linearized logit/log plot of the same data (values ± SE)

Step 2. Sigmoid curves for standards and cross-reacting compounds need to be linearized by the log-logit transformation:

$$\text{logit } B/B_0 = \ln \{(B/B_0)/(100\text{-}B/B_0)\}$$

Process the resulting data using the ImmunoRustregAnalyser software developed in-house (Laboratory of Growth Regulators, Olomouc, Czech Republic). Fig 3 shows a typical calibration curve obtained for 24-epibrassinolide ELISAs and linearized logit/log plot of the same data.

Experiment 3.2. HPLC-ELISA protocol

Materials and equipments

The HPLC system (Alliance 2695 Sepatarions module – Waters, USA) linked in series to a PDA 2996 (Watrs,USA) and Frac-100 fraction collector (Pharmacia, Sweden); analytical reversed-phased column (150 mm x 2.1 mm, 5 μm particles; Symmetry C18, Waters); glass vials and inserts; plastic tubes.

Reagents

100% methanol. PBS solution: Dissolve 2.7 g $Na_2HPO_4.12H_2O$, 0.3 g of NaH_2PO_4. 10 H_2O and 8.5 g of NaCl in 1 litre of distilled water, adjust pH to 7.2 with HCl or NaOH; **5 mM formic acid**.

Procedure

(i) Dissolve the samples in 100% methanol and separate 5 μl (25% of the total sample volume) on an analytical reversed-phased column (C18, Waters). The column thermostat should be set at 30 °C. The mobile phase is the following binary gradient of solvent A (methanol) and solvent B (5 mM formic acid) at a flow rate of 0.25 ml/minute: 0 minute, 70% A; 0-12 minutes, 75% A, 12-16 minutes, 100% A (Swaczynová *et al.* 2007). The separation is combined with testing of HPLC-fractionated extracts by ELISA specific for 24-epiCS.

(ii) For the ELISA analysis, collect fractions of 0.5 minutes, evaporate to dryness *in vacuo* and re-dissolve in 15 μl 100% methanol and 185 μl PBS buffer.

(iii) Investigate aliquots of 50 μl in duplicate by scintillation counting and ELISA. Asses content of individual brassinosteroids in the collected fractions using a series of different ELISA analyses including dilution and internal standarization (Weiler, 1982; Badenoch-Jones *et al.*, 1984).

Calculations

See equations in Experiment 3.1, *Calculations*.

The brassinosteroid values obtained by the ELISA of fractions are calculated from the brassinosteroid calibration curves and corrected by the appropriate

recovery values to obtain estimates of BR levels in plant tissues (expressed as BRs equivalents).

Statistical analysis

Prepare at least three (N=3) samples for each plant tissue assayed and calculate standard deviation for each mean.

Suggested Readings

Badenoch-Jones, J., Letham, D.S., Parker, C.W. and Rolf, B.G. 1984. Quantification of cytokinins in biological samples using antibodies against zeatin riboside. *Plant Physiology* **75:** 1117-25.

Fujioka, S., Noguchi, T., Takatsuto, S. and Yoshida, S. 1998 Activity of brassinosteroids in the dwarf rice lamina inclination bioassay. *Phytochemistry* **49:** 1841-48.

Kim, S.-K., Abe, H., Little, C.H.A. and Pharis, R.P. 1990. Identification of two brassinosteroids from the cambial region of scots pine (*Pinus sylverstris*) by gas chromatography-mass spectrometry, after detection using a dwarf rice lamina inclination bioassay. *Plant Physiology* **94:** 1709-13.

Khripach, V.A., Sviridov, O.V., Pryadko, A.G., Litvinovskaya, R.P., Drach, S.V., Matveentsev, V.D., Novik, T.V., Mikhailopulo, K.I., Zhabinskii, V.N., Zavadskaya, M.I., Aver'kova, M.A., Drachenova, O.A. and Chashchina, N.M. 2007. Enzyme Immunoassay of (24*R*)-Brassinosteroids. *Russian Journal of Bioorganic Chemistry* **33:** 347-53.

Khripach, V., Zhabinskii, V. and de Groot, A. 2000. Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for the XXI century. *Annals of Botany* **86:** 441-7.

Svatoš, A., Antonchick, A. and Schneider, B. 2004. Determination of brassinosteroids in the sub-femtomolar range using dansyl-3-aminophenylboronate derivatization and electrospray mass spectrometry. *Rapid Communications in Mass Spectrometry* **18:** 816-21.

Swaczynová, J., Novák, O., Hauserová, E., Funksová, K., Šíša, M. and Strnad, M. 2007. New technologies for estimation of naturally occurring brassinosteroids. *Journal of Plant Growth Regulation* **26:** 1-14.

Swaczynová, J., Šíša, M., Hnilièková, J., Kohout, L. and Strnad, M. 2006. Synthesis, biological, immunological and anticancer properties of a new brassinosteroid ligand. *Polish Journal of Chemistry* **80:** 629-635.

Wada, K., Marumo, S. Abe, H., Morishita, T., Kawamura, K., Uchiyama, M. and Mori, K. 1984. A rice lamina inclination test-A micro-quantitative bioassay for brassinosteroids. *Agricultural and Biological Chemistry* **48:** 719-726.

Weiler, E.W. 1982. An enzyme-immunoassay for cis-(+)-abscisic acid. *Physiologia Plantarum* **54:** 51-514.

Yokota, T., Watanabe, S. and Ogino, Y. 1990. Radioimmunoassay for brassinosteroids and its use for comparative analysis of brassinosteroids in stems and seeds of *Phaseolus vulgaris*. *Journal of Plant Growth Regulation* **9:** 151-9.

Yokota, T., Nomura, T. and Nakayama, M. 1997. Identification of brassinosteroids that appear to be derived from campesterol and cholesterol in tomato shoots. *Plant Cell Physiology* **38:** 1291-94.

Zullo, M.A.T. and Adam, G. 2002. Brassinosteroid phytohormones – structure, bioactivity and application. *Brazilian Journal of Plant Physiology* **14:** 83-121.