

Ascorbic acid induces furanocoumarin production in organ cultures of *Glehnia littoralis*

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Materials and methods

Callus cultures were induced from petiole segments of *Glehnia littoralis* and maintained on a solid Murashige and Skoog (MS) basal medium containing 2,4-D (1 mg/L) and kinetin (0.01 mg/L) at 25°C in the dark [1]. From the callus, cell suspension cultures were obtained and maintained with the same medium for 3-week periods on a reciprocal shaker at 80 rpm. Organ cultures were initiated from the early stage of petiole callus by transferring the calluses to MS medium with or without 0.01 mg/L of 2,4-D, where the calluses produced embryos as well as adventitious roots. The adventitious roots were propagated and maintained in liquid MS medium supplied with 5 mg/L of IBA in the dark. Shoot cultures were also established from the embryos on a solid MS medium containing 1 mg/L of IBA and 3 mg/L of BAP under continuous light (ca. 20 $\mu\text{mol}/\text{m}^2/\text{s}$) and maintained under the same conditions [2].

From preliminary examinations, cultures at the most sensitive stages were used for stress treatments. Cell suspension cultures were separated into cells and medium by vacuum filtration, then 1 g of cells was transferred to 100 mL flasks containing 25 mL of liquid fresh medium mentioned above, and 10-day-old suspension cultures were treated with stress inducers. In the case of root cultures, roots (120 mg) were inoculated in 100 mL flasks containing 25 mL of the same root culture medium and cultured for 10 days prior to stress treatment. In the case of leaves, shoots were divided into leaf and petiole sections with a knife and adequately-developed young leaves were used for the experiments. Isolated leaves (180-200 mg) were inoculated in a 100 mL flask containing 25 mL of liquid MS medium and then pre-incubated overnight for stress treatment.

Furanocoumarins were extracted from 0.2-0.5 g of frozen tissues (cells, leaves and roots) and from the culture medium [2]. Tissues were homogenized with 1.25 mL of methanol and incubated for 1 h at 80°C. The extract was vortexed for 2 min and then centrifuged at 6,600 x g for 10 min, after which the supernatant solution was removed. This procedure was repeated twice and the supernatant solutions were combined. After removal of the methanol by evaporation under vacuum, the residue was dissolved in 1 mL of water and then extracted with 1 mL of ethyl acetate by vortexing for 2 min.

After centrifugation at 6,600 x g for 10 min, the ethyl acetate fraction was collected, the solvent was removed under vacuum, and the residue was dissolved in 1 mL of methanol for HPLC analysis. Culture medium (*ca.* 20 mL) was extracted with 20 mL of ethyl acetate; 10 mL of this was removed and evaporated to dryness under vacuum, and the residue was redissolved in 1 mL of methanol for analysis. 4-Methylumbelliferone (5 mM, 50 μ L) was added to each sample as an internal standard before extraction.

Furanocoumarins were separately quantitated by HPLC (Shimadzu LC-10). A Finepak SIL C18S column (4.6 mm i.d. x 150 mm; Jasco, Japan) was used and the column was eluted with a step gradient of water (A) and acetonitrile (B), starting at 20 % B for 10 min, and followed by 30 %, 35 %, 40 % and 20 % B, respectively, after 10 min intervals. Elution, at a flow rate of 1 mL/min at 40°C, was monitored with a UV-visible detector at 254 nm and a photodiode array detector. Retention times of furanocoumarins under these conditions were as follows: umbelliferone (internal standard), 14.2 min; psoralen, 24.2 min; xanthotoxin, 26.3 min; bergapten, 30.8 min.

References

- ¹Kitamura Y, Ikenaga T, Ooe Y, Hiraoka N, Mizukami H. Induction of furanocoumarin biosynthesis in *Glehnia littoralis* cell suspension cultures by elicitor treatment. *Phytochemistry* 1998; 48: 113-7.
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Figure legends

Figure 1S: Changes of culture-medium pH in response to AsA concentration, monitored over 24 h. AsA solution, adjusted to pH 5.5 with 4M HCl and filter-sterilized, was added to the medium of root- and leaf-organ cultures; 200 μ L aliquots were withdrawn at intervals for determination of pH.

