Secretion of Allelochemicals from the Cultured Suspension Cells of *Marchantia polymorpha*

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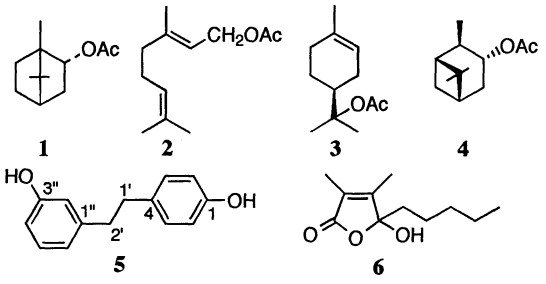
4-[2-(3-Hydroxyphenyl)ethyl]phenol and 4-hydroxy-2, 3-dimethyl-2-nonen-4-olide were isolated from the medium of *Marchantia polymorpha* cell cultures as allelopathic substances.

Many phytopathogenes make higher plants (vascular plants) produce biologically active substances which affect the plant growth, e.g. plant growth regulators, phytoalexins and allelochemicals.[1] Especially, potato phytoalexins, represented by rishitin and phytuberin, have been well known.[2,3] Recently, Murai and his co-workers showed the mechanism of the formation of phytoalexins.[4,5] However, in contrast to the study of the defense mechanism of higher plants, the defense mechanism of lower plants (non vascular plants), e.g. liverworts and algae, are unclearly understood. Question has been raised whether plants such as liverworts have an ability to respond with defense reactions against higher plants. In this paper, we will show the cultured cells of *Marchantia polymorpha* display a defense reaction against monoterpenoids, which are formed in higher plants as secondary metabolites.

The cultured suspension cells (25 g) of *M. polymorpha* were preincubated for 2 weeks in 300-ml conical flasks containing 100 ml of fresh MSK-II medium at 25 °C.[6,7] Monoterpenoids such as bornyl acetate (**1**), geranyl acetate (**2**), α-terpinyl acetate (**3**) and isopinocampheyl acetate (**4**), (125 μmol each) were administered to the flasks. As shown in Table 1, after about 24 h incubation, the cultured cells of *M. polymorpha* turned brown, particularly with bornyl acetate, which was used as stress substrate in subsequent experiments, unless otherwise noted. Growth of the cultured cells on the treatment with bornyl acetate was slower than that of the control cultures. However, microscopic examination revealed that more than 90% cells were alive.



The cultured medium (10000 ml) in the stimulation experiment by bornyl acetate (12.5 mmol) was extracted with ethyl acetate to give an ethyl acetate soluble extract. The crude extract was subjected to column chromatography on silica gel with hexane/ethyl acetate (7:3 v/v) and reversed phase HPLC with water/methanol/2-propanol/acetonitrile (5:1:2:2 v/v) to give 4-[2-(3-hydroxyphenyl)ethyl]phenol (lunularin, **5**)[8,9] (3.2 mg) and 4-hydroxy-2,3-dimethyl-2-nonen-4-olide (**6**)[10,11] (2.7 mg). These compounds were not present in the control

Table 1. Substrate specificity on the browning (A_{600}) and the release of H_2O_2 in the medium of the cultured cells of *M. polymorpha*

Substrate	A_{600}	Relative H_2O_2 concentration (%)
(±)-Bornyl acetate (**1**)	0.680	100
Geranyl acetate (**2**)	0.653	104
(+)-α-Terpinyl acetate (**3**)	0.454	95
(-)-Isopinocampheyl acetate (**4**)	0.461	74
Control	0.255	3

experiment. Lunularin and its derivatives are well known as secondary metabolites of liverworts and are also known to be fungitoxic.[12] 4-Hydroxy-2, 3-dimethyl-2-nonen-4-olide was identified from butter fat[11] and fermented tobacco leaves,[13] but has not been found in liverworts.

In order to clarify that these compounds are secreted in the medium of *M. polymorpha* cell cultures when they are stimulated with bornyl acetate, the time courses of these compounds were followed. Since Murai and his co-workers reported that H_2O_2 was generated before the secretion of phytoalexins,[4] the H_2O_2 concentration in the cultured medium was also examined in our experiments by a method using *N,N*-dimethyl-1,4-phenylenediamine and horseradish peroxidase.[4,14] The result is that H_2O_2 was released from the cultured cells of *M. polymorpha* when stimulated with bornyl acetate. As shown in Figure 1, the H_2O_2 concentration reached its maximum value

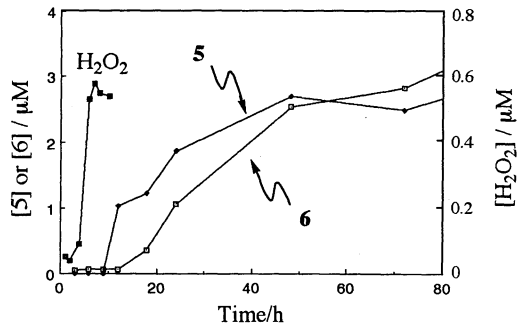


Figure 1. Amounts of H_2O_2 generated from and lunularin (**5**) and 4-hydroxy-2,3-dimethyl-2-nonen-4-olide (**6**) accumulated in the culture medium of *Marchantia polymorpha* cells.

6 h after the bornyl acetate stimulation, and then compounds **5** and **6** were released into the cultured medium. On the other hand, when the cultured cells of *M. polymorpha* were not stimulated with bornyl acetate, the color of the cultured cells was unchanged and no remarkable release into the cultured medium of H_2O_2, compound **5** or **6** was found.

Since compounds **5** and **6** are known as fungal toxins,[12,13] these compounds may play a role as phytoalexins or allelochemicals in *M. polymorpha* cells. Thus, the growth

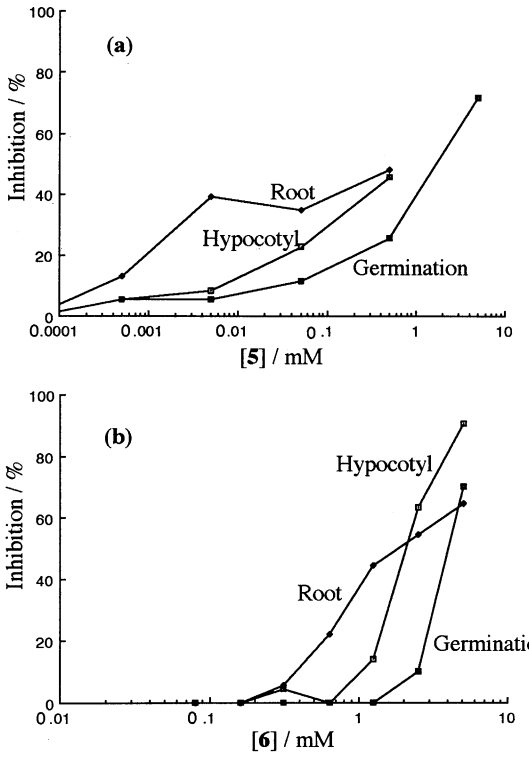


Figure 2. Activities of (**a**) lunularin (**5**) and (**b**) 4-hydroxy-2,3-dimethyl-2-nonen-4-olide (**6**) against germination of *Lactuca sativa* seeds and elongation of their roots and hypocotyls.

regulation activities of these compounds against higher plants were also investigated. Compounds **5** and **6** were synthesized as described in refs. 11 and 15, respectively. Biological activities of compounds **5** and **6** were measured using three different toxin bioassays. The results of the bioassays of *Lactuca sativa* (lettuce) seeds germination and elongation of root and hypocotyl of lettuce are shown in Figure 2. Compounds **5** and **6** were found to heve the inhibitory activities for germination and growth. This result suggests that compounds **5** and **6** cause cell death in higher plants. To clarify the cell toxicity of compounds **5** and **6**, they were administered to the cultured suspension cells (1 g) of *Nicotiana tabacum*. The treated cells were subjected to the phenosafranine staining reaction and then the number of stained cell was monitored with a microscope. As shown in Figure 3, the cultured cells of *N. tabacum* responded to the treatment with compounds **5** and **6** in a dose-dependent manner. Compound **5** showed no activity, but 5 mM of compound **6** induced the complete death of the cultured cells of *N. tabacum*. In the case of cultured cells of *M. polymorpha*, more than 80% of the cells were still alive though.

These results suggest that compounds **5** and **6** may be allelopathic substances, but further works are necessary to clarify their physiological function.

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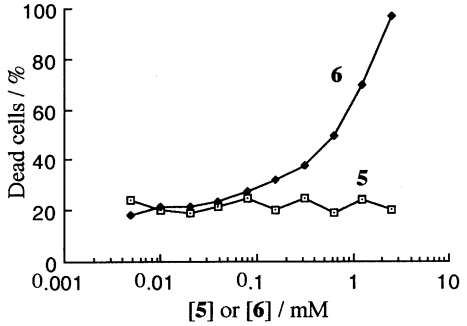

Figure 3. Population of the dead cells of *Nicotiana tabacum* in function of the concentration of added lunularin (**5**) and 4-hydroxy-2,3-dimethyl-2-nonen-4-olide (**6**).

References and Notes

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