한국환경농학회지 제16권 제3호(1997)
Korean Journal of Environmental Agriculture
Vol. 16, No. 3, pp. 264~268

Uptake of Soilmicrobial Metabolites and Allelochemicals in Plant Root System

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식물의 뿌리에 의한 토양 미생물 대사산물 및 Allelochemicals의 흡수

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Abstract : Microbial metabolites from rhizosphere soil samples mainly inhabitated by Streptomyces are selectively uptaken into plants. The culture broth of a Streptomyces strain K9301 showed a major metabolite which disappeared in the medium 24hrs after planting of seedlings. This metabolite was selectively uptaken in the rice plants as well as the wheat plants. We identified the targeted metabolite showing a strong UV-absorbing spot at Rf 0.6 on TLC to be 2-aminobenzamide.

Key words : Allelochemicals, 2-aminobenzamide, sea-urchin

Introduction

Plant-bacteria interactions begin with distant chemical signaling in the rhizosphere. Substanecs interchanged at this stage modulate the early response and set conditions for the initiation of symbiosis. Plants release a variety of organic compounds into the environment by way of leaf leachates, root exudates, and through decomposition of litter. The growth of microorganisms in the rhizosphere can be profoundly controlled by these compounds. These compounds, predominantly phenolic acids, have been shown to be allelopathic to seed germination, seedling growth, and soil bacteria. However, little is known about the other biologically active compounds concerning the relationship between plants and microorganism. Simple phenolic compounds enjoy widespread distribution in nature. It has become evident in recent years that they occur in great variety and often in high concentration in plant tissue[1], microorganisms[2], and soils[3]. A voluminous literature described the chemistry and biochemistry[4,5] and natural distribution of phenolic compounds in plants[6,7]. Soil inhabiting, fluorescent pseudomonas are known to exhibit chemotaxis toward a range of chemical stimuli, including sugars, amino acids, organic acid and aromatic acids[8~11]. Many studies have been focused on the plant nutrition, which deals mainly with uptake of inorganic elements into plants, and rather a small number of research papers have been published on the uptake of organic compounds. We carried out preliminary study to indicate the occurrence of a variety simple phenolics in the microbial metabolites, and the uptake of some of them by plant roots. In this study, we focused on the uptake-selectivity toward simple metabolites of soil-microorganisms, and their physiological effects on the plants.

Materials and Methods

Preparation of microorganisms.

Microoganisms were isolated from soil samples collected from the farm field of Faculty of Agriculture, Okayama University, in the following procedures ;

Soil samples(about 1g) were taken into a tube containing 10ml of sterilize water. After shaking, 1ml of the soil solution was poured into a tube containing 9ml of sterilize water to give a diluted solution(1/10). This solution was then diluted twice in the same way to give a diluted solution(1/1000). A drop

of the finally diluted soil solution was spread on a LB agar plate in a Petri-dish(φ9cm). The plate was incubated at 28℃ for 4 days. Colonies of microoganisms appeared on the plates were observed under a microscope to distinguish the colonies of bacteria, fungi and Streptomycetes. Each of the individual colony of Streptomycetes was transferred to a fresh plate containing the same medium, and incubated under the same condition as described above. The isolated strains of Streptomycetes were cultured on a Bennett's agar slant in a 18mm test tube, and kept in a refrigerator at 4℃. For uptake experiment with rice and wheat, each of the Streptomycetes strains were incubated in a 25mm test tube containing 10ml of Bennett's liquid medium at 26℃ in darkness for 5 days. The Streptomycetes cells and culture broth were separated by centrifugation(5 min.×10000rpm).

Plant materials.

The seeds were sterilized by immersing 70% EtOH for 10min., followed by 5% H_2O_2 for 20min. and rinsed three times with sterilized water. Five surface-sterilized seeds were transferred onto the semisolid medium containing 0.1%(w/v) $MgCl_2$ and 0.2%(w/v) Gelrite in a test tube and incubated in the light at 26℃ for 10 days. The roots of 10-day-old rice seedling were soaked in a test solution of 3ml culture broth(pH 7.0) of Streptomycetes strans in a φ16×100mm test tube in light at 26℃ for 24 hrs. The culture broth without seedlings soaking were used as controls. After 24h, the plants were removed from the test solution and then the resulting solution was partitioned with EtOAc and *n*-BuOH. The EtOAc and *n*-BuOH layers from the solutions were examined by TLC analysis and the constituents of the treated solution were carefully compared with the control. After the treatment, the culture broth of a Streptomycete strain K9301 conatining a constituents which showed Rf value of 0.6 at 254nm on TLC(Benzene : Acetone : MeOH, 7 : 2 : 1) was disappeared. This means that this constituent was uptaken by rice plant. We carried out the uptake experiment in the culture broth of strain K 9301 with wheat plants. The culture broth of a Streptomycete strain K9301 contained a constituent which showed Rf value of 0.6 on TLC(Benzene : Acetone : MeOH, 7 : 2 : 1). After treatment with roots, this spot disappeared, suggesting that this spot was also selectively uptaken by the wheat plant.

Antimicrobial activity and phytotoxicity.

2-aminobenzamide was evaluated for an antimicrobial activity against *Bacillus subtilis* and *Escherichia coli*, a spore germination inhibitory activity against *Aspergillus candidus* and *Cladosporium herbarum*, a cytotoxicity against sea-urchin eggs and seed germination inhibitory activity against *Medicago sativa*. Table 1 indicates the minimum inhibitory concentration(MIC ; μg/ml) of the compound in the biological tests. MK-1 showed antifungal activity against *Aspergillus candidus* and *Cladosporium herbarum* and showed a weak phytotoxicity against *Medicago sativa*.

Table 1. A variety of inhibitory activities of compound MK-1 in some biological tests.

Test organisms	MIC(μg/ml)		
	MK-1	tetracycline	cycloheximide
Bacillus subtilis[a]	>1,000	3	
Escherichia coli[a]	>1,000	6	
Aspergillus candidus[b]	>1,000		25
Cladosporium herbarum[b]	>1,000		12
Sea urchin eggs	100		
Medicago sativa	500		

[a]Dilution method
[b]Spore germination tests

Seed germination inhibition test for Medicago sativa.

Various amounts of the samples in methanol were applied to paper disks(18mm O.D.) and dried in vacum. The paper disks were transferred to small beakers(20mm O.D.) and 200μℓ of sterilized H_2O was added. Seven seeds of Medicago sativa soaked in advance for 12 hrs in sterilized H_2O were then placed on each disk. After 58 hr incubation at 25℃, the seed germination was checked and the MIC was determined by comparison with the control.

Assay of Cytotoxic Activity.

Sexually matured sea urchin(*Hemicentrotus pulcherrimus*) was collected during the breeding season (January-March) from the coastal water near Ushimado Marine Laboratory in Okayama Prefecture. The eggs and sperm were obtained by KCl-shedding. The sperm shaded from the genital papilla was collected with the glass capillary and stored in a

refrigerator(4℃). Eggs were agitated and left for 3 minutes. The eggs near the surface and the bottom were removed by decantation and suction. This process promised better fertilization and synchronous development of the eggs. Throughout this experiments, more than 90% of fertilization rate was obtained. Eggs and sperm thus obtained were used for following experiments.

The eggs(*ca.* 4×10^3 eggs) were inseminated in the sperm suspension(1ml, *ca.* 1000×dilution of dry sperm). The cytotoxic activity of the plant extract and every fraction in the course of separation was measured by adding one drop of fertilized eggs(c.a 100 eggs) of *H. pulcherrimus* to a serially diluted sample solutions in a flat bottom 96-well microplate. The plate was then incubated at 18℃. The initial concentration used was 1,000μg/ml. After 90 minutes, the inhibition of embryonic development at the first cell division was examined using a phase-contrast microscope as described before[6].

Antimicrobial Assay.

Antimicrobial tests against bacteria and fungi were performed by the two-fold dilution method described by Kobayashi *et al.*[6].

Results

Extraction and isolation of chemical compounds.

Clear difference was seen between the original broth and the broth treated with plant. The strong UV-absorbing spot at Rf 0.6 disappeared in the treated fraction, suggesting that this spot was selectively taken into the rice and wheat plants. Repeated column chromatography with silica gel and ODS achieved the isolation of the targeted compound. The compound MK-1 has a strong fluorescence on silica gel TLC under UV light(360nm).

The EIMS spectral analysis of the compound gave a molecular ion at m/z 136. The fragment ions m/z 119(M-17) and 102(M-34) suggested the presence of two amino groups. The ^{1}H-NMR spectrum suggested the presence of ortho-substituted benzene. The IR spectrum suggested the presence of carbonyl group. Based on these spectral data, MK-1 was identified with 2-aminobenzamide(Fig. 1).

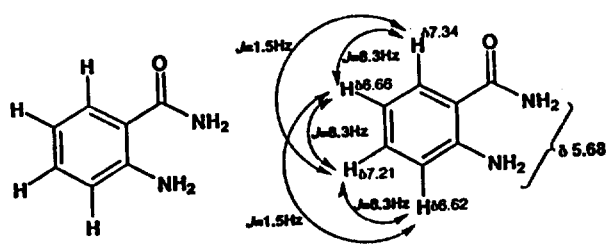

Fig. 1. Chemical structure of MK-1.

Structural determination of 2-aminobenzamide.

Compound MK-1 was identified as 2-aminobenzamide(Fig. 1). The spectral data are shown as below ;

EIMS(direct inlet.) 70 eV m/z(rel. int.) : 136 [M^+], 119(17), 92(44).

^{1}H NMR(500MHz, $CDCl_3$) : 5.68(4H, b), 6.62 (1H, t, J=8.0Hz), 6.66(1H, d, J=8.3Hz), 7.21(1H, t, J=8.3Hz), 7.34(1H, d, J=8.0 Hz).

FT-IR ν max(KBr)cm^{-1} : 3197-3412, 2768, 16 59, 1628, 1609, 1588, 1545, 1496, 1405, 1124, 755, 631.

UV ν max(MeOH) nm(ε) : 224(10,300), 38(3,800).
(MeOH+HCl) : 248(9,250), 330(5,600).

Antibacterial Assay.

Escherichi coli and *Bacillus subtilis* were used in this bioassay. The bacteria were precultured in 10ml of nutrient broth medium for 12 hrs at 27℃ on a shaker, and then diluted 100-fold with the same medium. Two hundred microliters of liquid cultures of bacteria containing various concentrations of serially diluted test materials(1μg/ml~1000μg/ml) were placed in the wells of a round bottom 96-well microplate and incubated at 27℃ for 24 hrs. The growth of bacteria was evaluated by the degree of turbidity of the culture with the naked eye. The MIC values were determined by comparison with the control. In the controls, tetracycline inhibited the growth of *B. subtilis* and *E. coli* at MICs of 3μg/ml and 6μg/ml, respectively.

Antifungal Assay.

Aspergillus candidus and *Cladosporium herbarum* were used in the spore germination test. Fungi were inoculated into 10ml of a potato-malt-extract-sucrose agar medium, and incubated at 27℃ for seven days to form a well-expended fungal mat with spores. These spores were collected by filtration and suspe-

nded in 50ml of a medium(0.2% glucose, 0.1% yeast extract, 0.1% citric acid, and 0.37% $Na_2HPO_4.12\ H_2O$). Two hundred microliters of the spore suspension of fungi containing various concentrations of serially diluted test materials(1μg/ml~1000μg/ml) were placed in the wells of a flat bottom 96-well microplate and incubated at 27℃ for 24 hrs. The spore germination was examined under a microscope. The MIC values were determined by the comparison with the control. In the control, cycloheximide suppressed the spore germination of *A. candidus* and *C. herbarum* at MICs of 25μg/ml and 12.5 μg/ml, respectively.

Biological Activities of the Compound MK-1.

Compound MK-1 was subjected to various biological tests such as a cytotoxicity test with sea urchin eggs, an antimicrobial test with *Escherichia coli* and *Bacillus subtilis* and a spore germination test with *Aspergillus candidus* and *Cladosporium herbarum* and seed germination inhibition test with *Medicago sativa*. Table 1 indicates the minimum inhibitory concentration(MIC, μg/ml) of MK-1 in the 4 biological tests mentioned above. Compound MK-1 prevented the first cleavage of fertilized *Hemicentrotus pulcherrimus* eggs at an MIC of 100μg/ml. However, compound MK-1 was not active against bacteria and fungi.

2-aminobenzamide uptake in wheat plant.

Wheat seeds were used for the uptake experiment. The seeds were peeled and sterilized by immersing in 70% EtOH for 8 min, followed by treatment with 5% H_2O_2 for 15 min. The seeds thus treated were rinsed three times with sterilized water. Five surface-sterilized seeds were transferred onto a medium containing 0.1%(w/v) $MgCl_2$ and 0.2% (w/v) Gelrite in test tubes and incubated in light at 20℃ for 8 days. The roots of 8-day-old plants were soaked into a test solution containing 500μg/ml of 2-aminobenzamide at 20℃ for 24h. The plants were removed from the test solutions and extracted with 50% aqueous MeOH. These extracts were subjected to HPLC analysis under the following conditions ;

HPLC(L-6200 Intelligent Pump ; L-4200 UV-VIS Detector ; AS-2000 Autosampler ; L-5020 Column Oven ; D-2500 Chromato-Integrator ; HITACHI, Tokyo) with Inertsil ODS(φ4.6×250mm ; GL Sciences, Tokyo) and a flow rate of 0.6ml/min. A linear gradient of 30% MeOH containing 1% AcOH to 90% MeOH containing 1% AcOH in 45 min. was employed. Fig. 2 shows HPLC chromatogram of MeOH-H_2O extract obtained from the wheat plant harvested 24 hrs after 2-aminobenzamide treatment. Several unidentified compounds were induced in the feeding experiment, but have not yet identified.

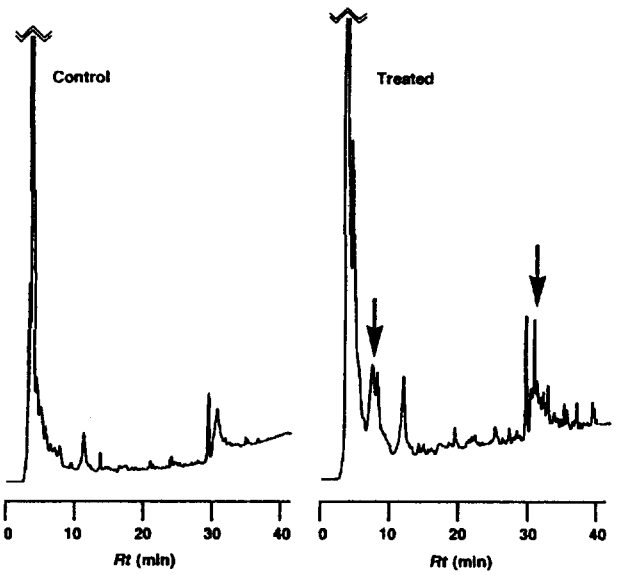

Fig.2 HPLC profiles of unidentified compounds induced in rice plants treated with 2-aminobenzamide(500μg/ml) for 24h.
Rt, retention time

Discussion

We are interested in compounds taken by plants which affect not only plant physiology but also microflora in the rhizosphere. In this study, we found that the root of higher plants exuded amino acid and sugars in response to the stimulating factors like phenolics. Amino acids and sugars might stimulate the growth of microorganisms in soil, to form favorable rhizosphere for healthy plant growth. Further studies on the compounds affecting plant-microbe interactions will provided us with another interesting information about a complicated phenomenon occurring in rhizosphere.

We focused on microbial metabolites which are selectively uptaken into plant. Firstly, Streptomycetes spp. were isolated from collected soil samples in rhizosphere. The culture broth of Stereptomycetes strain K9301 showed a major metabolite which disappeared in the medium 24 hrs after planting

of seedlings. This suggested that this metabolite was selectively taken in the rice plants as well as the wheat plants. We identified the targeted metabolite showing a strong UV-absorbing spot at Rf 0.6 on TLC to be 2-aminobenzamide. This compound or related compounds might play an important role in the metabolism and rhizosphere of rice plant/wheat plant. However, the significance of the compounds selectively taken up by the plant is not thoroughly investigated. This compound itself has no biological significance in appearance.

A survey of the literature reveals a variety of compounds exuded from roots. These include sugars, amino acids, enzymes, vitamins, organic acids, nucleotides, fungal stimulators, inhibitors and attractants, and eelworm hatching and attracting factors[8]. On TLC plate sprayed with diphenylamine and nynhydrin, we found several spots in the solution applied and identified these spots to be amino acids and sugars. 2-aminobenzamide noticeably induced amino acids in a remarkable amount(data not shown). At the same time, we measured sugar content in the medium. The result that 2-aminobenzamide induced the exudation of much sugars is a noteworthy finding(data not shown). These findings suggest that plants may take up the external compounds from the roots and use them positively as allelochemicals.

요 약

식물이 토양미생물 유래 유기화합물의 선택적인 흡수에 주목하여 근권주위의 토양시료로부터 방선균을 중심으로 분리하였다. 방선균 K9301주 배양액에 식물유묘를 24시간 처리후하였다. 처리후의 배양액중의 하나의 대사산물이 소식됨을 알수 있었다. 이 대사산물은 벼뿐만아니라 밀에서도 선택적으로 흡수 되었다. TLC상의 Rf 0.6의 대사산물 spot를 분리하였으며 2-aminobenzamide로 동정되었다.

References

1. Barber D.A..(1968). Microorganisms and the inorganic nutrition of higher plants. *Annual Review of Physiology* **19** : 71~88.
2. Bockers M., Rivero C., Thiede B., Jankowski T., and Schmidt B.(1994). Uptake, translocation, and metabolism of 3,4-dichloroaniline in soybean and wheat plants. *Z. Naturforsch.* **49c** : 719~726.
3. Fried M. and Shapiro R.E.(1961). Soil-plant relationships in ion uptake. *Annual Review of Plant Physiology* **12** : 91~112.
4. Glass A.D.M. and Dunlop J.(1974). Influence of phenolic acids ion uptake. *Plant Physiol.* **54** : 855~858.
5. Glass A.D.M. and Bohm B.A.(1971). The uptake of simple phenols by barley roots. *Planta(Berl.).* **100** : 93~105.
6. Kobayashi A., Kajiyama S., Inawaka K., Kanzaki H., and Kawazu K.(1994). Nostodione A, a novel mitotic spindle poison from a blue-green alga *Nostoc commune. Z. Naturforsch.* **49c** : 464~470.
7. McFarlane J.C., Pfleeger T., and Fletcher J.(1987). Transpiration effect on the uptake and distribution of bromacil, nitrobenzene, and phenol in soybean plants. *J. Environ. Qual.* **16**(4) : 372~376.
8. Perez F.J. and Nunez J.O.(1991). Root exudates of wild oats : allelopathic effect on spring wheat. *Phytochemistry.* **30** : 2199~2202.
9. Quastel J.H.(1965). Soil metabolism. *Annual Review of Plant Physiology* **16** : 217~204.
10. Jones D.L., Edwards A.C., Donachie K. and Darrah P.R.(1994). Role of ptoteinaceous amino acids released in root exudates in nutrient acquisition from the rhizosphere. *Plant and Soil.* **158** : 183~192.
11. Kobayashi A., Kim M.J. and Kawazu K.(1996). Uptake and exudation of phenolic compounds by wheat and antimicrobial components of the root exudate. *Z. Naturforsch.* **51c** : 527~533.