Studies on the metabolic regulation of denitrifying bacteria and phytopathogenic microorganisms using chemical agents found in chemical ecology-based phenomena

Yasuyuki Hashidoko

Research Faculty of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita-ku, Sapporo 060–8589, Japan

(Accepted October 16, 2017)

An interaction between two different living creatures is often mediated by a chemical substance, along with metabolic or morphological differentiation. Such phenomenon-based investigation of chemical substances sometimes leads to the discovery of a novel signaling substance associated with biological pest control, including pinpoint regulation of fundamental metabolisms. In studies on the metabolic regulation of denitrifying bacteria and phytopathogenic microorganisms, such chemicals linked to the introduction of new ideas and unique approaches for biorational pest controls are described. © Pesticide Science Society of Japan

Keywords: biocontrol agent, anti-bacterial rice seedling blight, tropolone biosynthetic inhibitor, excessive hyphal branching factor, sporulation-inducing factor.

Introduction

In terrestrial ecosystems, diverse living creatures, including plants, animals, and microbes, form a complicated network via communication signals. This two-way communication involves many regulation systems to stabilize such ecosystems with recognition, acceptance, and harmonization, while suppression, exclusion, and detoxification against invasive living creatures also function in the ecosystem. An academic research field to understand the essence of biological and ecological phenomena via chemical signals that mediate interactions between or among different living creatures is called “Chemical Ecology” or “Ecological Chemistry.” Many famous and lesser known scientists and agronomists have discovered techniques to avoid continuous cropping hazard and for effective crop protection against several pests, including herbivorous insects, pathogenic microorganisms, and weeds. Professor J. B. Harborne has established the research field of Chemical Ecology as a section of modern science by publishing “Introduction to Ecological Biochemistry” 1977 (Japanese language version published in 1981, translated by E. Takahashi and H. Fukami). As shown in this book, biologically active chemical substances were searched for, and the signaling factors dominating biological and ecological phenomena are addressed as a chemical tool for integrated pest management (IPM). Such chemical signals have long been proposed for “biorational pest control” to regulate the action, response, life cycle, and metabolism of pest organisms, and these approaches are also linked to new pesticide development.

In our laboratory, antagonistic microorganisms that can control intractable phytopathogens or soil microorganisms including N₂O emitters were researched, and the chemical principle(s) produced by the antagonistic microorganisms were investigated in association with pest management using biological agents. To control N₂O emitters in farmland soils, we constructed new culturing bioassay systems using a soil-mimicking gellan gum soft gel medium or the farmland soil itself. Using this bioassay, characteristic secondary metabolites of green manure, or widely used herbicides and insecticides, particularly those of electron transport inhibitors, were searched for powerful N₂O emission-regulative agents. In addition, we have chased and purified a Bacillus sporulation–inducing factor from methanolic extracts of okara and determined its chemical structure. In this review, I wish to introduce a part of our studies and describe the importance of developing a new bioassay system to search for effective chemical/biological controlling agents for practical use. Candidates for such biological control agents attracted us by their unique response to naturally occurring problematic conditions as well as those artificially prepared. I will also introduce our current studies on microbial control via the naturally occurring and artificial chemical substances listed in Fig. 1.
1. Essence of an ideal biological pesticide and new biological resources for antagonistic agents

Many attempts to utilize antagonistic microorganisms have been made due to great need for IPM. However, such antagonistic microorganisms are limited in their practical use due to the long period required for settlement and low survival in infected areas of plants and to the production of antipathogenic metabolites against competitive phytopathogens in vitro. Thus, many antagonistic, antibiotic-producing microorganisms selected by in vitro assay were often ineffective against plant pathogens because of rapid exclusion as an alien from the microbial communities or because their growth was retarded or suppressed by pathogens and native community members. What, then, are the most appropriate natures or behaviors for practical and effective antagonistic microorganisms in field or farmland use?

Of course, a remarkable antagonistic activity against phytopathogens and a high ability to produce antagonistic chemical substances in vitro are the basic properties required for biological control agents. However, in the micro-ecosystems of the rhizosphere, more important qualities of biological control agents raised for the practical suppression of symptom development in farmlands are 1) colonizing and surviving abilities in the rhizosphere, rhizoplane, or phylosphere; 2) insusceptibility to toxins produced by phytopathogens; and 3) a harmless or even growth-promoting nature with regard to host plants. Particularly, 1) and 2) have often been overlooked during the screening process for biological control agents.

In fact, many observations have indicated that certain plants show high acceptability of or tolerance to some microorganisms. For example, South Kalimantan local rice varieties highly adapted to non-fertilized, medium-strongly acidic paddy field (pH 3.0–3.7 in the stagnant water, while pH 2.1–3.3 in post-harvest dry soil in the local paddy field) possessed specified acid-tolerant eubacteria of *Sphingomonas* spp., *Burkholderia* spp., and *Flavobacterium* spp. as major culturable isolates. These local rice variety–associated rhizobacteria had the ability to neutralize the rhizoplane and serve as nitrogen fixers. Some of the *Burkholderia* spp. were also capable of solubilizing unavailable aluminum phosphate to a soluble phosphate. Of course, these were non-pathogenic bacteria. Thus, the rhizosphere of many crops frequently harbors their own root- or leaf-associating eubacteria in a high population density. If we could find antagonistic microorganisms among such crop-associating microorganisms, they would be powerful candidates for practical biological control agents. As describe above, we have isolated acid-tolerant microorganisms from the rhizosphere of acid-tolerant plants, including local varieties of paddy rice. What, then, would be a target for the screening of the antagonistic agent, using these isolates as the library?

2. Study on antagonistic bacteria against bacterial rice seedling blight-causative *Burkholderia plantarii*

Bacterial rice seedling blight disease is caused by virulent strains of *Burkholderia plantarii*, and yellowing, stunting, and pigmen-
tation are recognized as its main symptoms. Phytotoxin produced by highly phytopathogenic *B. plantarii* is known to be a seven-membered aromatic compound, tropolone (1), which can strongly chelate with an Fe$^{3+}$ cation, leading to severe iron deficiency in the microenvironment.\(^8\) This phytotoxin also shows strong toxicity against bacteria and fungi with a broad antimicrobial spectrum. Hence, tropolone-producing *B. plantarii* not only kills the rice seedling but also sweeps microbial communities in the rhizosphere.

As the toxicity of tropolone is due to its powerful chelating nature toward Fe$^{3+}$, powerful antibiotic-producing bacteria or fungi are easily defeated by *B. plantarii*. If susceptible to tropolone, such antagonistic microorganisms would be eliminated before the antagonistic microorganism started to produce the antibiotic. Hence, *B. plantarii* emerged in 1982 as a synthetic bactericide-tolerant seed-born bacterium and was used as a targeted phytopathogen for biological control.\(^7\) We first started screening practically antagonistic microorganisms from the library of acid-tolerant bacteria collected from Indonesian rice in 1998–2001.

This acid-tolerant bacterial library was used for the following reasons: the soil is moderately to highly acidic; particularly woody tropical peat soil is Fe-deficient soil; and such peat soil-adapting plant roots may harbor bacteria that are estimated to be relatively tolerant to Fe-deficient conditions or the Fe chelator itself. Consequently, some isolates showed a pronounced suppression of symptom development on *B. plantarii*-infested rice seedlings. However, some others accelerated the symptom development in the pot experiment. All of the tested bacteria were harmless toward growth of the rice seedlings when inoculated to the seedlings without infestation of *B. plantarii*.\(^7\)

One of the most effective antagonistic isolates from the Indonesian rice varieties was *Burkholderia mimosarum* strain 901-5B, which had been isolated from the rhizoplane of acid-tolerant local paddy rice, Siam Unus, in South Kalimantan, Indonesia. As we developed a new combined assay for rice seedling growth and symptom development on a Hoagland’s medium-based gel bed, together with growth performance of the above-ground part of the rice seedlings, *B. plantarii*-infested rice seedlings (Koshihikari) showed drastic stunting not only above ground but also in the roots. However, when 5- to 10-fold higher cell numbers of strain 901-5B was co-inoculated to the seeds inoculated with the *B. plantarii* cells, this antagonistic bacterium completely suppressed the symptom development, and more importantly, this antagonistic bacterium never eliminated or killed *B. plantarii* but completely calmed its phytotoxicity.\(^8\)

In this rice seedling assay system, co-inoculated with *B. plantarii* and *B. mimosarum* 901-5B during the pre-germination stage, tropolone production was selectively suppressed, and the pinpoint metabolic regulation by *B. mimosarum* strain 901-5B was predicted to be the main reason for the antagonism.

Although we attempted to set up a field or greenhouse experiment using nursery box of rice seedlings, such a field experiment for these overseas antagonistic bacteria was not permitted by Plant Protection Station. Thus, we eventually gave up on this bacterium. Since then, we have spent more than 10 years trying to understand the molecular mechanism of symptom suppression by the antagonistic *Burkholderia* sp., including characterization of the signaling molecules.

In 2010, we restarted a similar research program using rice-rhizosphere-associating microorganisms collected from post-harvest paddy fields in Central Hokkaido. As the paddy field soil is not moderately to highly acidic, it was necessary and significant to perform another pre-screening, in which tolerance to tropolone (1) was first planned. In this step, we encountered a cost-management problem because tropolone was too expensive to use as a supplement to the screening medium. Fortunately, it was already known that the toxicity of tropolone is due to its high Fe-chelating ability, leading to severe Fe deficiency in the microenvironment around the tropolone producers. Hence, we replaced tropolone with catechol (1,2-dihydroxybenzene), which is also known as an aromatic Fe chelator toxic to both fungi and Gram-negative bacteria and is inexpensive (less than 1/100 price of tropolone) and environmentally friendly, and attempted preliminary screening for tropolone-tolerant and antagonistic microbes against *B. plantarii* on a catechol-containing potato-dextrose agar (PDA) medium. Using catechol in the range of 0.2–20 mM in PDA plates, catechol-tolerant microorganisms were thus pre-screened.

Among the 183 microbial isolates (14 fungi and 169 eubacteria), 16 isolates (four fungi and 12 eubacteria) were culturable on a 10 mM catechol-containing PDA plate, showing that these are highly tolerant to catechol and probably to tropolone too.\(^9\) Using this small library of 16 isolates, we have done a second screening for a *B. plantarii*-infested rice seedling growth assay on a gellan gum bed to search for microorganisms antagonistic to symptom development. In this small library, *Burkholderia helieia* strain PKA1-2 was found to be a powerful antagonistic bacterium against the bacterial rice seedling blight disease.\(^10\)

We also developed a simple paper disc assay to search for a tropolone production-accelerating/inhibitory principle on a 1 mM FeSO$_4$-containing PDA or gellan gum plate impregnated with *B. plantarii* cells. Using this bioassay system, a tropolone production inhibitory factor was chased in the culture fluid of *B. helieia* PKA1-2. Eventually, as an active principle in the extract, indole-3-acetic acid (IAA, 2) was isolated, and it was confirmed that IAA can suppress tropolone production but not the cell growth of *B. plantarii* at 1 µmol/disc.\(^10\) Although several indole derivatives were tested for similar activity to suppress tropolone production, none except IAA showed such activity. As those indole derivatives included some IAA analogs that possessed auxin-like biological activity, it was pronounced that the auxin-like biological activity and tropolone production–inhibiting activity are not linked.\(^10\)

For tropolone biosynthesis, Thiel and colleagues have reported a biosynthetic pathway of tropolone from l-phenylalanine via phenylacetic acid (PAA, 3) in 2010.\(^11\) We also tried a feeding
experiment for *B. plantarii* to add 1 mM \(1-[\text{ring-}^3\text{H}_3]\)-phenylalanine\(^{12}\) to the culture medium, and consequently, deuterated tropolone \(([\text{ring-}^2\text{H}_5,\text{H}_3]\text{)}\) and deuterated PAA \(([\text{ring-}^2\text{H}_5]\text{)}\) were obtained. When \([\text{ring-}^2\text{H}_5]\text{-PAA}\) was added as the sole precursor, deuterated tropolone was also produced. Thus, *B. plantarii* produced tropolone from PAA as its direct precursor.\(^{10}\)

When looking at the chemical structures of IAA and PAA, I have noticed their structural similarity. Hence, a tropolone-production inhibition assay was conducted for several analogous compounds of PAA. At 1 \(\mu\text{mol/paper disc}\) on the *B. plantarii-*impregnated gel plates, \((\pm)-2\)-methylphenylacetic acid (4) and \(p\)-isopropylphenyl acetic acid (5) showed pronounced inhibitory activity against tropolone production by *B. plantarii*, both of which activities were higher than those for IAA. When exposed to 0.5 mM IAA in a potato-dextrose broth medium, the accumulation of PAA in the culture fluid of *B. plantarii* was suppressed, while both compound 5 and \((S)-\text{and } (R)-\text{forms of compound 4}\) at the same concentration of IAA rather promoted the accumulation of PAA in the culture. Hence, it was predicted that IAA inhibits the conversion of \(l\)-phenylalanine to PAA via deamination of the \(\alpha\)-amino acid, while 4 and 5 suppress the decarboxylation of PAA followed by ring-rearrangement on the benzene ring into a seven-membered ring of tropolone (Fig. 2).\(^{10}\)

Both *B. mimosarum* strain 901-5B and *B. heleia* strain PKA1-2 that inhibit tropolone production of *B. plantarii* never repressed cell growth of other bacteria; however, both generously regulated the metabolic behavior of *B. plantarii*. As a result, these antagonistic bacteria maintained stable microbial consortia in the rhizosphere, and never allowed monopolization by *B. plantarii*. This event may have been established under an old nursery system for paddy rice, known as *nawashiro* in Japanese., and never allowed monopolization by *B. plantarii*

Fig. 2. Estimated interference of the tropolone biosynthetic pathway of *B. plantarii* by indole-3-acetic acid and its analogous compounds

Hence, we searched for antagonistic secondary metabolites that were produced and extracellularly released into the culture fluid only under exposure to such chemical stresses, and we successfully isolated it as colorless needles. Consequently, we have identified it as daucane-type sesquiterpene diol caf-603 (we temporally named carot-4-en-9,10-diol, 6) based on spectroscopic analyses.

In the carbon skeleton of daucane sesquiterpene from Apiaceae and Rosaceae plants,\(^{13}\) quaternary carbon at the C-7 position that is connected to the C-15 bridge-headed methyl group is always in an \(S\) configuration. However, this compound from the fungus possessed an \(R\) configuration at the bridge-head carbon.\(^{14}\) Thus, it is predicted that the farnesyl diphasphate cyclase of *T. virens* strain PS1-7 is pronouncedly different from the cyclase \(R.\) *rugosa* and Apiaceae that plants harbor. Our trials of RNA-seq analysis in the mycelial cells cultured in the 0.5 mM catechol-present or catechol-absent liquid medium and the differential display for gene expression under the same culturing conditions showed that the farnesyl diphasphate cyclase gene \(\text{vir}4\) gene is a bottleneck gene.

This substance showed an autoregulatory factor-like biological activity to induce conidiospore formation in *T. virens* PS1-7 when this fungus is highly exposed to chemical stresses. Indeed, *T. virens* PS1-7 also produced this compound under exposure to 0.05–0.2 mM tropolone, and the productivity of 6 was enhanced in a dose-dependent manner, similar to the response to catechol at concentrations of 0.1–1 mM. Indeed, some Fe chelators at 0.5 mM, such as pyrogallol, gallic acid, salicylic acid, ethylenediaminetetraacetic acid, and citric acid, also activated the production of 6 by *T. virens* PS1-7. Conversely, we also chased the tropolone production–inhibitory factor in the culture fluid of *T. virens* PS1-7 mycelium, and the isolated substance was unexpectedly compound 6. In the presence of 6 at a final concentration of 200 \(\mu\text{M}\), the tropolone production of *B. plantarii* was suppressed to 1/3 of the untreated control, and more importantly, this sesquiterpene diol induced a pronounced pseudo-biofilm in the planktonic state of *B. plantarii* cells. Unlike the normal biofilm induced by exposure to 20–50 \(\mu\text{M}\) tropolone, which is highly tolerant to environmental stresses and survives for a relatively long time in the culture (1–2

3. A sesquiterpene diol produced by bacterial rice seedling blight disease—antagonistic

*Trichoderma virens* PS1-7

To screen biocontrol agents against *Burkholderia plantarii*, we employed a bioassay that resulted not only in highly catechol-tolerant bacteria but also in fungi. Particularly, *Trichoderma virens* strain PS1-7 was the most catechol-tolerant microorganism (against 20 mM catechol) among the rhizosphere microorganisms we have collected and tested, and this fungus exhibited antagonistic efficacy as a biocontrol agent for rice seedling blight. Hence, we searched for antagonistic secondary metabolites that were produced and extracellularly released into the culture fluid under exposure to such chemical stresses, and we successfully isolated it as colorless needles. Consequently, we have identified it as daucane-type sesquiterpene diol caf-603 (we temporally named carot-4-en-9,10-diol, 6) based on spectroscopic analyses.

In the carbon skeleton of daucane sesquiterpene from Apiaceae and Rosaceae plants,\(^{13}\) quaternary carbon at the C-7 position that is connected to the C-15 bridge-headed methyl group is always in an \(S\) configuration. However, this compound from the fungus possessed an \(R\) configuration at the bridge-head carbon.\(^{14}\) Thus, it is predicted that the farnesyl diphasphate cyclase of *T. virens* PS1-7 is pronouncedly different from the cyclase \(R.\) *rugosa* and Apiaceae that plants harbor. Our trials of RNA-seq analysis in the mycelial cells cultured in the 0.5 mM catechol-present or catechol-absent liquid medium and the differential display for gene expression under the same culturing conditions showed that the farnesyl diphasphate cyclase gene \(\text{vir}4\) gene is a bottleneck gene.

This substance showed an autoregulatory factor-like biological activity to induce conidiospore formation in *T. virens* PS1-7 when this fungus is highly exposed to chemical stresses. Indeed, *T. virens* PS1-7 also produced this compound under exposure to 0.05–0.2 mM tropolone, and the productivity of 6 was enhanced in a dose-dependent manner, similar to the response to catechol at concentrations of 0.1–1 mM. Indeed, some Fe chelators at 0.5 mM, such as pyrogallol, gallic acid, salicylic acid, ethylenediaminetetraacetic acid, and citric acid, also activated the production of 6 by *T. virens* PS1-7. Conversely, we also chased the tropolone production–inhibitory factor in the culture fluid of *T. virens* PS1-7 mycelium, and the isolated substance was unexpectedly compound 6. In the presence of 6 at a final concentration of 200 \(\mu\text{M}\), the tropolone production of *B. plantarii* was suppressed to 1/3 of the untreated control, and more importantly, this sesquiterpene diol induced a pronounced pseudo-biofilm in the planktonic state of *B. plantarii* cells. Unlike the normal biofilm induced by exposure to 20–50 \(\mu\text{M}\) tropolone, which is highly tolerant to environmental stresses and survives for a relatively long time in the culture (1–2
weeks or more), pseudo-biofilm-forming bacterial cells inside the extracellular polymeric substances died within a short time (3–4 days). Therefore, the antagonistic action of \( T. \ virens \) PS1-7 against bacterial rice seedling blight disease is likely to be the suppression of tropolone production by a molecular mechanism different from the IAA produced by \( B. \ helicin.^{13} \)

Compound 6, which we isolated as the conidia formation-inducing factor or the tropolone production-suppressing factor, was most actively produced in 1.0 mM catechol-supplemented potato-dextrose medium, and its productivity for 3-day incubation was 13 mg L\(^{-1}\).\(^{16} \) Although the acute bactericidal or fungicidal activity of this compound was weak, marked biological activities to induce conidium formation on the mycelia of \( T. \ virens \) PS1-7 and pseudo-biofilm formation on phytopathogenic \( B. \ plantarum \), along with the suppression of tropolyte production, were confirmed.\(^{17} \) However, the main role of the sesquiterpene diol in this saprofungus remains a mystery.

I have long been familiar with daucane-type sesquiterpenes due to phytochemical studies on the leaves of \( R. \ rugosa. \)\(^{13} \) After 20 years in the research field of ecological chemistry and the biological control of pests, I again met a compound possessing the same carbon skeleton of daucane sesquiterpene. Due to this “reunion for the first time in 20 years,” this series of research seems to me a very impressive one.

### 4. Secondary metabolites produced by \( \textit{Pseudomonas jessenii} \), an antagonistic rhizospheric bacterium against sugar beet root rot-causative \( \textit{Aphanomyces cochlioides} \)

\( \text{Peronosporomycetes Aphanomyces cochlioides} \) is a root-rot-causative fungus that infects \( \textit{Amaranthaceae} \) crops, including sugar beets, spinach, and edible amaranthus. In the 1980s, our laboratory discovered cochlilipha in A, 5-hydroxy-6,7-methylene-dioxylavone, as a chemoattractant and a host-recognition signal compound toward the zoospores of \( A. \ cochlioides. \)\(^{18} \) Recently, we also found a highly attractive response of \( A. \ cochlioides \) zoospores to cAMP, and an activator of AMP cyclase, forskolin, led to a two-fold increase of zoospore population density during the morphodifferentiation induction. In addition, 1 \( \mu M \) adenine in an \( A. \ cochlioides \) zoospore suspension cancelled the zoospore attraction toward cochlilipha A.\(^{19} \)

Antagonistic rhizospheric bacteria against this soil-born pernosporomycte phytopathogen were screened from the rhizosphere of host and non-host plants from Hokkaido. Accordingly, 150 isolates thus obtained were subjected to a dual-culturing bioassay against \( A. \ cochlioides \), and eight isolates showed remarkable mycelial growth inhibition. Among them, \( \textit{Pseudomonas jessenii} \) strain EC-S101 isolated from a spinach root administered an induction of unique hyperbranching toward \( A. \ cochlioides \) mycelia.\(^{20} \)

To isolate this hyperbranch-inducing factor and elucidate its chemical structure, we first attempted a shake culture of \( P. \ jessenii \) strain EC-S101 in potato-dextrose broth medium at a scale of several liters. However, neither the concentrated culture fluid thus obtained nor the extract showed any hyphal hyperbranching-inducing activity. Conversely, when \( P. \ jessenii \) strain EC-S101 was allowed for the culturing on solid medium of potato-dextrose agar plates for 2–3 days, a small cube (2×2×2 m\(^3\)) of solid agar from the cultured plate placed on a new plate culturing \( A. \ cochlioides \) mycelia showed a pronounced hyperbranching induction. Hence, an extract of the cultured agar plate with MeOH was subjected to the paper disc assay against \( A. \ cochlioides \) mycelia, and hyperbranching induction was reproducibly observed. This preliminary experiment clearly showed that a solid plate culture is necessary to obtain the active substance.

Isolation of the active substance was a physical labor. Namely, potato-dextrose agar plates totaling 7.5L (250 plates of ø 9 cm plastic petri dishes, each containing 30 mL of PDA) were inoculated with \( P. \ jessenii \) EC-S101 and incubated for 2 days at 25°C in the dark. After incubation, this agar medium was frozen at −30°C, followed by further treatment to be defrosted and squeezed in cotton cloth to obtain culture fluid, and the solid residues were re-extracted with 1.5 L of Milli-Q water to obtain total of 5.5 L culture fluids. The resulting 5.5 L of culture fluid was, after the removal of bacterial cells and agar flakes by centrifugation, extracted with the same volume of EtOAc three times to administer 250 mg of the crude extract. As this extract showed hyperbranching activity in the paper disc assay on the PDA plate, silica gel column chromatography for the crude extract followed by HPLC for the active fraction administered two active principles, substance A (30.4 mg) and substance B (8.0 mg). Their structures were elucidated to be (−)-4,5-didehydroacaterin (7) and its homolog that possessed the alkyl chain being shortened by the C\(_2\)H\(_4\) unit (8), respectively. The major active principle 7 accounted for more than 10% of the crude extract, while the minor principle 8 was a novel compound.\(^{21} \)

\( A. \ cochlioides \) mycelia exposed to the paper disc that contained 1 µg of each compound showed hyperbranching reproducibly along with accelerated mitosis and rapid nuclei degradation. Hence, we concluded that compounds 7 and 8 are main pernosporomycte hyperbranch-inducing factors produced by \( P. \ jessenii \) EC-S101 (Fig. 3). Generally, phytopathogenic perno- sporomycetes acquire tolerance to fungicides by gametangial fusion for the transmission of fungicide tolerance–associated genes. However, mycelia exposed to these chemical compounds are strongly inhibited formation of zygospore (or chlamydo- spore), and this antagonistic bacterium that produces hyper-

![Fig. 3. Morphodifferentiation-inducing furanone derivatives on pernosporomycetes mycelia. (−)-Didehydroacaterin (7) induce hyperbranching of A. cochlioides hyphae. Along this morphodifferentiation, cell mitosis and senescence of the nuclei are accelerated.](image)
branch-inducing 7 and 8 suppresses not only mycelial growth but also the emergence of fungicide-tolerant mutants in agricultural fields. These furanone derivatives harbor a basic carbon skeleton of 1-hydroxyalkyl-5-methylene-2(5H)-furanone, the same as that of 3-(1-hydroxy)butyl-4,6-dibromo-5-methylene-2(5H)-furanone (9), which is a well-known quorum-mimicking substance produced by a red sea alga, Delisea pulchra. This compound was first reported as a red alga substance that defends against pathogenic bacteria by disturbing the bacteria’s quorum-sensing systems.

Hence, we also attempted to demonstrate the quorum-like activities of 7 and 8 toward a Burkholderia cepacia isolate B with an active swarming nature on a nutrient broth agar (NBA) plate and toward P. jessenii EC-S101 itself, which had shown no swarming capability, on an NBA plate. As a result, at a final concentration of 178 μM on the NBA plate, compounds 7 and 8 clearly induced active swarming of P. jessenii EC-S101 with a 4 cm diameter, spreading after 24 hr incubation, while these furanone compounds completely cancelled the swarming of B. cepacia isolate B without any cell growth inhibition at a final concentration of 22 μM in the NBA medium.

To determine the mode of action for hyperbranching induction by compounds 7 and 8, we attempted fluorescence staining of the hyperbranched mycelial cells using rhodamine phalloidin, a fluorescent probe for actin filaments. Normal hyphal cells possessed evenly placed actin plaques along the inner surface of the biomembrane in the hyphal cells that maintained the tubular shape of the hyphal cell. Only at the hyphal tip, actin filaments are oriented maintaining flexibility of hyphal extension or allowing normal branching. In contrast, in the hyperbranching cells, the formation of actin plaques is unregulated, and their distribution is irregular. The actin plaque shapes are also unstructured, non-spherical shapes. Hence, it is predicted that a mode of action of 7 and 8 in hyphal hyperbranching induction is inhibition of the normal assembly of actin filaments.

5. Association between herbicides and N₂O-emitting microorganisms

Nitrogen is an essential element for plants and other living creatures. In many cases, nitrogen is provided as amino acid, ammonium (NH₄⁺, stable in soil), or nitrate (NO₃⁻, easy to lose from soil via leaching and denitrification) released from degraded organic substances. Plants or fungi inhabiting nitrogen-poor soil acquire available nitrogen as follows: 1) establishing symbiosis with free-living nitrogen-fixing bacteria, 2) decomposing organic substances actively, or 3) suppressing ammonia oxidation by nitrification bacteria or archaea, known as biological nitrification inhibition (BNI). Some Poaceae (also known as Gramineae) plants are known to release BNI principles. Brachytria humidi cola releases diterpene (brachialactone), while Sorghum bicolor produces paraquinoine derivatives (soliqoquinoines) from plant roots. Such BNI principles are expected to biologically suppress nitrification in agricultural farmlands. Conversely, neither natural products to suppress denitrification in natural soil nor synthesized chemical agents to suppress N₂O emission in farmland are known.

After clear cutting of tropical swampy forests established on acidic woody tropical peatlands, the reclaimed lands used for agriculture or oil palm plantation often turn into the most powerful hot spots of N₂O emission. However, oil palm plantations, which are thought to produce the highest economic value from the reclaimed tropical peatland, harbor relatively low N₂O-emitting soils despite a large N input in the farmland. We thus investigated the suppressing principle using our culturing assay, including some herbicides, e.g., glyphosate and paraquat, which were used there. Some other herbicides selected for the N₂O emission suppression assay using the denitrifying eubacteria were those of electron transport inhibitors that may block the reduction process of denitrification.

In consequence, paraquat (10) showed a remarkable suppressing effect on N₂O emission via a hyperactive N₂O-emitting Pseudomonas sp. at 2.5 μM in 10 mL of soft gel media and at 50 μM in 5 g of Andisol, without any bacterial growth inhibition (Fig. 4). Isouron (11) also showed inhibitory activity against N₂O emission, similar to paraquat, while diquat, a N-cationic herbicide similar to paraquat, did not show any suppressive effect on the N₂O emitter used for the N₂O emission assay. In addition, dicyandiamide, which is approved in the USA for use as a nitrification-inhibiting agrochemical, did not inhibit but rather accelerated N₂O emission. Allyl isothiocyanate, a representative volatile secondary metabolite of Brassicaceae, including mustard, rape seed, radish, and horseradish plants, completely inhibited N₂O emission at 30 μM in the N₂O suppression assay, but the effect was often unstable due to its volatile nature. For instance, when a whole seedling of yellow mustard autoclaved in the soft gel medium was added, N₂O emission was accelerated.

In these studies, we showed that some chemical agents that
have been approved to spray over farmlands, namely legal “pesticides,” are effective agents to suppress N₂O emission from fertilized farm soils. Thus, some structure–activity correlations in the electron-transport inhibitors are predicted from our results. We wish further development of a “herbicide that possesses the binary effect” to practically utilize such chemicals for suppression of N₂O emission from agricultural farmlands along with weed control.

6. Searching for the sporulation-inducing factor in okara for Bacillus eubacteria

A group of genus Bacillus spp. are spore-forming, culturable Gram-positive bacteria commonly distributed throughout soil, the phyllosphere, and fresh and marine water. In many cases, sporulation of Bacillus spp. is highly linked to metabolic differentiation. During spore formation, B. subtilis known as a bacterium for natto (a fermented traditional Japanese food made from steamed soybeans) produces lipopeptide antibiotics, while B. thuringiensis yields insecticidal protein Cry toxin along with the sporulation event.

It had been recognized that okara induces sporulation in bacilli and enhances antibiotic production. However, the sporulation-inducing factor in okara had not yet been identified when we began our study. We extracted 10 kg of okara with 10 L of MeOH and found the activity in the concentrated methanolic extract. Hence, using a LIVE/DEAD BacLight assay to check sporulation, the sporulation-inducing principle was chased by several chromatographic fractionations, followed by the sporulation assay. Eventually, we successfully isolated the active substance from a water-soluble fraction of okara. The fraction at the final step (450 mg) contained 40 mg of the active substance, and the active principle was isolated as an acetylated compound from 45 mg of the final fraction (1/10 of the mixture) treated with pyridine/acetic anhydride. Acetate of the sporulation-inducing substance led to structure elucidation of the active principle as diacetonamine (4-amino-4-methylpentan-2-one, 12) (Fig. 5).

Diacetonamine (12) is a primary amine produced by the Michael addition of an ammonia on mesityl oxide (4-methyl-3-penten-2-one) that had been formed by the aldol condensation of two acetone molecules. Hence, we could not eliminate the possibility that 12 is an artifact during the final purification process, but it was reproducibly confirmed that this compound was contained in the active fraction before the final steps. We thus concluded that compound 12 is, at least, not an artifact during the purification process.

An amide derivative of 12 with N-acrylic acid, diacetone acrylamide (13), is an industrial product for a co-polymerization agent with urethane to increase the wettability of polyurethane foam. This monomeric vinyl compound was characterized as a stable sporulation-inducing chemical agent that is more active than 12. These sporulation-inducing chemicals, particularly 12 which is contained in a food material okara, can be applied to a sporulation inducer for Bacillus probiotics. In addition, stable hydrochloride of 12 is possibly usable for antibiotic production of Bacillus species. This study linking to the observation of a phenomenon is also a good example of Chemical Ecology-based pesticide study.

Conclusion

Since I engaged in natural product chemistry 30 years ago and joined the Pesticide Science Society of Japan 10 years ago, I have felt strongly that pesticide science is a practical science in agricultural pest control. Several signaling chemicals discovered in our laboratory are neither bactericidal nor fungicidal compounds but serve as a pinpoint metabolism inhibitor or a sharp differentiation inducer. Similar to anti-cancer drugs, molecular targeted pesticides are the current fashion to the high selectivity of pesticides in modern pesticidal drug design and innovation, including in silico assays and target protein identification using chemical biology techniques. In this sense, signaling compounds that we could find based on our careful observation and newly designed bioassay probably show new horizons for biologically active chemicals against phytopathogens difficult to control by pathogen-killing pesticides. In addition, some widely used herbicides not only sweep weeds but also regulate denitrification along with the suppression of active N₂O emission. Hence, those binary herbicides should be re-evaluated in light of global warming issues. Furthermore, the discovery of a Bacillus sporulation-inducing principle from edible okara may lead us to new approaches to antibiotic production by Bacillus eubacteria or some other microorganisms.

Acknowledgements

All of the studies introduced in this review were done in the Laboratory of Molecular and Ecological Chemistry, Research Faculty/Graduate School of Agriculture, Hokkaido University, where I belonged and started my study life. I thank emeritus professor of Hokkaido University Dr. Junya Mizutani, who provided me with an opportunity and place to start my career; emeritus professor of Hokkaido University Dr. Satoshi Tahara, who taught me the basic concepts of science and the excitement of small but new discoveries; and emeritus professor of University of Alberta, Canada, Dr. Chuij Hiruki, who taught me that the most powerful tool for science is a new idea. I also wish to express deep thanks to Dr. Mengcen Wang (lecturer at Zhejiang University), who performed research on antagonistic microorganisms against bacterial rice seedling blight disease; Dr. Abhinandan Deora (Syngenta Canada), who did pioneer work in rhizospheric microorganisms antagonistic to phytopathogens; and many other grad-
uate students and collaborating researchers who worked on the related studies. Last but not least, I also thank Prof. Kazuhideki Matsuda (Kindai University) for his recommendation of my achievements for the Society Awards 2017 (on prominent achievement) of the Pesticide Science Society of Japan.

References

2) S. Tsushima: Nishokubyouho 80, 188–196 (2014).