J. Res. Fac. Agr. Hokkaido Univ., Vol. 72, Pt. 2 : 1～19 (2008)

Screening of rhizobacteria possessing phenolic acid-decarboxylation abilities from several plant families

Albert Asante, Satoshi Tahara and Yasuyuki Hashidoko

Laboratory of Ecological Chemistry, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Kita-Ku, Sapporo 060-8589, Japan

Keywords: bacterial 4-hydroxycinnamate decarboxylase, decarboxylation of phenylpropanoic acids, phenolics of plant origin, rhizosphere bacteria, root epiphytes

ABSTRACT

We isolated 332 rhizoplane bacteria from 74 plant samples consisting of 38 different plant species that belong to 20 different families. The bacterial isolates were screened for their decarboxylation activities on common phenolics of plant origin. Screening tests using *Klebsiella oxytoca* JCM 1665 as a positive control involved substrate-specificity assay and 4-hydroxycinnamate decarboxylase (4-HCD) induction assay. Most isolates (57%) were observed to have 4-HCD activity on both (*E*)-3,4-dihydroxycinnamic acid (synonym of (*E*)-caffeic acid, CFA) and (*E*)-4-hydroxycinnamic acid (4-HCA). Substrate-specificity of CFA-decarboxylative rhizobacteria was tested on 24 positive isolates, and they generally accepted (*E*)-ferulic acid (FRA) as well as CFA. However, some bacteria could not decarboxylate 4-HCA. Neither 6-hydroxynaphthoic acid (6-HNA) nor (*E*)-2-hydroxycinnamic acid (2-HCA) was accepted as the decarboxylative products. These results suggested that 4-HCDs in bacteria have wide range of enzymatic variation to show alternative substrate-specificity. Some few rhizobacterial isolates (2%) undergo peroxidase- or tyrosinase-associating melanin production. This study has suggested that metabolic properties of many eubacteria inhabiting the rhizosphere environments are dependent on polyphenolic secondary metabolites accumulated in the plant roots. It was thus found that phenylpropanoid acid-decarboxylative bacteria are widely distributed in the terrestrial ecosystem.

INTRODUCTION

In a typical agro-ecosystem, large concentrations of phenolic acids are

produced in the soil as a result of litter made during the cropping season. The phenolic acids may affect the persistence of the rhizobacteria in the soil and in the rhizosphere and their symbiotic efficiency with the plant hosts. Phenolic acid utilization by rhizobacteria leads to changes in their biochemical and physiological features[1–3]. Phenolics of plant origin eventually influence interactions between plants and pathogens, mutualists and competitors[4,5]. It is incumbent on the rhizobacteria to devise ways that could enhance their survival in the soil and in the rhizosphere. Soil microorganisms rapidly decompose many of these phenolic compounds or tolerate them. The rhizospherous community therefore undergoes an induction and/or selection from these phenolics accumulated in the soil and the rhizosphere of roots[6], during which the rhizobacteria are naturally screened for their ability to degrade phenolic acids of plant origins.

The production of phenolic metabolites is considered to be involved in the plant defensive reaction as they are generally toxic to microorganisms[7]. The type and magnitude of their effects vary with the situation (e.g. rhizosphere/phylloplane environments, concentration and chemical structures of phenolics) and the properties of organism. Due to these variations, it has been difficult to predict when and where phenolics will be active. Variations in the ecological activity of phenolics are as a result of 3 main reasons: (1) differences in the tolerance levels of phenolics among organisms, (2) variations in the structure and concentration of the phenolics exposed to the organisms, and, (3) the conditions affecting the mode of action of the phenolics in the organism[4,5].

Some microorganisms however, have evolved ways of suppressing the plant defense system and other microorganisms are able to tolerate these plant defense chemicals by detoxification of the phenolic acids. In some cases, chemical metabolites of such root-associating microorganisms play mutualistic roles with the plant hosts, for example by increasing the concentration of defensive metabolites potentially active against a group of pathogens[8]. Decarboxylation by microorganisms is one of those important processes of detoxification and decomposition of phenolic acids and predominant representative plant secondary metabolites exuded from plant roots and litters. Hashidoko *et al*. reported that 4-hydroxycinnamate decarboxylase (4-HCD) of Gram-negative Enterobacteraceae *Klebsiella oxytoca* and *Erwinia uredovora*, which are phylloepiphytic bacteria on the leaves of *Polymnia sonchifolia* (family Asteraceae), show high substrate-specificity for 4-hydroxylated (*E*)-cinnamates to produce 4-hydroxylated styrenes effectively[9,10]. Crude protein extracts from the bacteria cells of *K. oxytoca* had proven to have abilities as substrate-inducible hydroxycinnamate decarboxylase (4-HCD)[9–11]. The 4-HCD of *K. oxytoca* is a substrate-inducible soluble protein showing *E*-isomer specificity. *K. oxytoca* was concluded by the authors to possess not only detoxifying enzyme for this phenolic compound of host plant but also act as host defensive principle. Phenolics have also been proposed by several researchers ecologically as defense chemicals in plants including disease

resistance, signal molecules in rhizospherous bio-complex formation and protection from UV-B light [12,13]. Organisms that are evolutionarily linked and have a history of association with phenolics have evolved the ability to tolerate them and even to some extent benefit from them[14−16]. Ecological responses are invariably threshold phenomena with ranges of sensitivity and tolerance[4,5]. The mechanism for organisms to tolerate and hence benefit from their association with the phenolics is however largely unknown[17]. The vast diversity of phenolics, comprising of different groups of compounds with different modes of action, also accounts for some variations in the activity of phenolics in organisms[16,18]. Many microorganisms including fungi and bacteria are known to decarboxylate carboxylic acids of the phenylpropanoid class to produce the corresponding 4-hydroxystyrenes. Such cinnamate decarboxylases are usually classified according to their substrate specificity[10,19]. This confirms the assumption made by Goodey and Tubb[20] in 1982 that hydroxystyrenes resulting from decarboxylation of hydroxycinnamates by microorganisms is a process of detoxifying the phenolics into other metabolic forms that they may be able to tolerate.

This experiment was devised to investigate plant phenolics accumulated in plant tissues and how these chemicals affect microfloral composition with particular reference to the metabolic and/or decomposing abilities of rhizoplane bacteria on common phenolics of plant origin. We therefore isolated 332 rhizoplane bacteria from 38 different plant species consisting of 20 plant families. These bacterial isolates were tested for decarboxylation ability, using *K. oxytoca* JCM 1665 as the reference bacterium. Our main objectives were to isolate, screen and select rhizospherous bacterial isolates from specific plant/families that had metabolic abilities on common phenolics of plant origin. We characterized their decarboxylation activity on common phenolic acids in order to understand their ecological significance.

Material and Methods

1. Chemicals used in decarboxylation assay

Chemicals used in this study are shown with their structures in Fig. 1. (*E*)-Caffeic acid (CFA, Wako Pure Chemical Industries Ltd., Osaka, Japan), (*E*)-4-hydroxycinnamic acid (4-HCA, Sigma-Aldrich, St. Louis, MO), and (*E*)-ferulic acid (FRA) were all used as substrates and/or 4-HCD inducers. 6-Hydroxynaphthoic acid (6-HNA, Wako Pure Chemical Industries Ltd.) and (*E*)-2-hydroxycinnamic acid (2-HCA, Wako Pure Chemical Industries Ltd.) were used as a 4-HCD-inducer and a substrate analogue, respectively.

2. Culture media

The culture media used for isolation and purification of rhizoplane bacteria were modified Winogradsky's agar (MWA) medium of pH 6.0 and commercial

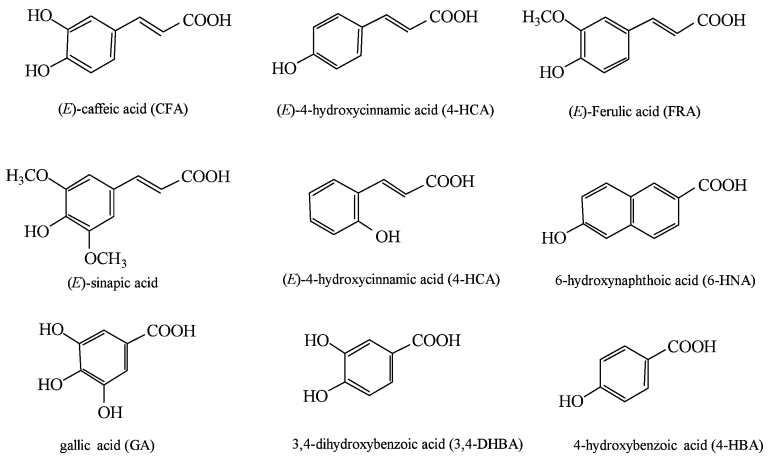

Fig. 1 Chemical structures of substrates and substrate analogs

potato-dextrose agar (PDA, 24 g l^{-1} potato-dextrose broth, Difco, Sparks, MD, plus 1.5% w/v agar, Wako Pure Chemical Industries Ltd.) medium. The MWA was nitrogen poor medium supplemented with 1% sucrose as the sole carbon source and 0.005% yeast extract[11]. Subsequent screening of bacterial isolates for their decarboxylation activities on common phenolics of plant origin was done on PD broth. The bacterial isolates in the PD broth were incubated in a rotary shaking culture at 25°C in the dark (100 rpm) for 4–5 days.

3. Plant samples and bacterial isolation process

From 10 different sites on Hokkaido University campus (Fig. 2), during the spring to summer (May-August) of 2004, 74 plant samples, belonging to 37 different plant species (19 plant families) were collected in the field. Three samples were collected for each plant species. All the plant samples used for the collection of bacterial isolates were identified by the use of some picture books of Japanese wild plants and particularly from "Plants in Sapporo" a book written by Hara that documented all wild plants in selected areas of Sapporo, including Hokkaido University Campus[21]. The fresh roots of all the plant samples were thoroughly and carefully washed with 200 ml of sterile water using bottled hand spray. When judged to be free of rhizosphere soil, they were cut into several pieces of about 1–3 cm under aseptic condition. Subsequently, they were placed into sterile 50 ml conical tubes (Blue Max™, Becton Dickison Labware, Franklin Lakes, NJ) containing 5 ml of sterile water. The tubes were then vortexed for 30 sec. A 100 μl of the resulting water suspension was then inoculated onto the MWA culture medium (pH 6.0) to allow incubation for 3–4 days at 28°C in the dark. The root residues were next placed in another sterile 50 ml conical tubes containing 5 ml of sterile water. They were then scraped with a sterilized spatula to detach the root surface-adhesive bacteria, and also vortexed for 30 sec.

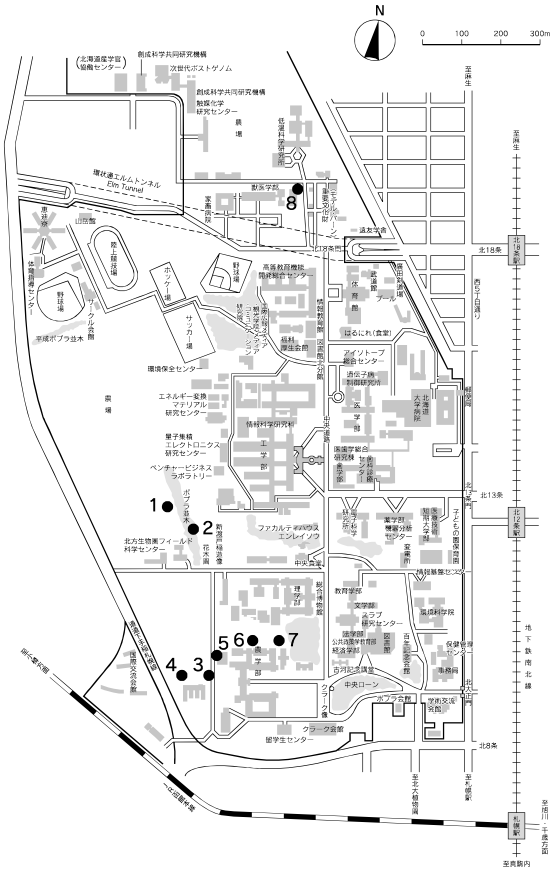

Fig. 2 Map of plant sampling sites for bacterial isolation and on Hokkaido University campus

Location of the sampling sites and code of the plants collected from each site are as follows: **1**, site near rice experimental field in experimental farm of Hokkaido University (sample code 41–47); **2**, another site next to the rice experimental field in the experimental farm (48–50); **3**, site around the greenhouse of Research Faculty of Agriculture (12–20); **4**, another site around the greenhouse of Research Faculty of Agriculture (1–11); **5**, site behind the new building of Research Faculty of Agriculture (21–30); **6**, site along the roadside along Library Building, Research Faculties of Agriculture (31–35 and 57–64); **7**, site behind old library house of Research Faculty of Agriculture (36–40); and **8**, site in the open fields near the Veterinary Hospital of the Hokkaido University (65–75). Major plants grown in each site are as follows: **1**, *Chenopodium album* and *Helianthus tuberosus*; **2**, *Polygonum lapathifolium* and *Equietum arvens*; **3**, *Rudbeckia laciniata* and *Sonchus oleraceus*; **4**, *Aegopodium podagraria* and *Plantago lanceolata*; **5**, *Trifolium repens* and *Rumex acetosella*; **6**, *S. oleraceus* and *Plantago asiatica*; **7**, *E. arvens* and *Erygeron annuus*; and **8**, *R. laciniata* and *Mentha spicata*. Some bacterial codes were shown in Tables 3–7.

The water suspension was again inoculated onto MWA medium plates as done previously in root washings. Purification of bacterial isolates was done on both MWA and PDA. The purified bacterial cultures were kept at −84°C in 10% glycerol for bioassays done hereafter. An average of 6–7 different bacteria strains per sample were isolated and purified from each plant sample, thus giving a total of 332 rhizoplane bacteria.

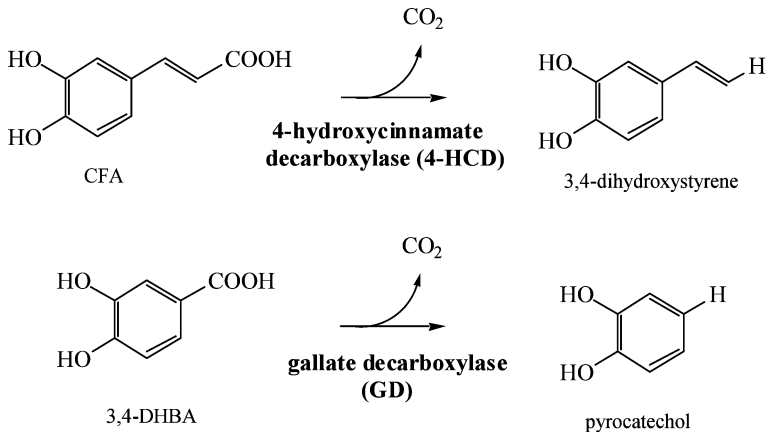

Fig. 3 Non-oxidative decarboxylation on CFA and 3,4-DHBA

3. Thin layer chromatography (TLC)

Analytical and preparative TLC was carried out on thin layer plates of Merck Art. 5715 Kieselgel 60 F_{254} (0.25 mm thickness). The decarboxylated products (Fig. 3) developed on TLC in $CHCl_3$: MeOH: HCOOH 50: 5: 1 were detected under UV_{254} light as a quenching spot. Gibbs reagent[22,23] (0.2% w/v of 2,6-dichloro-4-(chloroamino)-2,5-cyclohexane-1-one 0.2% w/v in $CHCl_3$) was also used to determine the presence of phenolic compounds. On the developed TLC plates that were sprayed Gibbs reagent followed by exposing to concentrated ammonia vapour, the phenolic products showed a dark brown (*p*-substituted) or purple (*p*-non-substituted) color.

4. Process of primary screening for CFA decarboxylation

The isolated rhizoplane bacteria were primarily tested for their decarboxylation abilities on (*E*)-3,4-dihydroxycinnamic acid (= (*E*)-caffeic acid, CFA). Each bacterial isolate, pre-incubated for 2 days at 28°C on PDA (Difco) slants, was inoculated to 10 ml of PD broth medium for 2–3 days (25°C, 100 rpm) in a rotary shaker. For primary screening test, 50 μl of the bacterial cultured medium was inoculated into freshly prepared PD broth (10 ml) containing 1 or 2 mM (180 or 360 mg/l) of CFA as the test substrate. One test tube contained the test phenolic substrate but with no bacterium (blank), and another test tube contained only the bacterium culture without the test substrate to serve as the control. The test tubes with their liquid PD medium were then incubated for 4–5 days in the dark by two-way shaking in the shaker at 25°C and at 100 rpm. After incubation, 1.5 ml of EtOAc was added to the test tubes and thoroughly vortexed to extract the metabolites from the bacterial culture. Subsequently, each tube was left to stand for a while and the EtOAc layer was subjected to TLC (Merck Kieselgel 60 F_{254}, 0.25 mm thick) using $CHCl_3$: MeOH: HCOOH, 50: 5: 1 as the developing solvent system. The identified quenching spots on TLC under UV_{254} were sprayed with

Gibbs reagent[22] followed by exposure to vapor of concentrated ammonia solution. A blue/purple/brown color indicated a presence of phenolic substance. In comparison with standard substance on TLC, each bacterial isolate was judged to be decarboxylation-positive or negative, according to the pattern of the appearing spots.

Appropriate inferences were made about the decarboxylation abilities of the test bacteria compared to the known decarboxylation activity of the reference bacterium *K. oxytoca* JCM 1665. The classification scale was as follows: (++) strong decarboxylation activity in which all test substrates was converted into the single decarboxylated product with no occurrence of other metabolic compounds, (++) strong decarboxylation ability in which all or most of test substrate was converted to the corresponding decarboxylated product along the occurrence of other metabolic compounds, (+) moderate decarboxylation ability (more than a trace amount of the test substrate remained), (±) trace decarboxylation strength and (−) no decarboxylation ability (none of the substrate was converted), depending on the intensity of quenching spots observed under UV_{254} illumination on TLC. A reference bacterium, *K. oxytoca* JCM 1665 (origin: pharyngeal tonsil, reported as inducible under exposed to CFA[24]), was always tested together for a standard 4-HCD-positive bacterium to show (++).

5. Bioassay in secondary screening (substrate-inducible or constitutive 4-HCD)

The bacterial isolates were tested to determine whether the CFA-decarboxylase is substrate-induced or constitutive. The test was done so as to enable us make inferences about whether the bacterial decarboxylation activity in resting cells pre-cultured in PD-broth medium without any 4-HCD inducers are observed.

Each active (++) bacterial isolate selected after the primary screening (Table 3) was cultured in 50 ml of PD broth in Erlenmeyer flask with (0.5 mM for 6-HNA or 2.0 mM for FRA) or without 4-HCD inducers in a rotary shaker (25°C, at 100 rpm) for 2–3 days. The bacterial cell culture (a portion of 25 ml) was then centrifuged at 6000×*g* at 4°C for 10 min. The resulting cells once washed with 20 ml of the Tris-HCl buffer were re-suspended into a 25 ml of the Tris-HCl buffer as described above. To the bacterial cell suspension, 12.5 μl of 10 M CFA in dimethyl sulfoxide (DMSO) was added and made a final concentration of 5 mM of the test chemical substrate. The resting cell suspension with the test substrate was then shaken overnight at 100 rpm in the dark at 25°C. After the incubation, the pH of the culture broth was often acidic in the range of 3.0–5.0, and if the pH was over 5.0, it was adjusted to pH 4.0 using 1 M HCl, and then extracted with 4 ml of EtOAc. The resulting EtOAc solution was then developed on silica gel TLC in $CHCl_3$: MeOH: HCOOH 50: 5: 1. The spots on TLC were analyzed under UV_{254} illumination and/or Gibbs reagent, for each rhizoplane bacterium tested.

6. Bioassay in secondary screening (substrate specificity and decarboxylase induction by substrate analogues)

The bacterial isolates were tested for their decarboxylase-types and to determine whether the CFA-decarboxylative bacteria have narrow or broad range of substrate acceptance and they change substrate acceptability by different 4-HCD inducers. The decarboxylation abilities of the bacteria isolates were also compared to a reference (positive) bacterium, *K. oxytoca* JCM 1665 whose decarboxylation abilities on common plant phenolics are known to be limited to 4-hydroxylated cinnamates, including CAF, 4-HCA and FRA[24]. JCM 1665 is also known to have decarboxylation ability on gallic acid (GA), protocatechuic acid (PA), and 4-hydroxybenzoic acid (4-HBA)[9] (Fig. 1).

Preparation of the cell suspension of CFA-exposed bacteria was the same as described above. When FRA was used as a 4-HCD-inducer, its final concentration was 2 mM, same as that of CFA. In the case of 6-hydroxynaphthoic acid (6-HNA)[24], however, final concentration of the non-substrate type 4-HCD inducer was reduced to 0.5 mM, because 2 mM of 6-HNA was toxic to many of tested bacterium to suppress their cell growth. To the bacterial cell suspension in a conical tube, several phenolic compounds and the substrate analogs (4-HCA, 3, 4-DHBA, 2-HCA and (*E*)-sinapic acid) were added.

RESULTS

1. Frequency of 4-HCD-positive bacteria in the rhizosphere of plants

During primary screening for decarboxylation activity of the rhizoplane bacterial isolates on CFA, 57 (17.2%) isolates showed clearly, positive metabolic activity on the test substrate, and these were selected for secondary screening process for their constitutive/inducible abilities on other phenolics of plant origin (Table 1). A large total of 173 (52.1%) bacterial isolates showed trace (±) or none (−) of decarboxylation activity when tested on CFA. They were subsequently rejected in the current study. The individual decarboxylation activities of bacterial isolates from the plant samples/families used for the study are shown in Table 2. The number of isolated rhizobacteria from family Asteraceae was the highest (89 isolates) followed by that of Umbelliferae (40 isolates). Although the numbers of plant species and specimens tested are not enough for statistical analysis, 4-HCD-positive rhizobacteria to CFA (++, and +) were found more frequently from the plant families Polygonaceae (18 out of 28 isolates, 64%) and Fabaceae (20 out of 33 isolates, 61%) compared to the major families Asteraceae (39%) and Umbelliferae (45%) in this screening. *Arctium lappa* (Asteraceae), *Oxalis corymbosa* (Oxalidaceae), *Polygonum lapathifolium and Polygonum pubescens* (both Polygonaceae) were all frequent host of rhizobacterial isolates possessing CFA decarboxylation abilities. All the rhizobacteria isolated from *Matricaria matricarioles* (Asteraceae), *Rumex obtussifolius* (Polygonaceae), and *Agrimonia*

Table 1 Percentage (%) distribution of decarboxylation abilities of bacterial isolates on *E*-caffeic acid from primary screening

Decarboxylation on CFA	No. of isolates (%)	Subtotal (%)	
++ sp	57 (17.2)	76 (22.9)	159 (47.9)
++ mp	19 (5.7)		
+	83 (25.0)		
±	42 (12.7)		173 (52.1)
−	131 (39.5)		
Total	332		

(++ sp): Strong decarboxylation activity in which all test substrates was converted to corresponding non-oxidative decarboxylative products without any other metabolite. Code sp means "single product". (++ mp): Strong decarboxylation ability in which all or almost all test substrate was converted to the corresponding decarboxylated compound with the occurrence of other metabolic compounds on TLC. Code mp means "multi-products". (+): Weak to medium decarboxylation ability (more than a trace amount of the test substrate remained within on TLC). (±): Weak decarboxylation strength (only a trace amount of decarboxylative product accumulated). (−): No decarboxylation activity (none of the substrate was converted). *Klebsiella oxytoca* JCM 1665 (reference bacterium) shows (++ sp) activity.

pilosa (Rosaceae), showed no or trace decarboxylative abilities when they were tested on CFA in primary screening. Out of 332 bacterial isolates that were primarily screened, 159 isolates (48%) somehow showed positive activities on CFA, and among them, 57 isolates (17%) were (++) active same as that of the reference bacterium *K. oxytoca* JCM 1665 (Table 1). These (++) isolates were selected for the secondary screening.

2. Results from secondary screening

Fifty-seven out of 332 rhizobacterial isolates showing (++) decarboxylation activities on CFA (Table 1) proceeded to be determined whether the decarboxylation abilities were due to constitutive or inducible enzymatic process, except 12 isolates because subculture of them was a failure. In the assay for judgment of substrate-inducible or constitutive 4-HCD, 19 out of 45 that were pre-cultured in PD broth without CFA completely decarboxylated the substrate to give 3,4-dihydroxystyrene. Hence, it was obvious that these 19 isolates possessed constitutive 4-HCDs (Table 3). Whereas, 13 isolates (1I, 5N, 5T, 21B, 35A, 36G, 59B, 59N, 60I, 65L, 65Q, 66G, and 70D) grown under the same condition did not show any decarboxylative activity in the resting cells, indicating that they produced absolute substrate-inducible 4-HCDs under an exposure to CFA. Other remaining 13 isolates (19K, 31G, 37E, 37M, 46A, 49B, 49G, 58B, 58F, 59P, 59X, 60E, and 72N) had the same response and ability as those of reference bacteria, *K. oxytoca* JCM 1665. Among the 45 isolates that were exposed to 2 mM CFA and used as resting cell suspension, 23 isolates were also (++) on 4-HCA, while 16 and 6 isolates were (+) and inactive (−) toward 4-HCA respectively. In the secondary screening, 16 out of the 45 isolates showed decarboxylation on 3,4-DHBA as

Table 2 Pooled data of bacterial isolates from the plant species/families and their decarboxylation abilities at primary screening on CFA

Origin		Number of isolated bacteria		Number of isolates that showed positive metabolic activity on CFA		
families	species			(++ sp)	(++ mp)	(+)
Asteraceae	*Arctium lappa*	11	89	2	0	6
	Artemisia montana	4		0	0	2
	Erigeron annuus	8		1	0	0
	Helianthus tuberosus	2		0	0	1
	Matricaria matricarioides	2		0	0	0
	Rudbeckia laciniata	37		5	3	4
	Senecio vulgaris	9		1	0	2
	Sonchus olaraceus	16		4	1	3
Boraginaceae	*Omphalodes* sp.	13		1	3	3
Caryphyllaceae	*Sagina* sp.	6		1	1	0
Chenopodiaceae	*Chenopodium album*	12		4	1	4
Commelinaceae	*Commelina communis*	6		1	2	0
Davilliaceae	*Davillia* sp.	4		0	0	1
Equisetaceae	*Equisetum arvense*	4	14	2	0	2
	Equisetum hyemale	10		3	0	2
Fabaceae	*Trifolium pratense*	13	33	2	1	6
	Trifolium repens	20		5	0	6
Geramiaceae	*Geranium robertianum*	10		0	0	2
Labiatae	*Mentha spicata*	6		1	0	4
Onagraceae	*Epilobium* sp.	11		1	0	2
Oxalidaceae	*Oxalis corymbosa*	7		1	0	4
Plantaginaceae	*Plantago asiatica*	10	15	1	1	3
	Plantago lanceolata	5		0	0	2
Polygonaceae	*Polygonum aviculare*	6	28	0	0	1
	Polygonum lapathifolium	8		4	0	4
	Polygonum pubescens	6		3	1	2
	Polygonum sp.	6		1	0	2
	Rumex obtusifolius	2		0	0	0
Rosaceae	*Agrimonia pilosa*	6	17	0	0	0
	Aruncus dioicus	6		3	0	0
	Potentilla freyniana	5		1	0	2
Salicaceae	*Salix bakko*	4		2	0	0
Scrophulariaceae	*Antirrhinum majus*	5		1	0	2
Simaroubaceae	*Ailanthus altissima*	8		0	2	1
Umbelliferae	*Aegopodium podagraria*	8	40	1	0	3
	Anthriscus sylvestris	11		1	1	1
	Coelopleurum lucidum	8		0	1	3
	Cryptotaenia japonicum	13		3	1	3
Vitaceae	*Vitis coignitae*	1		1	0	0
20 families	38 species	Total	332 isolates	57	19	83

observed in *K. oxytoca* JCM 1665 (Table 3).[9]

Taken together, bacteria possessing *K. oxytoca*-type decarboxylation activity were not often found in the rhizoplane of wild plants. Many of (++) CFA decarboxylating bacteria did not accept either 4-HCA or 3,4-DHBA. In particular, 5 isolates (35A, 46A, 66M, 1I, and 25P), all of which the resting cells collected and suspended in Tris-HCl buffer (pH 7.2), could not accept 4-HCA as the decarboxylative substrate after overnight incubation. These isolates were further investigated for their response to inducers 6-hydroxynaphthoic acid (6-HNA) and (*E*)-ferulic acid (FRA), together with randomly selected (+) CFA decarboxylating bacteria (61G, 63Q from *Geranium robertianum* and *Arctium lappa* respectively) for comparisons. As shown in Table 4, 6-HNA known to be active on *K. oxytoca* JCM 1665[24] was not sufficient to induce 4-HCD on isolates 35A and 1L, as on 63Q and 61G. Although 46A, 66M and 25P showed 4-HCD activity in the cells, decarboxylation activity on 4-HCA were not observed to any of these 3 isolates (46A, 66M and 25P), same as other 4 isolates. Our screening also involved the exposure of these isolates to FRA in PD broth. As shown in Table 5, 3 isolates (1L, 63Q, and 66M) actively converted FRA into 3-methoxy-4-hydroxystyrene as (++)-positive, but they were all weakly active on 4-HCA (+). Among 7 isolates, 25P and 46A showed almost the same decarboxylating activities on 4-HCA as *K. oxytoca* JCM 1665 did, while these two isolates showed (+) decarboxylation activity on FRA.

When (*E*)-sinapic acid and (*E*)-2-hydroxycinnamic acid (2-HCA) were used as the substrates for decarboxylation assay, most bacterial cell suspensions positive to 4-HCD activity (++, both constitutive and inducible) were unable to accept these derivatives. Observing the TLC patterns of the decarboxylation products of this group of bacterial isolate, 26P and 68I, however, had inducible-activity on 2-HCA due to the detection of a weak positive spot of decarboxylation product upon spraying Gibbs reagent to detect phenolic substances (Table 6).

3. Observations of other metabolic properties during primary screening

During primary screening of the rhizoplane bacteria, 12 isolates produced dark-brown coloring matter in their culture medium after 3–5 days of shaking incubation in the dark at 25°C, 100 rpm (Fig. 4). The bacterial isolates that showed the most active pigmentation (+++) in the cultured medium originated from the family Asteraceae, Onagraceae, and Geraniaceae (Table 7). These bacterial isolates were observed to have weak or no abilities to metabolize CFA. The other medium-browning bacteria (++ and +) had also relatively weak or no abilities to metabolize the test substrate, with a similar tendency to that reported by Hashidoko *et al*.[25] Exceptionally, both 30B and 31E possessed (++) decarboxylation abilities on CFA by converting all the test substrate (CFA) into single metabolite (3,4-dihydroxystyrene).

Table 3 Ability of rhizoplane bacteria to metabolize phenolics of plant origin in constitutive/inductive bioassay

Bacterial isolates	Origin		Cultured in PD broth		CFA-exposed cells in Tris- HCl	
	species	families	+ CFA	− CFA	4-HCA	3,4-DHBA
50F	*Arctium lappa*	Asteraceae	++	++	++	−
26P	*Artemisia montana*	Asteraceae	++	++	++	−
65L	*Rudbeckia laciniata*	Asteraceae	++	−	++	−
65Q			++	−	+	−
70D			++	−	++	−
70S			++	++	++	+
70T			++	++	++	−
32E			++	++	++	−
19K	*Omphalodes* sp.	Boraginaceae	++	+	++	+
21B	*Chenopodium album*	Chenopodiaceae	++	−	++	−
46A			++	+	−	−
60E			++	+	++	+
60I			++	−	++	+
5N	*Commelina communis*	Commelinaceae	++	−	+	+
5T			++	−	++	+
36G	*Equisetum hyemale*	Equisetaceae	++	−	+	−
1I	*Trifolium pratense*	Fabaceae	++	−	−	−
66G			++	−	++	−
66M			++	++	−	−
25P	*Trifolium repens*	Fabaceae	++	++	−	+
54A	*Mentha spicata*	Labiatae	++	++	++	+
54F			++	++	++	+
72N	*Epilobium* sp.		++	+	++	+
37E	*Oxalis corymbosa*	Oxalidaceae	++	+	+	−
37M			++	+	+	−
31F	*Plantago asiatica*	Plantaginaceae	++	++	+	−
31G			++	+	++	−
49B	*Polygonum lapathifolium*	Polygonaceae	++	+	+	−
49F			++	++	−	−
49G			++	+	+	−
59B	*Polygonum pubescens*		++	−	+	−
59C			++	++	++	−
59N			++	−	++	+
59P			++	+	++	+
59X			++	+	++	+
11L	*Aruncus dioicus*	Rosaceae	++	++	++	+
68H	*Ailanthus altissima*	Simaroubaceae	++	++	+	+
58B	*Salix bakko*	Salicaceae	++	+	+	−
58F			++	+	+	−
64J	*Aegopodium podagrari*	Umbelliferae	++	++	+	−
35A	*Anthriscus sylvestris*		++	−	−	+
18E	*Coelopleurum lucidum*		++	++	+	−
18R	*Cryptotaenia japonicum*		++	++	+	−
14F			++	++	+	−
14G			++	++	++	−
K. oxytoca JCM 1665 (Reference bacterium)			++	+	++	+

Signs (+ and −) have same meanings as described in Table 1. (++) are involved in (++) in this table. (− CFA) is the bacterial cells cultured in PD without any 4-HCD inducer. When resulting resting cell in Tris-HCl buffer showed (++) decarboxylation on CFA, the bacterial 4-HCD is constitutive, while (−) is absolutely inducible and (+) is weakly inducible. For each resting cell suspension, decarboxylation assays on 4-HCA and 3,4-DHBA were done. CFA: caffeic acid, 4-HCA: 4-hydroxycinnamic acid, 3,4-DHBA: 3,4-dihydroxybenzoic acid. Code in the left column showed specimen of source plants (as a number) and bacterial isolates (as an alphabet) in the combination. Locations of the source plants are described in figure legend of Fig. 3.

Table 4 Decarboxylation activity of some selected bacterial isolates that had been exposed to 6-HNA to induce 4-HCD and then suspended in Tris-HCl buffer to react with CFA or 4-HCA

Bacterial isolates	Origin		Decarboxylation activity	
	Plant species	Plant families	CFA (in Tris-HCl)	4-HCA (in Tris-HCl)
35A	*Anthriscus sylvestris*	Umbelliferae	+	−
46A	*Chenopodium album*	Chenopodiaceae	++	−
1I	*Trifolium pratense*	Fabaceae	+	−
66M	*Trifolium pratense*	Fabaceae	++	−
25P	*Trifolium repens*	Fabaceae	++	−
63Q*	*Arctium lappa*	Asteraceae	+	−
61G*	*Geranium robertianum*	Geraniaceae	+	−
K. oxytoca JCM 1665 (Reference bacterium)			++	+

Bacterial isolates selected in this experiment were those shown clear activity (++) to 5 mM CAF but no response (−) to 5 mM of 4-HCA, after exposure to 2 mM CFA as a 4-HCD-inducer. In this experiment, concentration of 6-HNA, non-substrative 4-HCD-inducer, was set at 0.5 mM in PD broth and incubated for 24 h, and then exposed to 5 mM CFA (center) or 4-HCA (right). Signs (+ and −) have the same meanings as described in Table 1. Left column was to check decarboxylation of 6-HNA itself, while center and right columns showed decarboxylation activities on CFA and 4-HCA, respectively, of the resting cells previously exposed to 6-HNA. (++): All the substrate was decarboxylated to give corresponding non-oxidative decarboxylation product. (+): Decarboxylative, but the substrate remains unchanged. (−): No active decarboxylation. Asterisk shows (+) 4-HCD-positive bacteria used for comparisons. 6-HNA: 6-hyroxynaphthoic acid, CFA: caffeic acid, 4-HCA: 4-hydroxycinnamic acid.

Table 5 Decarboxylation activity of some selected bacterial isolates exposed to FRA

Bacterial isolates	Origin		Decarboxylation activity	
	Plant species	Plant families	FRA (inducer, in PD)	4-HCA (in Tris-HCl)
35A	*Anthriscus sylvestris*	Umbelliferae	no growth	NT
46A	*Chenopodium album*	Chenopodiaceae	+	++
1I	*Trifolium pratense*	Fabaceae	++	+
66M	*Trifolium pratense*	Fabaceae	++	+
25P	*Trifolium repens*	Fabaceae	+	++
63Q*	*Arctium lappa*	Asteraceae	++	+
61G*	*Geranium robertianum*	Geraniaceae	no growth	NT
K. oxytoca JCM 1665 (Reference bacterium)			++	++

Signs (+ and −) have the same meanings as described in Table 1. (++): All the substrate was decarboxylated to give corresponding non-oxidative decarboxylation product. (+): Decarboxylative, but the substrate remains unchanged. (−): No active decarboxylation. Isolates 35A and 61G did not grow in 2 mM FRA-containing PD broth. NT: not tested. Asterisk indicates (+) 4-HCD-positive bacteria used for comparisons. FRA: ferulic acid, 4-HCA: 4-hydroxycinnamic acid.

DISCUSSION

The investigation of over 330 rhizobacteria from 37 different plant species and 20 different families have offered us the opportunity to screen and classify these rhizobacteria on the basis of their decomposition of common phenolics of plant origin. Nearly 1/4 of the bacterial collections showed marked decarbox-

Table 6 Decarboxylation ability of some decarboxylation-positive (++) isolates that also showed (++) on 4-HCA

Bacterial isolates	Origin		Decarboxylation activity		
			CFA	2-HCA	Sinapic acid
	Plant species	Plant families	(inducer, in PD)	(in Tris-HCl)	(in Tris-HCl)
50F	*Arctium lappa*	Asteraceae	++	−	−
26P	*Artemisia montana*		++	+	−
70D	*Rudbeckia laciniata*		++	−	−
70S			++	−	−
60E	*Chenopodium album*	Chenopodiaceae	++	−	−
60I			++	−	−
5N	*Commelina communis*	Commelinaceae	++	−	−
54A	*Mentha spicata*	Labiatae	++	−	−
54F			++	−	−
59N	*Polygonum pubescens*	Polygonaceae	++	−	−
59P			++	−	−
59X			++	−	−
72N	*Epilobium* sp.	Onagraceae	++	−	−
11L	*Aruncus dioicus*	Rosaceae	++	−	−
68H	*Ailanthus altissima*	Simaroubaceae	++	−	−
68I			++	+	−
K. oxytoca JCM 1665 (Reference bacterium)			++	−	−

Signs (+ and −) have the same meanings as described in Table 1. Bacterial isolates tested here are all those accepted 4-HCA as the substrate (++). Bacterial cells were exposed to 2 mM CFA in PD during the cell growth, and tested for 2-HCA and sinapic acid in Tris-HCl buffer. Response (+) defined as giving weak but a Gibbs reagent-positive (reddish purple) spot. This spot did not appear on TLC in resting cell suspension of the −CAF 26P and 68I. 2-HCA: 2-hydroxycinnamic acid.

Table 7 Strength of metabolism of CFA by plant samples and their families that gave dark-brown colored PD medium after shaking culture in the dark

Bacteria	Plant species	Plant families	Pigmentation	4-HCD	4-HCD in rhizomicrofloral community (++ frequency)
63I	*Arctium lappa*	Asteraceae	+++	−	*a* (5/7)
33D	*Epilobium cephalostigma*	Onagraceae	+++	+	*c* (1/3)
72I	*Epilobium* sp.	Onagraceae	+++	−	*c* (2/8)
40D	*Erigeron annuus*	Asteraceae	++	+	*b* (1/2)
61K	*Geranium robertianum*	Geramiaceae	++	−	*c* (2/6)
31E	*Plantago asiatica*	Plantaginaceae	++	++	*b* (3/6)
22K	*Rudbeckia laciniata*	Asteraceae	+	−	*c* (0/9)
22P	*Rudbeckia laciniata*	Asteraceae	+	−	
24H	*Rumex obtusifolius*	Polygonaceae	++	+	*c* (0/2)
30B	*Sagina* sp.	Caryphyllaceae	++	++	*c* (2/6)
25H	*Trifolium repens*	Fabaceae	+	−	*b* (1/5)
8H	*Trifolium pratense*	Fabaceae	+	+	*b* (2/8)

For browning or darkening culture pigmentation (left column), (+++) signifies dark PD culture medium, (++) light dark pigmentation, and (+) brown pigmentation. In terms of 4-HCD activities (center column), signs (+ and −) have the same meanings on CFA as devised previously in Table 1. In the right column, frequency of 4-HCD-positive bacteria in the rhizobacterial microflora was shown by numbers of (++) 4-HCD-positive isolates out of the total rhizobacterial isolates in the rhizomicrofloral community of each host specimen. For the frequency of 4-HCD-positive bacteria, (*a*), (*b*) and (*c*) were defined as follows: (*a*), over 50% (++) 4-HCD isolates and no inclusion of (−), (*b*), no inclusion of (−) or the same or more numbers of (++) than (−), and (*c*), predominant (−) soil bacteria.

ylation capability on CFA (57 isolates as ++ sp, and 19 isolates as ++ mp) (Table 1). These 4-HCD-positive rhizobacteria may tend to be enhanced by metabolic properties of some plant species and families, due to regular exposure to the phenolic acids highly accumulated in the root tissues. Accumulation of chlorogenic acid and CFA in roots has been reported in several allelopathic plants[26] and also several crop and herb[27,28]. Our second screening processes of the (++) isolates on CFA enabled us to determine whether or not the (++) 4-HCD-positive rhizobacteria have constitutive or inducible 4-HCD activities. These bioassays gave us a broader understanding of the mutual association between phenolic acid-decarboxylating rhizobacteria and host that exists within the soil community.

In our previous 4-HCD (synonym of phenylacrylic acid decarboxylase, PAD) research, *K. oxytoca* JCM 1665 was capable of inducing 4-HCD under exposure to CFA, FRA, 4-HCA and 6-HNA,[24] and it also had a remarkable gallate decarboxylase (GD) activity to convert 3,4-DHBA into the corresponding non-oxidative decarboxylated compound, pyrocatechol[9] (Fig. 3). Decarboxylation of the carboxy group with 4-HCD is probably an important decomposing process of plant polyphenolic acids exuded and/or accumulated in plant roots. Moreover, it is also likely that 4-HCD-possessing rhizobacteria are players to determine the microfloral composition that are selected and survived in the plant root community[25].

During secondary screening of selected and cultured 45 rhizobacteria positive as (++ single product) 4-HCD that showed positive activity on CFA, in a constitutive/inductive assay using *K. oxytoca* JCM 1665 as a positive control, many of the tested bacteria could not decompose 4-HCA, unlike *K. oxytoca* JCM 1665 (Table 3). This assay result suggested two possibilities as follows: 1) some bacterial isolates that were able to decompose only CFA but not 4-HCA possessed or produced a different type of 4-HCD, and 2) CFA changed permeability of the cell membrane or the cell wall. This narrow substrate-acceptability in the decarboxylation of 4-hydroxylated cinnamic acids probably leads to higher susceptibility of the CFA-specific 4-HCD producing bacteria to 4-hydroxystyrene than 3,4-dihydroxystyrene. In fact, when these bacterial isolates were exposed to an active 4-HCD inducer 6-HNA, instead of CFA, decarboxylation activity of the resting cells to 4-HCA was all negative (−), same as 4-HCD-induced resting cells by CFA (Table 4). Hence, 4-hydroxycinnamate decarboxylase of the (++) 4-HCD-positive isolates were presumed to be a different type of 4-HCD from that of *K. oxytoca* JCM 1665[29]. As 4-HCD activities of 35A, 46A and 1I were substrate-inducible, molecular information of their 4-HCDs should be focused in future. Exposure to 2 mM FRA made isolates 25P and 46A possessed (++) decarboxylating activity on 4-HCA but not clear on FRA (as +), while other three 66M, 1I, and 63Q showed a completely opposite response to FRA and 4-HCA (++ and +, respectively) (Table 5). Substrate-acceptability study, including that shown in

Table 6, showed us a high diversity of bacterial 4-HCDs toward several substrates and enzyme production. We are yet to find out the reasons for these observations.

The melanization-like pigmentation in some cultured medium may either be the result of peroxidase or tyrosinase activity (Fig. 4). Hashidoko *et al*. have reported that melanization-occurring bacterium *Pseudomonas viridiflava* can share substrate with another 4-HCD-producing bacterium[25]. In their experiment, it has been shown that substrate-specificity of the bacterial peroxidase to CFA was unexpectedly lower than that of horseradish peroxidase[25]. As shown in Table 7, almost all the melanization-like pigmentation-positive bacteria are 4-HCD negative (− or +). Bacterial isolates 33D, 72I, 61K, 22K, 22P and 24H (a member of type *c* microflora, but they were negative of 4-HCD) may exclusively utilize the phenolic acids for their polymerization because of less competition among microfloral members for the substrate, similar to the case of *P. viridiflava*. In contrast, 8H, 63I, 40D and 25H probably utilize only decarboxylated products, because their microflora is comprised of powerful 4-HCD-positive bacteria. Furthermore, both 31E (from *Plantago asiatica*) and 30B (from *Sagina* sp.) possessed not only the enzymes for pigmentation and phenolic-polymerization but also the enzymes for CFA-decarboxylation. Above results showed that metabolic and ecological positions of the melanization-like pigmentation-positive bacteria are highly diverse.

This study suggested that 4-HCD-positive rhizobacteria inhabited certain plant species and/or certain conditioned soils with a high frequency. In particular, 8 isolates from a specimen of *Polygonum lapathifolium* in site 2 were all 4-HCD-positive, in which 4 isolates were (++) while others were (+). On the other hand, other observations rather suggested that not plant species but location and soil environments determine frequency of 4-HCD-positive bacteria. For example, *Rudbeckia lanciniata* in site 8 (Fig. 2) possessed 5 isolates that were all positive as (++ sp) or (++ mp), but another specimen in site 5 carried 12 of 4-HCD-negative isolates out of 15 bacteria (Table 7). In Hokkaido University

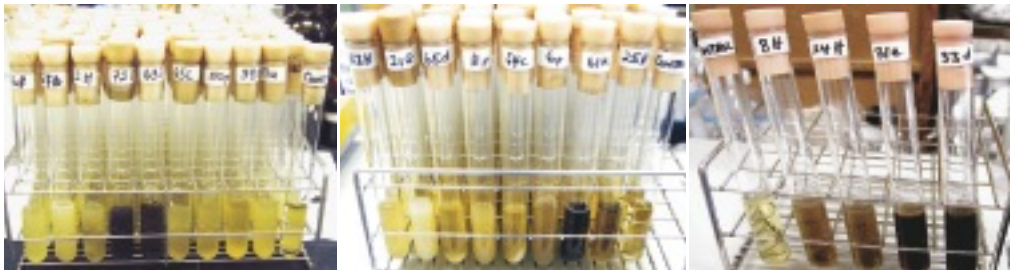

Fig. 4 Bacterial isolates that gave dark-brown pigmentation in the PD culture medium during primary screening for metabolic activity on CFA.
Pigmentation bacteria made 2 mM CFA-containing PD darken. As shown in Table 7, pigmentation was classified into (+++), (++), (+) and (−). Isolates 63I, 33D, and 72I are (+++), while 63I and 61K are (++). 25H, 8H and 24H were categorized as (+), showing transparent brown color.

Campus, natural weeds generally possessed 4-HCD-positive bacteria in the rhizoplane, but it is still difficult to find requirements for host plants to possess 4-HCD-positive rhizobacteria.

Plant species/families naturally select the dominant epiphytic bacteria that are likely to have tight relationship via plant-producing secondary metabolites, including polyphenolics and phenolic acids, to establish mutual benefits from each other[30]. Microorganisms that inhabit phenolics-rich plant species/families would have such versatile abilities to change the phenolics they encounter into detoxified forms that are not harmful to them[10]. In such phenolic acid-rich plants, rhizobacteria having an phenolic acid-conversible abilities may eventually modify the ecosystem to evolve new stages of the mutual relationships or to alter microfloral components. This later stage probably leads to stable microfloral communities to exclude phytopathogenicity of a member, as 4-hydroxylated styrene derivatives were more sensitive to phytopathogenic fungi than saprophytes[10]. Uchiyama *et al.* have recently developed PCR primers for specific detection of *Pad* gene from Gram-negative bacteria, and also revealed that *Pad* gene encoding 4-HCD had diversity in amino acid sequences[29]. This study certainly encouraged us to further investigate ecochemical significance of 4-HCD. Further studies are required for the statistical evaluation of the roles and meaning of *Pad* gene diversity on the prevalence of decarboxylation-positive or negative rhizobacteria in plant species/families.

In our another current report on screening of anti-*Fusarium oxysporum* rhizobacteria on PD agar medium, only 4 isolates out of 541 were active, and all the active bacteria were 4-HCD-negative.[31] It is, hence, awaited to investigate linkage between the decarboxylation ability on 4-hydroxycinnamates and other physiologically functional roles of rhizobacteria on host plant, such as IAA production, N_2-fixation, mycorrhizal association, and extracellular polymeric substance production. It should be discussed whether 4-HCD production would be an indicator for any types of rhizofunctional microorganisms.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan, by awarding Monbusho scholarship to the first author (A. A.). We also acknowledge Dr. Irnayuli R. Sitepu (Laboratory of Ecological Chemistry, Hokkaido University) for identification of some active rhizobacteria isolated during our screening works in the laboratory.

REFERENCES

1. Staman, K., Blum, U., Louws, F., and Robertson, D. (2001). Can simultaneous inhibition of seedling growth and stimulation of rhizosphere bacterial populations provide evidence for

phytotoxins transfer from plant residues in the bulk soil to the rhizosphere of sensitive species? *J. Chem. Ecol*., **27**: 807–829.
2. Sene, M., Dore, B., and Pellissier, F. (2000). Effect of phenolic acids in soil under and between rows of a poor sorghum (*Sorghum bicolor*) crops on germination, emergence and seedling growth of peanut (*Arachis hypogea*). *J. Chem. Ecol*., **26**: 625–637.
3. Gamini, S., and Jayasinghhearachi, J. S. (2003). Phenolic acids: possible agents of modifying N_2-fixing symbiosis through rhizobial alteration? *Plant Soil*, **252**: 385–395.
4. Appel, H. M., and Schultz, J. C. (1992). Activity of phenolics in insects may require oxidation. *In* R. W. Hemingway (ed). Plant Polyphenols: Biogenesis, Chemical Properties and Significance. Plenum Press, New York. pp. 609–620.
5. Appel, H. M. (1993). Phenolics in ecological interactions: the importance of oxidation. *J. Chem. Ecol*., **19**: 1521–1552.
6. Blum, U., Staman, K. L., Flint, L. J., and Shafer, S. R. (2000). Induction and/or selection of phenolic acid-utilizing bulk soil and rhizosphere bacteria and their influence on phenolic acid and phytotoxicity. *J. Chem. Ecol*., **26**: 2059–2078.
7. Schlösser, E. (1997). Performed structural and chemical barriers. *In* H. Hartleb, R. Heitefuss and H. H. Hoppe (eds). Resistance in Crop Plants against Fungi. Gustav Fischer: Jena Stuttgart, Lübeck, Ulm. pp. 84–99.
8. Schûlz, B., Rommert, A. K., Damman, U. H. J., and Aust Strack, D. (1999). Endophyte-host interaction: a balanced antagonism? *Mycol. Res*., **103**: 1275–1283.
9. Hashidoko, Y., Itoh, E., Yokota, K., Yoshida, T., and Tahara, S. (2002). Characterization of five phyllosphere bacteria isolated from *Rosa rugosa* leaves and their phenotypic and metabolic properties. *Biosci. Biotechnol. Biochem*., **66**: 2474–2478.
10. Hashidoko, Y., Urashima, M., Yoshida, T., and Mizutani, J. (1993). Decarboxylative conversion of hydroxycinnamic acids by *Klebsiella oxytoca* and *Erwinia uredovora*, epiphytic bacteria of *Polymnia sonchifolia* on damaged leaves. *Biosci. Biotechnol. Biochem*., **57**: 215–219.
11. Hashidoko, Y., Tada, M., Osaki, M., and Tahara, S. (2002). Soft gel medium solidified with gellan gum for preliminary screening for root-associating, free-living nitrogen-fixing bacteria inhabiting the rhizoplane of plants. *Biosci. Biotechnol. Biochem*., **66**: 2259–2263.
12. Schultz, J. C. (1989). Tannin-insect relationship, *In* R. W. Hemingway, and J. J. Karchesy (eds). Chemistry and Significance of Condensed Tannins. Plenum Press, New York. pp. 417–433.
13. Schlesinger, W. H. (1991). Biogeochemistry: An Analysis of Global Change. Academic Press, San Diego, California.
14. Austin, P. J., Suchar, L. A., Robbins, C. T., and Hagerman, A. E. (1989). Tannin- binding proteins in saliva of deer and their absence in saliva of sheep and cattle. *J. Chem. Ecol*., **15**: 1335–1347.
15. Steinberg, P. D., Edyvane, K., De Nys, R., Birdsey, R., and Van Altena, F. A. (1991). Lack of avoidance of phenolic rich brown algae by tropical herbivorous fishes. *Mar. Biol*., **109**: 335–343.
16. Steinberg, P. D., and Van Altena, F. A. (1992). Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecol. Monogr*., **62**: 189–222.
17. Harborne, J. B. (1988). Flavonoids in the environment: structure-activity relationships. *In* V. Cody, E. Middleton Jr., J. B. Harborne, and A. Beretz (eds). Plant Flavonoids in Biology and Medicine II. Biochemical, Cellular, and Medicinal Properties. A. R. Liss, New York. pp. 17–

27.
18. Nichols-Orains, C. (1991). Differential effects of condensed and hydrolysable tannin on polyphenol oxidase activity of attine symbiotic fungus. *J. Chem. Ecol*., **17**: 1811–1819.
19. Cavin, J. F., Barthelmebs, L., and Divies, C. (1997). Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, overexpression in *Escherichia coli*, purification and characterization. *Appl. Environ. Microbiol*., **63**: 1939–1944.
20. Goodey, A. R. and Tubb, R. S. (1982). Genetic and biochemical analysis of the ability of *Saccharomyces cerevisiae* to decarboxylate cinnamic acids. *J. Gen. Microbiol*., **128**: 2615–2620.
21. Hara, M. (1992). Plants in Sapporo, List of Species and Distribution, Hokkaido University Press, Sapporo, Japan (in Japanese).
22. Gibbs, H. D. (1927). Phenol tests III, the indophenol test. *J. Biol. Chem*., **72**: 649.
23. King, F. E., King, T. J., and Manning, L. C. (1957). An investigation of the Gibbs reaction and its bearing on the constitution of jacareubin. *J. Chem. Soc. Part 1*: 563–566.
24. Hashidoko, Y., Tanaka, T., and Tahara, S. (2001). Induction of 4-hydroxycinnamate decarboxylase in *Klebsiella oxytoca* cells exposed to substrate and non-substrate 4-hydroxycinnamate analogs. *Biosci. Biotechnol. Biochem*., **65**: 2604–2612.
25. Hashidoko, Y., Urashima, M., and Yoshida, T. (1994). Predominant epiphytic bacteria on damaged *Polymnia sonchifolia* leaves, and their metabolic properties on phenolics of plant origin. *Biosci. Biotechnol. Biochem*., **58**: 1894–1896.
26. Rice, E. L. (1984). Allelopathy, 2nd Ed., Academic Press, Orlando, FL.
27. Cheminat, A., Zawatzky, R., Becker, H., and Brouillard, R. (1988). Caffeoyl conjugates from *Echinacea* species: structures and biological activity. *Phytochemistry*, **27**: 2787–2794.
28. Truong, V.-D., Mcfeeters, R. F., Thompson, R. T., Dean, L. L., and Shofran, B. (2007). Phenolic acid content and composition in leaves and roots of common commercial sweetpotato (*Ipomea batatas* L.) cultivars in the United States. *J. Food Sci*., **72**: 343–349.
29. Uchiyama, H., Hashidoko, Y., Kuriyama, Y., and Tahara, S. Identification of 4-hydroxycinnamate decarboxylase gene of wild *Klebsiella oxytoca* originally isolated from damaged *Polymnia sonchifolia* leaves. *Biosci. Biotechnol. Biochem*., **72**: 116–123 (2008).
30. Hashidoko, Y. (2005). Ecochemical studies of interrelationships between epiphytic bacteria and host plants *via* secondary metabolites. *Biosci. Biotechnol. Biochem*., **69**: 1427–1441.
31. Asante, A., Hashidoko, Y., Deora, A., and Tahara, S. (2007) Antagonistic *Gluconobacter* sp. induces abnormal morphodifferentiation to *Fusarium oxysporum* hyphae. *J. Pestic. Sci*., **33**: in press (2008).