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Efficient Conversion of L-Tryptophan to Indole-3-Acetic Acid and/or Tryptophol by Some Species of *Rhizoctonia*

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In a study of various phytopathogenic fungi, we found that fungi that belong to the genus *Rhizoctonia* produce IAA efficiently from tryptophan. *R. solani* Kühn MAFF-305219, in particular, produced large amounts of tryptophol (Tol), which was assumed to be a specific by-product of the indole-3-pyruvate (IPy) pathway, in addition to IAA. Therefore, this fungus seemed suitable for analysis of the function and the regulation of the biosynthesis of auxin by a fungal pathogen. Under normal aerobic conditions, the ratio of IAA to Tol synthesized by this strain was higher than that under less aerobic conditions. In metabolic studies with various indole derivatives, *R. solani* converted L-tryptophan and indole-3-acetaldehyde to IAA and Tol, but other indole derivatives were scarcely metabolized. These results suggest that both IAA and Tol are synthesized from tryptophan through the IPy pathway in *Rhizoctonia*.

Key words: Auxin — Fungi — IAA — Indole-3-pyruvate pathway — *Rhizoctonia* — Tryptophol.

It is well known that not only the plants but also many microorganisms produce the plant hormones. Especially many of the microorganisms that induce hyperplasia and hypertrophy of plants have been reported to produce auxin. *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* are well-studied examples of such microorganisms.

A. tumefaciens induces tumors, called crown galls on a wide range of mainly dicotyledonous plant species. It has two genes for proteins that are involved in the formation of IAA from Trp, designated *tms*-1 and *tms*-2 (Schröder et al. 1984, van Onkelen et al. 1985). They encode tryptophan monooxygenase and indoleacetamide hydrolase, respectively. These two genes are on the T-DNA region of the Ti plasmid that is integrated into the plant chromosome after the bacterium has infected the host plant (Akiyoshi et al. 1983, Chilton et al. 1977, Thomashow et al. 1984). *P. savastanoi* also causes the gall and its genome includes genes that encode the same two enzymes (Comai and Kosuge 1980, 1982, Follin et al. 1985). *Bradyrhizobium japonicum*, which is a symbiotic bacterium that causes root nodules, has a gene for a protein with the same function as the protein encoded by *tms*-2 (Sekine et al. 1989).

The genes related to the production of IAA and the biosynthetic pathway to IAA have also been examined in the bacteria that are not strongly associated with plants, namely, *Enterobacter cloacae* (Koga et al. 1991b) and *Azospirillum brasilense* (Costacurta et al. 1994). In these bacteria, IAA is produced via a pathway different from that in *A. tumefaciens* and *P. savastanoi*. In *E. cloacae* and *A. brasilense* IAA is synthesized via the indole-3-pyruvic acid (IPy) pathway, from tryptophan (Trp), via IPy and indole-3-acetaldehyde (IAAld). Tol is also accumulated and has been reported to be a specific by-product of the IPy pathway. Indole-3-pyruvate decarboxylase (IPDC) has been clearly demonstrated to be the key enzyme of this pathway in *E. cloacae* (Koga 1995). This pathway has been considered to be the main pathway in plants for many years, however, recent analysis of mutants suggested that Trp is not an only precursor to IAA and that there exist pathways in plants other than the IPy pathway (Wright et al. 1991). The presence of the indole-3-acetamide (IAM) pathway, from IAM to IAA (Rajagopal et al. 1994) and from Trp to IAA via IAM (Kawaguchi et al. 1993), has also been reported. The pathway for the biosynthesis of auxin in higher plants remains to be clarified (Normanly et al. 1995).

We tried to detect a gene for IPDC in higher plant using a bacterial probe, but we were unsuccessful. As mentioned above, microorganisms that interact with plants often produce large amounts of auxin and the production of auxin plays an important role in the interactions of the plants with the microorganisms. Therefore, in this study, from among various phytopathogenic fungi, we chose as experimental material a fungus which efficiently produced both IAA and Tol and seemed suitable for investigations of the IPy pathway in eucaryotes. The conversion of Trp and various indole derivatives by the selected fungus, *Rhizoc-*

Abbreviations: FAA, formyl anthranilate; IAM, indole-3-acetamide; ILA, indole-3-lactic acid; IAAld, indole-3-acetaldehyde; IAN, indole-3-acetonitrile; IBA, indole-3-butyric acid; IPy, indole-3-pyruvic acid; IPR, indole-3-propionic acid; IPDC, indole-3-pyruvate decarboxylase; TNH_2, tryptamine; Trp, tryptophan; Tol, tryptophol; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; PDL, potato dextrose liquid medium.

tonia solani, and conditions for regulation of the production of tryptophol (Tol) and IAA are described.

Materials and Methods

Fungal strains and culture conditions—All fungal strains were obtained from the MAFF Gene Bank (National Institute of Agrobiological Resources, Tsukuba, Japan). They were cultured at 25°C in the dark on solid medium or in liquid medium as described below. The solid medium consisted of potato dextrose agar medium (Difco, Detroit) in 9-cm Petri dishes. The liquid medium was potato dextrose liquid medium (PDL) (Difco, Detroit) with or without 0.1% L-Trp. It was shaken at 140 rpm on a rotary shaker.

Detection by HPLC of the production of IAA and Tol by various kinds of fungi—The fungi (25 mm^2) were cultured in liquid medium with 0.1% Trp for 2 days. After incubation, each culture was diluted 10-fold with 0.1 M HCl and the supernatant after centrifuged at 18,000 × *g* was analyzed by HPLC (model CCPM, Tosoh, Tokyo) under the following conditions: column, Tosoh TSKgel-ODS-120A (4.6 mm i.d × 250 mm, Toyo Soda Co., Ltd., Tokyo); mobile phase, acetonitrile-water-acetic acid (38 : 61 : 1, v/v); flow rate, 1 ml min^{-1}; and detector, model FS-8000 spectrofluorometer (excitation wavelength, 280 nm; emission wavelength, 350 nm; Tosoh).

Extraction and purification of metabolic products of Trp—Fungal cultures were centrifuged at 18,000 × *g* for 20 min at 4°C. The supernatant was adjusted to pH 10 with Na_2CO_3 and extracted with diethylether (Tol fraction). This fraction was evaporated to dryness. The residue was dissolved in methanol and used for further purification by HPLC. The aqueous phase was adjusted to pH 2.5 with HCl and extracted with ethylacetate (IAA and peak 1 fractions (Fig. 1)). Each ethylacetate extract was evaporated to dryness. Each residue was dissolved in 25% methanol, 1% acetic acid (brought to pH 4.5 by the addition of NaOH) and used for further purification by HPLC. The purification of IAA and peak 1 by HPLC was performed three times as described above, with the exception that the mobile phase and the detector were changed for the third round. The mobile phase for the first and second rounds was 25% methanol, 1% acetic acid (pH 4.5) and for the third round it was 30% methanol, 3% acetic acid and the detector was a UV-spectrometer (Tosoh, detection at 280 nm). The fractions corresponding to IAA and peak 1 from each step were collected and subjected, separately, to the next step in the purification. The purification of Tol by HPLC was performed once as the same as that of IAA and peak 1, with the exception that the mobile phase was 38% acetonitrile, 1% acetic acid.

Mass spectrometry—IAA, peak 1 and Tol purified from the each fractions, were identified by the electron impact-mass spectra with Shimadzu QP5000 (ionization voltage 70 eV) (IAA and peak 1) or GC-MS with JOEL JMS-DX300 (ionization voltage 70 eV) (Tol).

Effects of oxygen on the production of IAA and Tol—Fully grown *R. solani* (MAFF 305219) was cultured in flasks of various sizes (30 ml, 50 ml, 100 ml, 300 ml and 500 ml) in 10 ml of PDL plus 0.1% Trp for 1 day at 25°C with shaking at 140 rpm for 1 day. The fungi were submerged when cultured in flasks with a volume of less than 100 ml. The flasks were tightly capped to prevent air exchange. IAA and Tol were analyzed by HPLC as described above.

Conversion of various indole derivatives to IAA and Tol—A piece of the fungi of 25 mm^2 (MAFF305219) was cultured in PDL supplemented with final 500 μM of various indole derivatives (Trp, IAM, ILA, indole, TNH_2, IAAld, IAN, IBA, IPy, IPR, IAA and Tol) for 2 days. IAA and Tol were analyzed by HPLC as described above.

Metabolism of IAAld by some strains of Rhizoctonia—Pieces of the fungi of 25 mm^2 (MAFF305231, MAFF305219, MAFF305277, K22) were cultured in PDL supplemented with final 500 μM IAAld for 15 h under less aerobic conditions (5 ml of medium/30-ml flask). IAA and Tol were analyzed by HPLC as described above.

Results

Production of IAA by various fungi—The metabolites of Trp in the supernatant of culture medium were surveyed by HPLC. Table 1 shows details of the production of IAA and Tol by the various fungi when they were cultured in medium with 0.1% Trp. IAA was detected in the culture medium of several fungi (*C. beticola, G. fujikuroi, G. singulata, R. solani, V. nishicola*), but a large amount of Tol was detected only in that of *R. solani*. No appreciable amounts of IPy, IAAld, TNH_2, IAM or IAN were detected as metabo-

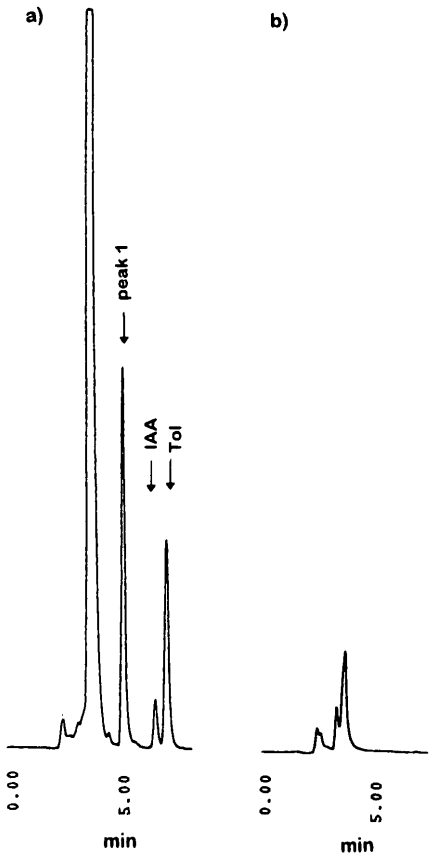

Fig. 1 Profile after HPLC of a culture of *R. solani* MAFF 305219. The fungi (25 mm^2) were cultured in liquid medium with 0.1% Trp or without Trp for 2 days. After incubation, each culture was diluted 10-fold with 0.1 M HCl and the supernatant was analyzed by HPLC. (a) cultured with 0.1% Trp; (b) cultured without Trp.

Table 1 Detection of the production of IAA by the various pathogenic fungi

MAFF	Fungus	Host plant	IAA	Tol
305023	*Ascochita pisi* Libert	*Glycine max* Merrill	−	−
305036	*Cercospora beticola* saccardo	*Beta vulgaris var. saccharifera*	+	−
305131	*Giberella fujikuroi* (Sawada) S. Ito	*Glycine max* Merrill	+	+
305137	*Glomerella singulata* (Stoneman) Spaulding et Schrenk	*Diopyros kaki* L.f.	+	−
712094	*Mycospherella melonis* (Parresini) Chiu et Walker	*Cucumis melo* L.	−	−
101001	*Pyricularia oryzae* Cavara	*Oryza sativa* L.	−	−
235002	*Pyricularia oryzae* Cavara	*Oryza sativa* L.	−	−
305637	*Pythium ultimum* Trow	*Fragaria grandiflora* Ehrh.	−	−
305003	*Rhizoctonia solani* Kühn	*Oryza sativa* L.	+	++
305216	*Venturia nashicola* Tanaka et Yamamoto	*Pyrus spp.*	+	+

The fungi (25 mm^2) were cultured in liquid medium with 0.1% Trp for 2 days.
++, more than 100 ng ml^{-1}; +, 10–100 ng ml^{-1}; −, less than 10 ng ml^{-1}.

lites of Trp in the culture of all fungi under these conditions. IAA nor Tol was detected in all the cultures without Trp (data not shown).

Detection of the production of IAA and Tol by the various species and strains of Rhizoctonia—In the cultures of all strains of *Rhizoctonia*, we detected IAA and/or Tol by treatment of 0.1% Trp (Table 2). However, the amount of each product was variable. MAFF305843 produced IAA most abundantly (about 400 ng ml^{-1}), and MAFF305219 produced the most Tol (about 250 ng ml^{-1}). The total

Table 2 Detection of the production of IAA and Tol by various species and strains of *Rhizoctonia*

MAFF	Strains	Host plant	IAA	Tol	IAA+Tol
235744	*R. cerealis* van der Hoevel	*Agrostis palustris* Huds.	+	+	+
235849	*R. oryzae* Ryker et Gooch	*Agrostis palustris* Huds.	−	++	++
235845	*R. solani* Kühn	*Agrostis palustris* Huds.	+	−	+
305204		*Beta vulgaris* L.	+	+	++
305206		*Solanum tuberosum* L.	++	+	++
305249		*Solanum tuberosum* L.	++	+	++
305259		Soil[b]	++	+	++
410375		*Betula ermanii* Cham.	+	+	++
305252		*Glycine max* Merrill	++	+	++
305255		*Archis hypogaea* L.	+++	−	+++
305220		*Lactica sativa* L.	+	+++	+++
305237		*Brassica oleaceae* L.	+	+	+
305843		*Solanum melongena* L.	+++	++	+++
305231		*Oryza sativa* L.	+++	+	+++
305219		*Oryza sativa* L.	++	+++	+++
K22[a]		*Oryza sativa* L.	+++	−	+++
235446	*R.* sp.		++	+	++
305267	*R.* sp. (binucleate)	*Arachis hypogaea* L.	+	+	+
305269		*Glycine max* Merrill	+	+	+
305277		*Oryza sativa* L.	+++	−	+++
305294		*Juncus decopiens* Nakai	++	−	++
305325		Soil[b]	++	+	++

The method was the same as that described in Table 1.
[a] Collected in Gifu prefecture. [b] Isolated from soil.
−, less than 10 mg ml^{-1}; +, 10–100 ng ml^{-1}; ++, 100–200 ng ml^{-1}; +++, more than 200 ng ml^{-1}.

amount of both IAA and Tol was about 550 ng ml^{-1} and 400 ng ml^{-1} in the culture media of MAFF305843 and MAFF305219, respectively. Considering that many indole derivatives are known to be converted non-enzymatically to IAA and that Tol is a component that is exclusively produced in the IPy pathway, we used MAFF305219, which had the highest Tol productivity, for further analysis.

Identification of IAA, Tol and Peak 1 by MS—IAA

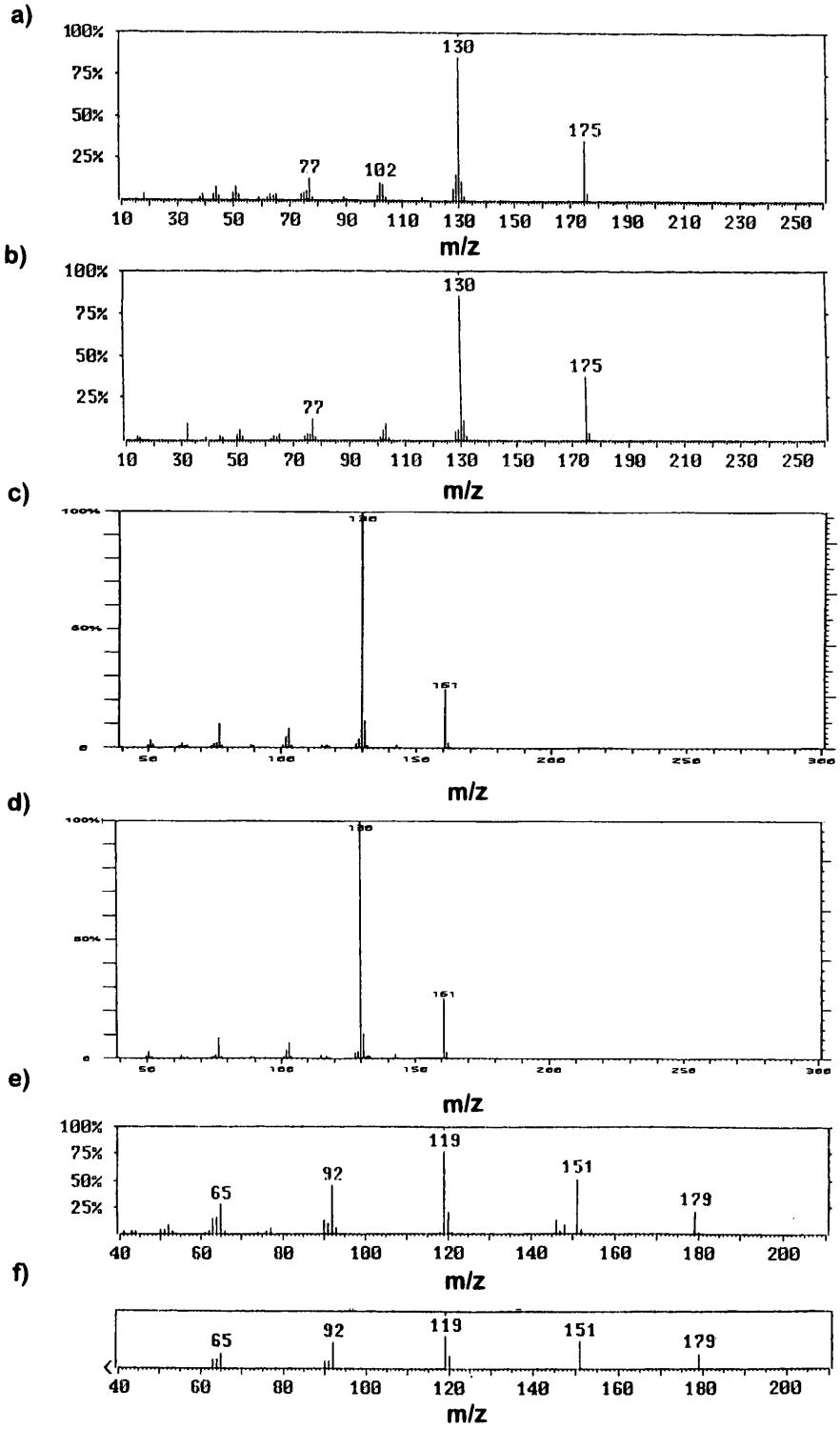

Fig. 2 MS of the IAA and Peak 1 fractions from a culture of *R. solani* MAFF305219 and GC-MS of Tol fraction from a culture of *R. solani* MAFF 305219. (a) Mass spectrogram of IAA fraction from the culture, (b) authentic IAA. (c) GC-MS of Tol fraction from the culture, (d) authentic Tol. (e) Mass spectrogram of peak 1 fraction from the culture, (f) authentic FAA referred to the database.

and Tol were purified as described in Materials and Methods and identified by direct mass-spectrometric analysis (IAA) or GC-MS (Tol) (Fig. 2a, b, c, d).

There was one major peak other than IAA and Tol when *R. solani* was cultured with Trp (Fig. 1). This peak was purified and identified as FAA by MS referring to the database (Fig. 2e, f).

Effects of oxygen on the production of IAA and Tol—The production of IAA and Tol by *R. solani* (MAFF 305219) was examined in flasks of various sizes with the same volume of medium. Since the total volume of oxygen was limited, the smaller the size of the flask, the less aerobic were the growth conditions. As shown in Fig. 3, under less aerobic conditions (30- to 100-ml flasks) the ratio of IAA to Tol was about 0.4, but under more aerobic conditions (200- to 500-ml flasks) that ratio increased to 0.8–1.1. This result suggests that the amount of oxygen in the culture influences the ratio. The total amount of Tol and IAA were also depended on the aerobic conditions (data not shown).

Conversion of various indole derivatives to IAA and Tol—The production of IAA and Tol was examined by adding various indole derivatives to the liquid medium to determine whether IAA and Tol were produced from the indole substrate or from other sources of nitrogen in the medium. Trp and IAAld were efficiently converted to IAA and Tol, and indole and IPy were converted to IAA and Tol to a limited extend (less than 10 ng ml^{-1}, Table 3). Neither IAA nor Tol was detected in the culture media that contained other indole derivatives. The conversion of IAAld to IAA and Tol was much more efficiently than that of Trp to IAA and Tol.

Conversion of IAAld to IAA and Tol by some strains of Rhizoctonia—Since the ratio of IAA to Tol produced by each strain of *Rhizoctonia* was rather variable and depended on aerobic conditions (Table 2, Fig. 3), the biotransformation of IAAld to IAA and Tol was examined under the less aerobic conditions for detection of Tol, with a short incubation time so that IAAld would not be oxidized to IAA

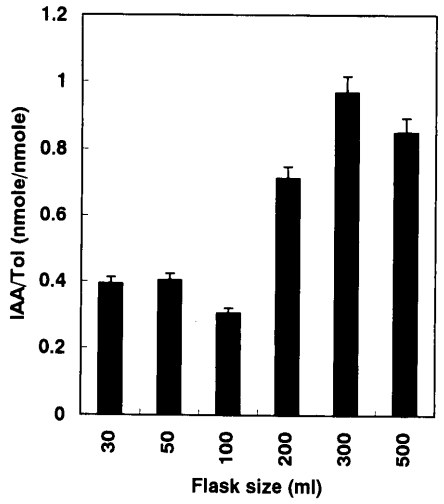

Fig. 3 Effect of aeration of the ratio of IAA to Tol. Fully grown *R. solani* was cultured in flasks of various sizes in 10 ml of PDL plus 0.1% Trp for 1 day at 25°C with shaking at 140 rpm.

Table 3 Conversion of tryptophan and other indole derivatives to IAA and Tol

	IAA (ng ml^{-1})	Tol (ng ml^{-1})
Trp	82	257
IAM	ND	ND
ILA	ND	ND
indole	±	±
TNH_2	ND	ND
IAAld	380	3,294
IAN	ND	ND
IBA	ND	ND
IPy	±	±
IPR	ND	ND
IAA	–	ND
Tol	ND	–

Pieces of the fungi of 25 mm^2 were cultured in PDL supplemented with final 500 μM of various kinds of indole derivaties for 2 days. Each value was the difference from the value of non-enzymatic conversion detected by the control experiments which was cultured without fungi. The value is an average of 3 replicates. ND, not detectable; ±, less than 10 ng ml^{-1}.

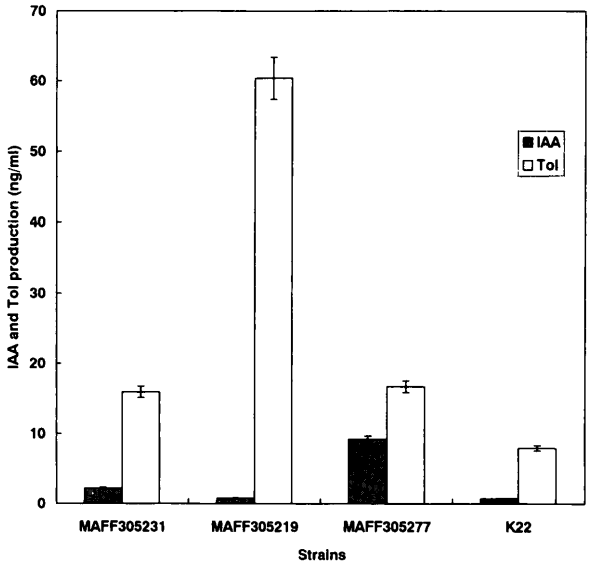

Fig. 4 Conversion of IAAld to IAA by some strains of *Rhizoctonia*. Pieces of the fungi of 25 mm^2 were cultured in PDL supplemented with final 500 μM IAAld for 15 h under less aerobic conditions (5 ml of medium/30-ml flask). Each value was the difference from the value of non-enzymatic conversion detected by the control experiments which was cultured without fungi. The value is an average of 3 replicates with a standard deviation.

automatically. All the fungal strains examined in this experiment produced detectable Tol and IAA (Fig. 4). However, the amounts of these two products and the ratio of these amounts were very different. The biotransformation by MAFF305219 of IAAld to Tol was the greatest of all (60 ng ml^{-1}), and the smallest was by K22 (8 ng ml^{-1}). The difference was more than 7-fold. The best product of IAA was MAFF305277 (9 ng ml^{-1}) and the worst was K22 (0.7 ng ml^{-1}). The difference was about 13-fold. In all cases, production of Tol and IAA was much higher when IAAld was added to the medium than when Trp was added (data not shown).

Discussion

To analyze the function of auxin and the regulation of the biosynthesis in eucaryotes in the pathogenecity, we surveyed the production of IAA by the various fungi. *Rhizoctonia* was found to produce much IAA and Tol among the tested genera. In particular, *R. solani* MAFF-305219 produced large amounts of Tol, which is a specific substance in the IPy pathway. *G. fujikuroi* and *V. nashicola* also produced small amounts of Tol. Therefore these two fungi may also have a functioned IPy pathway.

IAA and Tol were produced by *R. solani* when Trp and IAAld were added to the medium. It was unexpected that biotransformation of IPy to Tol and IAA was scarcely detectable. According to Koga et al. (1994), the K_m of the tryptophan aminotransferase that converts Trp to IPy is high (3.3 mM) in *E. cloacae* and the conversion of IPy to Trp can easily occur. However, no accumulation of Trp was observed in this case (data not shown). According to Koshiba and Matsuyama (1993), as IPDC was not detected in the plants, the conversion from IPy to IAA was unlikely to occur in them. And the reaction of the IAA-forming activity was apparently one step from Trp. But we have preliminarily detected the activity of IPDC in vitro, it is possible that IPy was not taken up by the fungi. Now the purification of this enzyme is in progress. Since small amounts of indole were converted to Tol and IAA, we cannot exclude the possibility that an IAA-producing pathway via indole exists. However, the major pathway is thought to be the IPy pathway, in view of the fact that Trp and IAAld were efficiently converted to IAA and Tol.

Concerning why Tol was not converted to IAA, some reasons were considered. (1) Tol was not the storage pool for IAA but excessive IAAld changed to Tol in this fungi. i.e. indole-3-ethanol oxidase doesn't exist. (2) Indole-3-ethanol oxidase exists but the condition such as oxygen concentration or pH was inhibitory for it. (3) Tol was not taken up by the fungi. The experiment in vitro is needed for answering this question.

The result that the ratio of IAA to Tol produced depended on aeration (Fig. 3) was consistent with results obtained by *E. cloacae* (Koga et al. 1991a). Furthermore, the conversion of IAAld to Tol appeared to be affected by the concentration of oxygen in the culture or by the concentration of IAAld because all the *Rhizoctonia* grown with IAAld in the medium produced Tol under less aerobic conditions (Fig. 4). Further experiments are needed to clarify that oxygen does actually affect this pathway and, if so, to find the target.

Two main pathways for synthesis of IAA have been found and well studied in microorganisms, in particular in bacteria. The IAM pathway is found in *A. tumefaciens* and *P. savastanoi,* which are tumorigenic bacteria. The IPy pathway is found in *E. cloacae, A. brasilense* and in some fungi, such as *Taphrina deformans* (Yamada et al. 1990) and *Physcomyces blakesleeanus* (Schramm et al. 1987, Ludwig-Müller et al. 1990). It is unclear why the IAM pathway is common to the tumorigenic bacteria while the bacteria that induce other symptoms have the IPy pathway. Manulis et al. (1991) proposed that the IPy pathway, which is commonly distributed in higher plants, is under strict regulation by plant metabolites to control the supply of IAA. Such metabolites might be present in infected wound tissues and, thus, regulate the bacterial IPy pathway as well. However, the ability of pathogens to overproduce IAA through the IPy pathway might possibly alter the physiological conditions in the host in favor of the pathogen.

Recently it has been shown that the nodule number on soybean roots by *Bradyrhizobium elkanii* was likely to correlated with IAA production in the culture. Inoculation with the IAA deficient mutants significantly reduced the nodule number as compared to that of the parent strain, and the addition of IAA restored that number to the original level (Fukuhara et al. 1994). Furthermore, the structure of the nodule was found to be different between the IAA positive strain and the negative one (Yuhashi et al. 1995). These results strongly suggest that IAA produced by *B. elkanii* is involved in soybean nodule formation.

We are now trying to purify the enzymes involved in this pathway and to isolate the corresponding genes in an attempt to clarify the role of IAA in *Rhizoctonia*. This work may also help to resolve issues related to the biosynthetic pathway to auxin in higher plants.

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