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Production of TMC-151, TMC-154 and TMC-171, a new class of antibiotics, is specific to '*Gliocladium roseum*' group

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A novel class of fungal metabolites, TMC-151, TMC-154, and TMC-171 series compounds, was found exclusively in *Gliocladium catenulatum*, *Clonostachys rosea* and closely related strains. These compounds were not detected in any other fungi examined. The production spectrum of each component was correlated to the morphology of the secondary conidiophores and the conidia. TMC-151 was limited to *Clonostachys rosea* (formerly *G. roseum*) forming navicular or reniform conidia or *G. catenulatum* with gray-green conidial masses, whereas TMC-154 and 171 were limited to the strains closely related to *Gliocladium roseum*, which grew more slowly and formed more symmetrical conidia.

Key Words——*Clonostachys compactiuscula*; *Clonostachys rosea*; *Gliocladium catenulatum*; *Gliocladium roseum*.

During screening of fungi for biologically active metabolites, we discovered a novel class of antibiotics designated as TMC-151, TMC-154, and TMC-171 (Kohno et al., 1999a, 1999b). They are characterized as possible polyketide-derived congeners containing sugars and hexitol or pentitol moieties (Fig. 1). This type of chemical entity had not previously been reported from fungi, although erythromycins from actinomycetes show some structural analogy to them. Taxonomic studies on three producers revealed that they should all be placed in *Gliocladium* Corda sensu lato (Domsch et al., 1980; here we use 'sensu lato' in contrast to the term *Gliocladium* sensu stricto used by Schroers et al., 1999), because the primary conidiophores are *Verticillium* Nees-like and the secondary conidiophores are densely penicillate with either oblique conidial columns or wet conidial heads as described and delimited by Domsch et al. (1980). Fungal metabolites are often produced by a restricted taxon. For example, isonitrile antibiotics are produced solely by *Trichoderma* Pers. (Okuda et al., 1982). Based on the production, *Trichoderma harzianum* Rifai has been divided into two groups. This division is well correlated with the morphology and minor genetic differences of *T. harzianum* strains (Fujimori and Okuda, 1994), and is further supported by the finding of Gams and Meyer (1998) that *T. harzianum* includes several types with distinct ribosomal DNA sequences. Since *Trichoderma* and *Gliocladium* are both anamorphs of *Hypocrea* Fr., we were prompted to examine the relationship between taxonomic characteristics and the production of the present TMC-series antibiotics. We therefore collected and examined a number of *Gliocladium* strains and related genera. Here we report the relationship between production and taxonomy.

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Materials and Methods

Strains used From our fungal library of tentatively identified strains preserved at $-80°C$, we selected mainly *Gliocladium* strains that appeared to be *Gliocladium roseum* sensu Domsch et al. (1980), and some other *Gliocladium* and *Trichoderma* strains. Most strains were isolated by us mainly from soil or plant samples collected in Japan (Table 1). *Clonostachys* Corda strains with CBS accession numbers were kindly supplied by Hans-Josef Schroers and Walter Gams of Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Three strains with IFO numbers were purchased from the Institute for Fermentation, Osaka, Japan.

Media Miura medium (LCA) was based on Miura and Kudo (1970). Oatmeal agar (OA) contained 23 g of Daigo Actino Medium No. 3 (Nihon Pharmaceutical Co., Tokyo, Japan) in 1,000 ml of distilled water. Two percent malt extract agar (2%MA) contained 20 g of malt extract and 20 g of agar in 1,000 ml of distilled water. The liquid medium used for seed cultures contained 10 g of glucose, 5 g of Polypepton (Nihon Pharmaceutical Co), 5 g of dried yeast (Wako Pure Chemical, Osaka, Japan), 200 ml of V-8 juice (Campbells Japan, Tokyo, Japan), 200 ml of apple juice (Kirin Tropicana Inc., Tokyo, Japan), and 5 g of $CaCO_3$ in 600 ml of deionized water, adjusted pH to 6.0 before autoclaving. The solid medium for production consisted of 10 g of rolled barley, 0.02 g of yeast extract, 0.01 g of Na-tartrate, 0.01 g

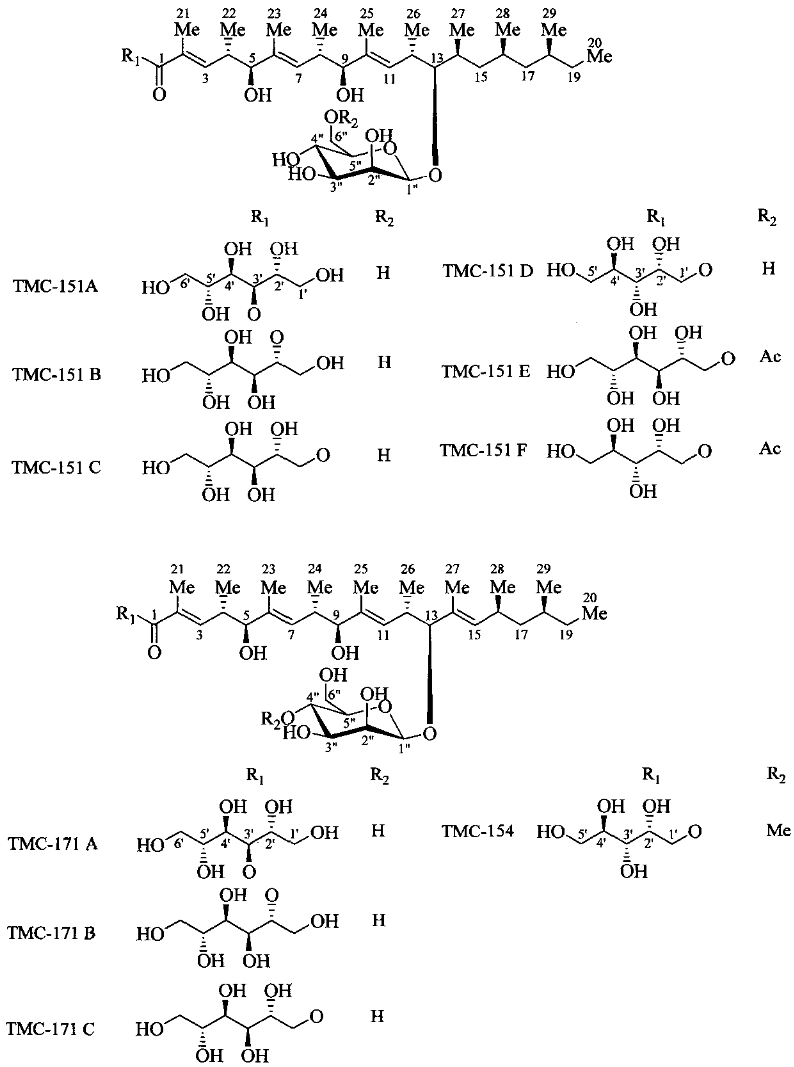

Fig. 1. Structures of TMC-151 A, B, C, D, E, F, TMC-171 A, B, C, and TMC-154.

of KH_2PO_4, and 20 ml of deionized water in a 250-ml Erlenmeyer flask.

Morphological characterization Each fungal strain was inoculated on LCA and OA at three points equidistant from the edge of the plate and from one another in a standard petri dish. The dishes were incubated at 25°C for 7 to 14 d under 12-h dark/12-h light conditions. To characterize conidial morphology precisely, we incorporated the parameters of area, circularity, and perimeter of conidia. These were calculated by using an image analyzing application, Optimas ver. 6.2 (Optimas Corp., Bothell, Washington). Area was the total area of conidia in two-dimensional lateral view in μm^2; perimeter was total length of the circumference of the area object (μm); and circularity was the ratio of the area perimeter length squared divided by the area. Conidial length, width, and the ratio of length/width were also measured with Optimas and expressed as follows. (n1)n2-n3-n4(n5) where n1=minimum value observed, n2=arithmetic mean minus standard deviation, n3=arithmetic mean, n4=mean plus standard deviation, and n5=maximum value observed (Table 4). More than 30 conidia were analyzed per strain. Technical terms used (such as primary and secondary conidiophores) are according to Domsch et al. (1980).

Production of the metabolites Each strain was inoculated in a test tube containing 6 ml of the liquid seed medium, which was shaken for 4 d at 27°C. The seed culture was transferred to a 250-ml Erlenmeyer flask containing the solid medium, and incubated under static conditions at 27°C for 12 d. Metabolites were then extracted with 20 ml of 1-butanol.

Analysis of the antibiotics by LC/MS Among a number of congeners of the antibiotics, production of the main components of TMC-151, TMC-154, and TMC-171 was examined. The productivity was analyzed by LC/MS (HP1100 Hewlett Packard, USA). Conditions for LC were as follows: column, YMC-Pack ODS-AM, AM-301-3 (4.6×100 mm, 37.0); eluent, CH_3CN-H_2O; time (min)/CH_3CN(%)=0/60, 10/60, 11/85, 16/85; flow rate,

Table 1. Strains examined.

Clonostachys compactiuscula (Saccardo) D. Hawksworth & W. Gams TC 1292 bark, Mt. Sekido, Kashima, Ishikawa Prefecture, Japan, 1996/5/1 → IFO 33101; IFO 7066; CBS 729.87; CBS 556.95.
Clonostachys sp. TC 1515 soil, Kashima Island, Tanabe, Wakayama Prefecture, Japan, 1998/5/18; TC 1516 soil, Kashima Island, Tanabe Wakayama Prefecture, Japan, 1998/5/18.
Gliocladium catenulatum Gilman & Abbott senseu Domsch et al (1980) TC 1280 soil from an asparagus field, Furumakibashi, Nakano, Nagano Prefecture, Japan, 1996/5/2 → IFO 33102; TC 1506 soil from a crop field, Tomino, Ishigaki Island, Okinawa Prefecture, Japan, 1997/3/7; IFO 31681; TC 1490 soil, Yotsukura, Iwaki, Fukushima Prefecture, Japan, 1999/2/7.
Gliocladium penicillioides Corda TC 1302 rotten wood, Yaku, Yakushima Island, Kumage, Kagoshima Prefecture, Japan, 1996/7/1.
Gliocladium roseum Bainier senseu Domsch et al (1980) and its closely related strains TC 1282 a fruiting body of *Tricholoma* sp., Kashiwabara Shrine, Kashiwabara, Nara Prefecture, Japan, 1995/11/15 → IFO 33104; TC 1294 soil, Kawagishi, Toda, Saitama Prefecture, Japan, 1995/11/21 → IFO 33099; TC 1295 soil, Hakone, Ashigarashimo, Kanagawa Prefecture, Japan 1995/11/26; TC 1296 soil, Hakone, Ashigarashimo, Kanagawa Prefecture, Japan 1995/11/26; TC 1297 living leaf, Mt. Tenran, Hannoh, Saitama Prefecture, Japan 1996/1/22 → IFO 33100; TC 1298 rotten bamboo, Shinsenko Temple, Tonami, Toyama Prefecture, Japan, 1996/5/2; TC 1299 rotten bamboo in a waterfall, Nakanogo, Hachijo, Hachijo Island, Tokyo, Japan, 1996/3/26; TC 1304 soil, location between Shahdol and Amarkantak, India, 1992/1/4; TC 1507 soil from a corn field, Kawagishi, Toda, Saitama Prefecture, Japan, 1997/5/22; TC 1508 stream water, Ohara, Taketomi, Iriomote Island, Okinawa Prefecture, Japan, 1997/7/3; TC 1509 stream water, Ohara, Taketomi, Iriomote Island, Okinawa Prefecture, Japan, 1997/7/3; TC 1510 stream water, Ohara, Taketomi, Iriomote Island, Okinawa Prefecture, Japan, 1997/7/3.
Gliocladium sagariense Saksena IFO 9080
Gliocladium viride Matruchot TC 1505 soil, Hie Shrine, Shuzenji, Takata, Shizuoka Prefecture, 1996/10/21; TC 1369 soil, Takeda Shrine, Kofu, Yamanashi Prefecture, 1997/1/24; TC 1368 stream water, Ohara, Taketomi, Iriomote Island, Okinawa Prefecture, Japan, 1997/7/3.
Trichoderma virens (J. Miller, Giddens & Foster) von Arx TC 1301 a fruiting body of discomycete, Oiwake, Karuizawa, Kitasaku, Nagano Prefecture, Japan, 1996/8/1.

1.0 ml/min; detection, UV 225 nm. Conditions for MS were: ionization mode, API-ES (electron spray); polarity, positive; MSD Mode, SIM (selected ion monitoring) 797, 799, 811, 829, 841, 871 $(M+Na)^+$, 813, 815, 827, 845, 857, 887 $(M+K)^+$; fragmentor, 200 V; drying gas flow, 10 L/min; nebulizer pressure, 50 psig; drying gas temp., 350°C; capillary voltage, 4000 V. Butanol extracts were concentrated to 1/2 volume, and samples of 5 μl were injected for analysis. The detection limit was 1 μg/ml.

Results and Discussion

Identification Our isolates were divided into seven groups. The first one was characterized by the pinkish shade of colonies, candelabrum branching pattern of secondary conidiophores, and navicular to ellipsoidal conidia of 3.5–7.2 × 2.1–3.7 μm, which were held in oblique chains or irregular slimy masses. The strains of this group, TC 1294, 1295, 1296, 1297, 1298, 1299, 1507, 1508, 1509, 1510, 1282, and 1304, were all assignable to *Gliocladium roseum* Bainier or its closely related strains according to the description by Domsch et al. (1980). *Gliocladium roseum* has recently been transferred to *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert & W. Gams (Schroers et al., 1999). This group was further divided into three subgroups, which will be discussed in a later section. The second group had almost the same characteristics as the first group except for gray to olive colonies. Based on Domsch et al. (1980), the strains, TC 1280, 1506, and 1490, were identified as *Gliocladium catenulatum* Gilman & Abbott. The third group contained one strain, TC 1292. It was characterized by pinkish colonies and more slender cylindrical conidia, and it lacked the primary verticillate conidiophores. Based on direct comparison with two CBS strains, it was identical to *Clonostachys compactiuscula* (Sacc.) D. Hawksworth & W. Gams. Strains of the fourth group, TC 1515 and TC 1516, were characterized by their whitish colonies and regularly cylindrical conidia, of which the L/W ratio was smaller than that of *C. compactiuscula*. The fifth group consisted of TC 1302. This strain showed yellowish colonies, totally appressed secondary conidiophores bearing almost parallel branches and phialides, and smaller cylindrical conidia. It was assignable to *Gliocladium penicillioides* Corda according to Matsushima (1975) and Schroers et al. (1999). The sixth group, comprising of TC 1301, was characterized by dark green spreading colonies, appressed short phialides, and large green broadly ellipsoidal conidia. It was identified as *Trichoderma virens* (J. Miller, Giddens & Foster) von Arx, formerly *Gliocladium virens* J. Miller, Giddens & Foster. The last group, containing TC 1368, 1369, and 1505, was characterized by densely penicillate conidiophores and slimy heads of dark green short cylindrical conidia. Strains were assigned to *Gliocladium viride* Matr. The isolates therefore covered pink and green *Gliocladium* strains of Hypocreales origin.

Production of the antibiotics TMC-151, TMC-154, and TMC-171 (Table 2) were not produced at the same time by any one of the 28 strains of 7 species examined. These compounds differ structurally in the presence or absence of a saturated bond at C14 position (Fig. 1).

Table 2. Production of the antibiotics and cultural characteristics.

Species	Strain	Production: TMC-151	Production: TMC-154	Production: TMC-171	LCA: Colony diameter (mm)	LCA: Texture	LCA: Color of conidial area (Munsell)	LCA: Color of conidial area	OA: Colony diameter (mm)	OA: Texture	OA: Color of conidial area (Munsell)	OA: Color of conidial area	OA: Pigment	OA: Reverse
Gliocladium catenulatum	TC 1280	++	−	−	39–41	Velutinous	5Y8/2	Grayish yellow to olive	40–43	Funiculose	5GY8/2	Grayish green		
	TC 1506	+	−	−	39–41	Velutinous funiculose	5GY9/2–8/2, 10Y7/4	Pastel green to grayish green	47	Funiculose	5GY8/2	Grayish green		Wax white
	TC 1490	+	−	−	34–37	Funiculose -velutinous	5Y7/2–10Y7/2	Grayish green to olive	43–48	Funiculose -floccose	10Y9/2–5GY9/2	Grayish yellow to pastel green		Grayish green
	IFO 31681	+	−	−	54–55	Funiculose	5Y9/2	Pale yellow	59	Funiculose	5Y9/2–10Y9/2	Pale yellow to grayish yellow		Dull yellow
Gliocladium roseum (=*Clonostachys rosea* typical strains)	TC 1294	+	−	−	43–48	Funiculose	10YR9/2	Cream	47–48	Funiculose	10YR9/2	Cream		
	TC 1295	+	−	−	33	Funiculose	10YR9/2	Cream	39–45	Funiculose	10YR9/2	Cream		
	TC 1296	+	−	−	30–32	Funiculose	10YR9/2	Cream	38–43	Funiculose	10YR9/2	Cream		
	TC 1297	++	−	−	36–38	Funiculose	10YR9/2	Cream	44–45	Funiculose	10YR9/2	Cream		
	TC 1298	+	−	−	30–34	Funiculose	10YR9/2	Cream	40–46	Funiculose	5–10YR9/2	Pale orange to cream		Grayish orange
	TC 1299	+	−	−	30–45	Funiculose	10YR9/2	Cream	38–44	Funiculose	10YR9/2–8/6	Cream to grayish orange		Grayish orange
	TC 1507	+	−	−	40–43		10YR9/2–8/8	Cream to melon yellow	45–48		10YR9/2–5Y9/2	Cream to pale yellow	Yellow	
	TC 1508	+	−	−	43–45	Funiculose	10YR9/2	Cream	53–55	Funiculose	10YR9/2	Cream		Cream
	TC 1509	+	−	−	35–40	Funiculose	10YR9/2–8/8	Cream to light orange	38–40	Funiculose	10YR8/4	Grayish orange		Grayish orange
	TC 1510	+	−	−	38–39	Funiculose	10YR9/2–8/6	Cream to grayish orange	40–43	Funiculose	5Y9/2	Pale yellow		Pastel yellow to grayish orange
(*G. roseum* Group B)	TC 1282	−	++	−	33–39	Floccose	5Y9/2	Pale yellow	30–35	Floccose	5Y9/2	Pale yellow	Pale yellow	
(*G. roseum* Group C)	TC 1304	−	−	++	30	Velutinous	10YR9/2	Cream	26–30	Velutinous	10YR9/2	Cream	Light yellow	Cream to light yellow
Clonostachys compactiuscula	TC 1292	−	−	−	24–29	Funiculose	5Y9/2	Pale yellow	23–25	Funiculose	5Y9/4	Pastel yellow		Vivid yellow to brownish yellow
	IFO 7066	−	−	−	40–55	Funiculose	5Y9/2	Pale yellow	40–50	Funiculose	5Y9/2–10Y9/2	Pale yellow to yellowish white		Cream to pastel yellow
	CBS 729.87	−	−	−	31–40	Funiculose	10YR9/2, 10YR8/2	Cream to orange-gray	30–36	Funiculose	5Y9/6, 10YR9/2, 10YR8/8	Light yellow		Light yellow to vivid yellow
	CBS 556.95	−	−	−	33–40	Funiculose -velutinous	10Y9/2–5GY9/2,	Yellowish white	33–48	Funiculose -floccose	10Y9/2–5GY9/2	Yellowish white		Grayish orange
Clonostachys sp.	TC 1515	−	−	−	25–38	Velutinous	5Y9/2–10YR9/2	Pale yellow	23–36	Velutinous	10YR8/8, 5Y9/2	Melon yellow to pale yellow		Brownish yellow to light yellow
	TC 1516	−	−	−	25–38	Funiculose	5Y9/2	Pale yellow	23–36	Funiculose	10YR9/2	Cream		Cream to grayish orange
Gliocladium sagariense	IFO 9080	−	−	−	25–38	Velutinous	5Y9/2	Pale yellow	23–36	Velutinous	5Y9/4	Pastel yellow		Light yellow
Gliocladium penicilloides	TC 1302	−	−	−	32–36	Velutinous		Colorless	26–31	Velutinous	5Y9/6	Light yellow		Vivid yellow
Trichoderma virens	TC 1301	−	−	−	>70	Floccose		Dark green	>70	Floccose		Dark green	Vivid yellow	
Gliocladium viride	TC 1505	−	−	−	>70	Velutinous		Dark green	>70	Velutinous		Dark green		
	TC 1369	−	−	−	>70	Velutinous		Dark green	>70	Velutinous		Dark green		
	TC 1368	−	−	−	>70	Velutinous		Dark green	>70	Velutinous		Dark green		

+, production at more than 1 mg/l; ++, production at more than 100 mg/l; −, not detected; colony diamter, diameter range after 7 days at 25°C; other characteristics were recorded after 7–14 days.

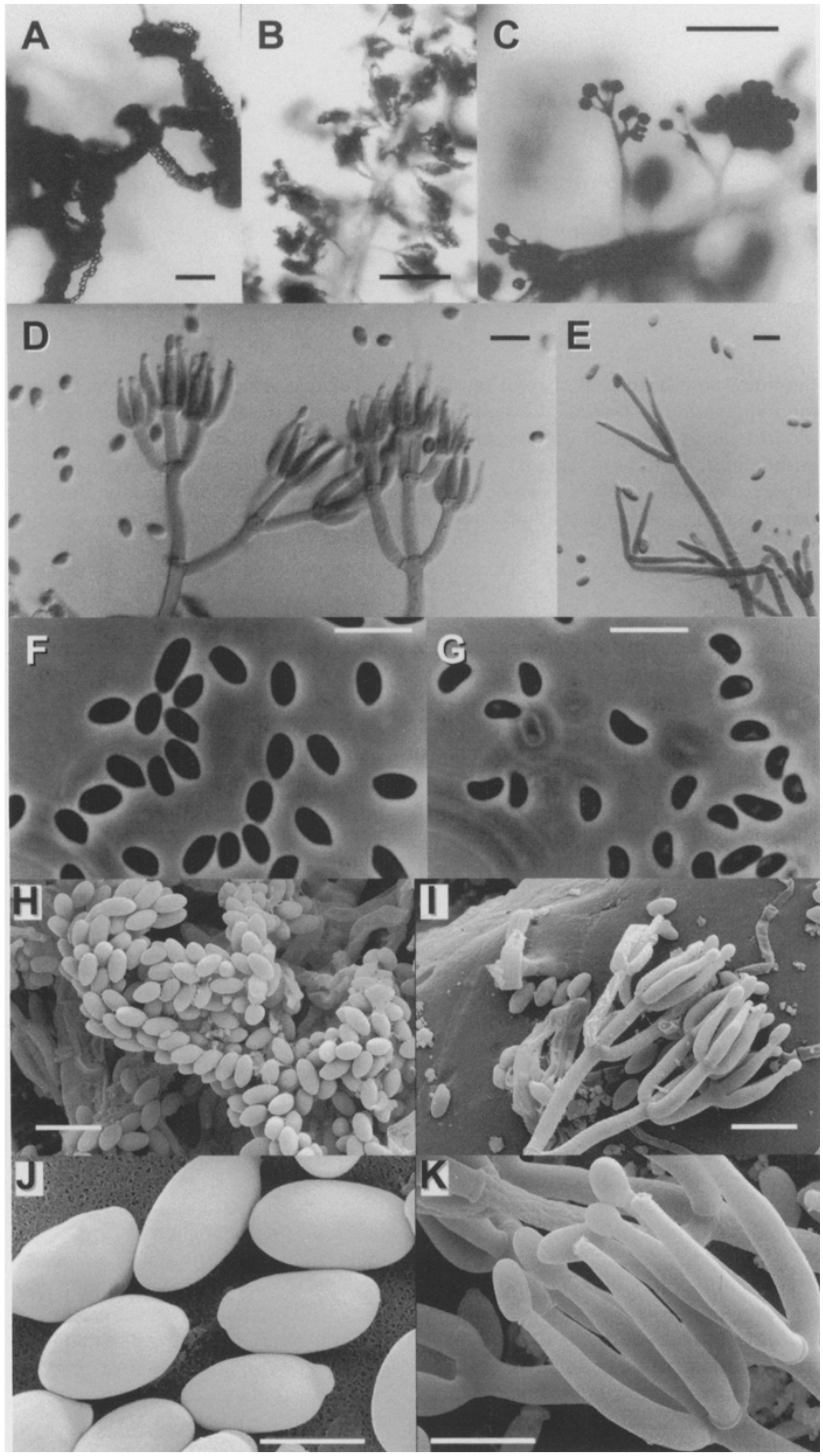

Fig. 2. Photomicrographs of *Gliocladium catenulatum* TC 1280. A, conidial chains from secondary conidiophores on LCA, bar=100 μm; B, secondary conidiophores on funicles on OA, bar=100 μm; C, conidial drops on primary conidiophores on OA, bar=100 μm; D, secondary conidiophores on OA, bar=10 μm; E, primary conidiophores on LCA, bar=10 μm; F, conidia from secondary conidiophores on OA, bar=10 μm; G, conidia from primary conidiophores on 2%MA, bar=10 μm; H, SEM photo of conidial chains on LCA, bar=10 μm; I, SEM of secondary conidiophore on LCA, bar=10 μm; J, SEM of conidia on LCA, bar=3 μm; K, phialides of secondary conidiophores on LCA, bar=6 μm.

Table 3. Comaprision of primary and secondary conidiophores and shape of conidia.

Species	Strains	LCA						OA						Major shape of conidia
		Sec			Prim			Sec			Prim			
		Frequency	Spore mass	Origin	Frequency	Spore mass	Origin	Frequency	Spore mass	Origin	Frequency	Spore mass	Origin	
Gliocladium catenulatum	TC 1280	+++	C	F	++	D	FS	++	C	F	+	D	FS	Navicular to ellipsoidal
	TC 1506	+++	C	FS	++	D	FS	++	C	FS	+	D	S	Broadly ellipsoidal to asymmetrically obovoidal
	TC 1490	+++	C	FS	+	D(C)	F	++	C	FS	+	D	FS	Navicular to broadly ellipsoidal
	IFO 31681	+++	C	F	++	D	FS	++	C	F	++	D	FS	Broadly ellipsoidal
Gliocladium roseum (=*Clonostachys rosea* typical strains)	TC 1294	+++	C	F	+	D	FS	++	C	F	++	D	FS	Navicular to cylindrical
	TC 1295	+++	C	F	+	D	FS	++	C	F	++	D	FS	Reniform to navicular
	TC 1296	+++	C	F	+	D	FS	++	C	F	++	D	FS	Navicular to cylindrical
	TC 1297	+++	C	F	++	D	FS	++	CD	F	++	D	S	Reniform, navicular to cylidrical
	TC 1298	++	C	FS	++	D	FS	++	C	FS	++	D	FS	Navicular to cylindrical
	TC 1299	+	D	FS	+++	D	FS	−			++	D	FS	Reniform to navicular
	TC 1507	+++	C	FS	++	D	FS	+	D	FS	++	D	FS	Reniform, navicular, cylindrical to broadly ellipsoidal
	TC 1508	++	CD	FS	+++	D	FS	++	D	FS	++	D	FS	Reniform, navicular to cylidrical
	TC 1509	++	CD	FS	++	D	FS	++	D	FS	++	D	FS	Reniform, navicular to cylidrical
	TC 1510	++	CD	F	++	D	F	+	D		++	D	F	Reniform, navicular to broadly ellipsoidal
(*G. roseum* Group B)	TC 1282	+++	C	FS	++	D	FS	++	C	FS	++	D	FS	Cylindrical
(*G. roseum* Group C)	TC 1304	++	D	FS	−			++	D	FS	−			Ellipsoidal
Clonostachys compactiuscula	TC 1292	+	C	F	−			++	C	F	+	D	F	Long cylindrical
	IFO 7066	−			+	D	FS	−			+	D	FS	Long cylindrical
	CBS	+++	C	FS	−			++	C	FS	−			Long cylindrical
	CBS	+	C	FS	−			++	C	FS	−			Long cylindrical
Clonostachys sp.	TC 1515	+++	C	S	++	D	S	++	C	FS	++	D	F	Cylindrical
	TC 1516	+++	C	S	++	D	S	++	C	FS	++	D	F	Cylindrical

Sec, secondary conidiophores; Prim, primary conidiophores; +++, abundant; ++, many; + rare; −, none; C, in chain; D, in wet drop; F, from funicles; S, from substrate.

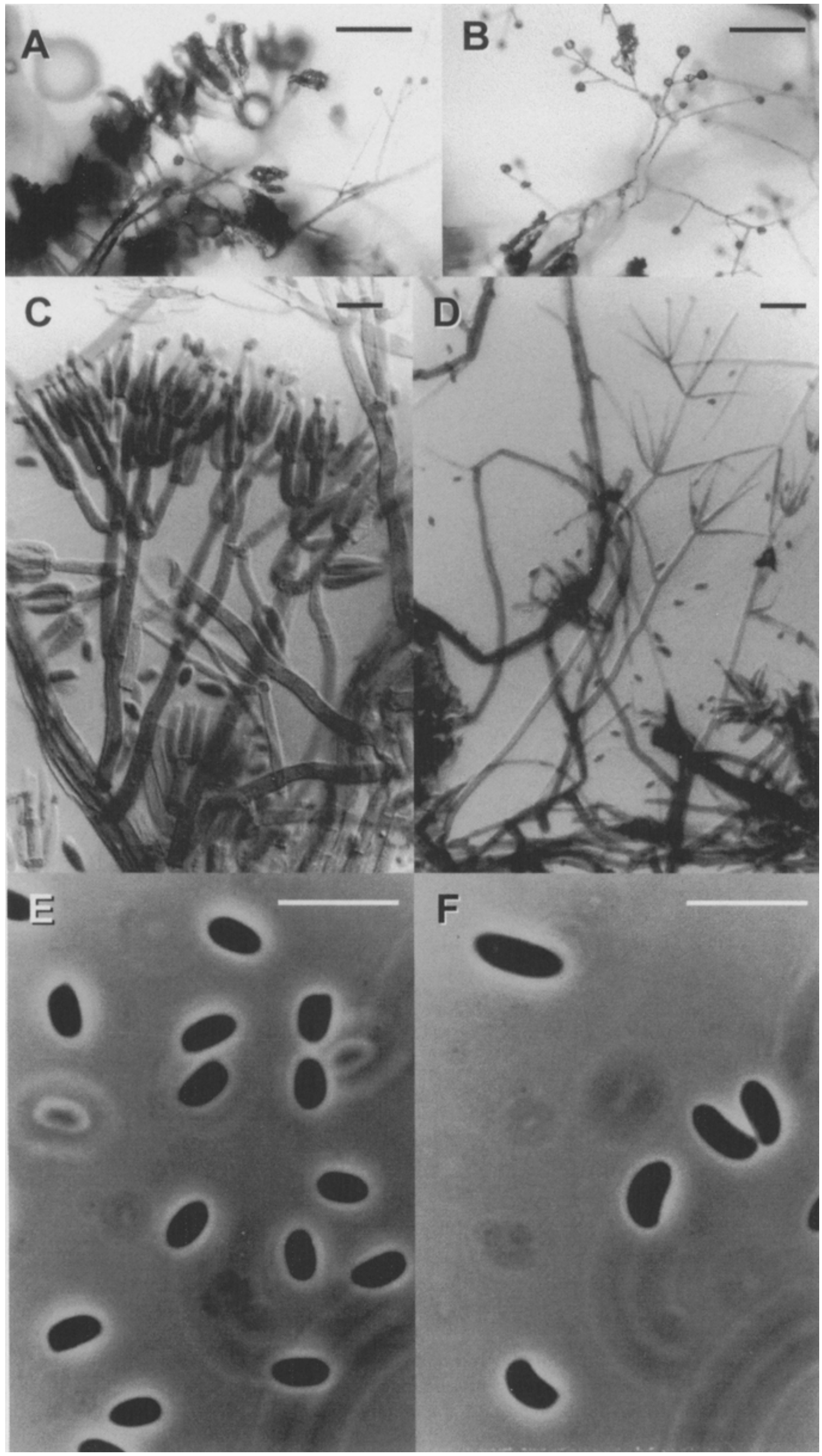

Fig. 3. Photomicrographs of *Clonostachys rosea* TC 1297 (typical strain, *G. roseum* Group A). A, conidial chains on secondary conidiophores on OA, bar=100 μm; B, conidial drops on primary conidiophores on OA, bar=100 μm; C, secondary conidiophore on LCA, bar=10 μm; D, primary conidiophores on OA, bar=20 μm; E, conidia from secondary conidiophores on OA, bar=10 μm; F, conidia from primary conidiophores on LCA, bar=10 μm.

Table 4. Comparison of conidial morphology.

Species	Strains	Length (μm)		Width (μm)	L/W	Area (μm^2)	Perimeter (μm)	Circularity
		Min Ave−SD Ave Ave+SD Max		Min Ave−SD Ave Ave+SD Max	Min Ave−SD Ave Ave+SD Max			
G. catenulatum	TC 1280	(4.5) 4.8 – 5.2 – 5.6 (6.4)	×	(2.8) 3.0 – 3.1 – 3.3 (3.4)	(1.4) 1.6 – 1.7 – 1.8 (2.0)	12.4	14.0	15.8
	TC 1506	(4.0) 4.6 – 5.2 – 5.8 (7.6)	×	(2.7) 3.1 – 3.2 – 3.4 (3.7)	(1.3) 1.4 – 1.6 – 1.8 (2.2)	12.4	14.2	16.3
	TC 1490	(3.5) 4.6 – 5.1 – 5.6 (6.9)	×	(2.4) 2.7 – 2.8 – 3.0 (3.2)	(1.3) 1.6 – 1.8 – 2.0 (2.6)	10.7	13.4	16.8
	IFO 31681	(4.0) 4.6 – 5.3 – 5.9 (6.7)	×	(2.5) 2.9 – 3.1 – 3.3 (3.3)	(1.4) 1.5 – 1.7 – 1.9 (2.4)	12.0	14.0	16.3
Gliocladium roseum (=*Clonostachys rosea* typical strains)	TC 1294	(3.6) 4.6 – 5.2 – 5.8 (7.4)	×	(2.4) 2.8 – 3.0 – 3.2 (3.5)	(1.3) 1.5 – 1.8 – 2.0 (2.5)	11.8	14.0	16.6
	TC 1295	(2.5) 4.2 – 5.2 – 6.2 (7.8)	×	(1.5) 2.1 – 2.4 – 2.8 (3.6)	(1.1) 1.7 – 2.1 – 2.5 (3.5)	8.8	12.9	19.3
	TC 1296	(5.2) 5.5 – 6.2 – 6.8 (7.7)	×	(2.4) 2.6 – 2.8 – 3.1 (3.5)	(1.7) 1.9 – 2.2 – 2.4 (2.7)	13.5	15.5	18.1
	TC 1297	(3.4) 4.4 – 5.5 – 6.5 (8.9)	×	(2.0) 2.5 – 2.8 – 3.1 (3.5)	(1.4) 1.7 – 2.0 – 2.2 (2.8)	11.5	14.4	18.4
	TC 1298	(3.7) 4.8 – 5.3 – 5.8 (6.6)	×	(2.0) 2.3 – 2.5 – 2.7 (2.9)	(1.6) 1.9 – 2.1 – 2.3 (2.7)	10.3	13.7	18.3
	TC 1299	(4.1) 4.8 – 5.7 – 6.7 (8.3)	×	(2.3) 2.5 – 2.9 – 3.3 (4.1)	(1.6) 1.8 – 2.0 – 2.2 (2.4)	12.5	14.9	18.1
	TC 1507	(3.3) 3.9 – 4.8 – 5.8 (7.2)	×	(2.4) 2.7 – 3.0 – 3.2 (3.5)	(1.2) 1.4 – 1.6 – 1.9 (2.3)	10.7	13.2	16.4
	TC 1508	(3.9) 4.2 – 4.7 – 5.3 (7.1)	×	(2.2) 2.5 – 2.7 – 2.9 (3.6)	(1.4) 1.6 – 1.8 – 2.0 (2.5)	9.4	12.6	17.0
	TC 1509	(4.3) 4.7 – 5.6 – 6.4 (8.4)	×	(2.4) 2.7 – 3.0 – 3.3 (3.8)	(1.4) 1.6 – 1.7 – 2.1 (2.6)	12.8	15.0	17.6
	TC 1510	(2.7) 3.5 – 4.8 – 6.1 (9.5)	×	(2.2) 2.5 – 2.8 – 3.1 (3.8)	(1.3) 1.4 – 1.7 – 2.0 (2.5)	10.2	12.9	16.7
(*G. roseum* Group B)	TC 1282	(4.3) 5.3 – 5.7 – 6.2 (6.7)	×	(2.2) 2.5 – 2.8 – 3.0 (3.4)	(1.6) 1.9 – 2.1 – 2.3 (2.6)	12.1	14.9	18.5
(*G. roseum* Group C)	TC 1304	(4.8) 5.4 – 6.3 – 7.2 (8.8)	×	(3.0) 3.3 – 3.5 – 3.7 (4.1)	(1.4) 1.6 – 1.8 – 2.0 (2.4)	17.0	17.0	17.1
C. compactiuscula	TC 1292	(3.7) 5.0 – 5.6 – 6.1 (7.9)	×	(1.4) 1.6 – 1.9 – 2.2 (4.7)	(1.3) 2.6 – 3.0 – 3.3 (3.9)	7.9	13.7	23.9
	IFO 7066	(4.9) 5.6 – 6.9 – 8.2 (10.4)	×	(2.3) 2.6 – 2.9 – 3.3 (3.8)	(1.8) 2.0 – 2.4 – 2.7 (3.3)	15.5	17.3	19.5
	CBS 729.87	(3.8) 4.5 – 5.2 – 5.9 (7.0)	×	(1.4) 1.6 – 1.9 – 2.1 (2.5)	(1.8) 2.4 – 2.8 – 3.2 (3.8)	7.1	13.5	25.8
	CBS 556.95	(4.2) 4.8 – 5.5 – 6.2 (8.6)	×	(1.4) 1.7 – 2.0 – 2.3 (2.6)	(2.1) 2.4 – 2.8 – 3.1 (3.4)	8.4	13.5	22.1
Clonostachys sp.	TC 1515	(4.5) 5.1 – 5.6 – 6.1 (7.2)	×	(2.2) 2.4 – 2.6 – 2.8 (3.3)	(1.7) 1.9 – 2.2 – 2.4 (2.8)	11.2	14.4	18.5
	TC 1516	(4.5) 5.0 – 5.5 – 6.0 (6.9)	×	(2.1) 2.3 – 2.5 – 2.7 (2.9)	(1.9) 2.0 – 2.2 – 2.5 (3.0)	10.6	14.1	18.8

Length, length of conidia in μm; Min, minimum size; Ave−SD, average size minus standard deviation; Ave, average size, Ave+SD, average size plus standard deviation; Max, maximum size; Width, width of conidia in μm; L/W, length to width ratio; Area, the total area of conidia in two-dimensional lateral view in μm^2; Perimeter, the total length of the circumference of the area object (μm); Circularity, the ratio of the area perimeter length squared divided by the area

Table 5. Comparision of production, growth, and conidia.

	Production					Conidia					
	TMC-151	TMC-154	TMC-171	Growth (mm)	Color of conidial area	Shape	Size (μm)	L/W	Area (mm²)	Perimeter (μm)	Circularity
Gliocladium catenulatum	+	−	−	40–50	Gray green	Asymmetrical ellipsoidal	5.1–5.3×2.8–3.2	1.6–1.8	10.7–12.4	13.4–14.2	15.8–16.8
Clonostachys rosea	+	−	−	40–50	Cream	Reniform to nvicular	4.7–5.6×2.5–2.8	1.6–2.2	8.8–12.8	12.6–15.0	16.4–19.3
Gliocladium sp. B	−	+	−	33–39	Pale yellow	Cylindrical	5.3–6.2×2.5–3.0	1.9–2.3	12.4	14.9	18.5
Gliocladium sp. C	−	−	+	30	Cream	Ellipsoidal	5.4–7.2×3.3–3.7	1.6–2.0	17	17	17.1
Clonostachys compactiuscula	−	−	−	23–50	Off-white	Long cylindrical	5.2–6.9×1.9–2.9	2.4–3.0	7.1–8.4(15.5)	13.5–13.7(17.3)	19.5–25.8
Clonostachys sp.	−	−	−	23–36	Off-white	Cylindrical	5.6–6.1×2.3–2.8	1.9–2.5	10.6–11.2	14.1–14.4	18.5–18.8

Growth, colony diameter after 7 d; L/W, Length/width ratio of conidia; area, the total area of conidia in two-dimensional lateral view in μm^2; perimeter, the total length of the conidial circumference of the area object (μm); circularity, the ratio of the area perimeter length squared divided by the area; exceptional values are in parenthesis.

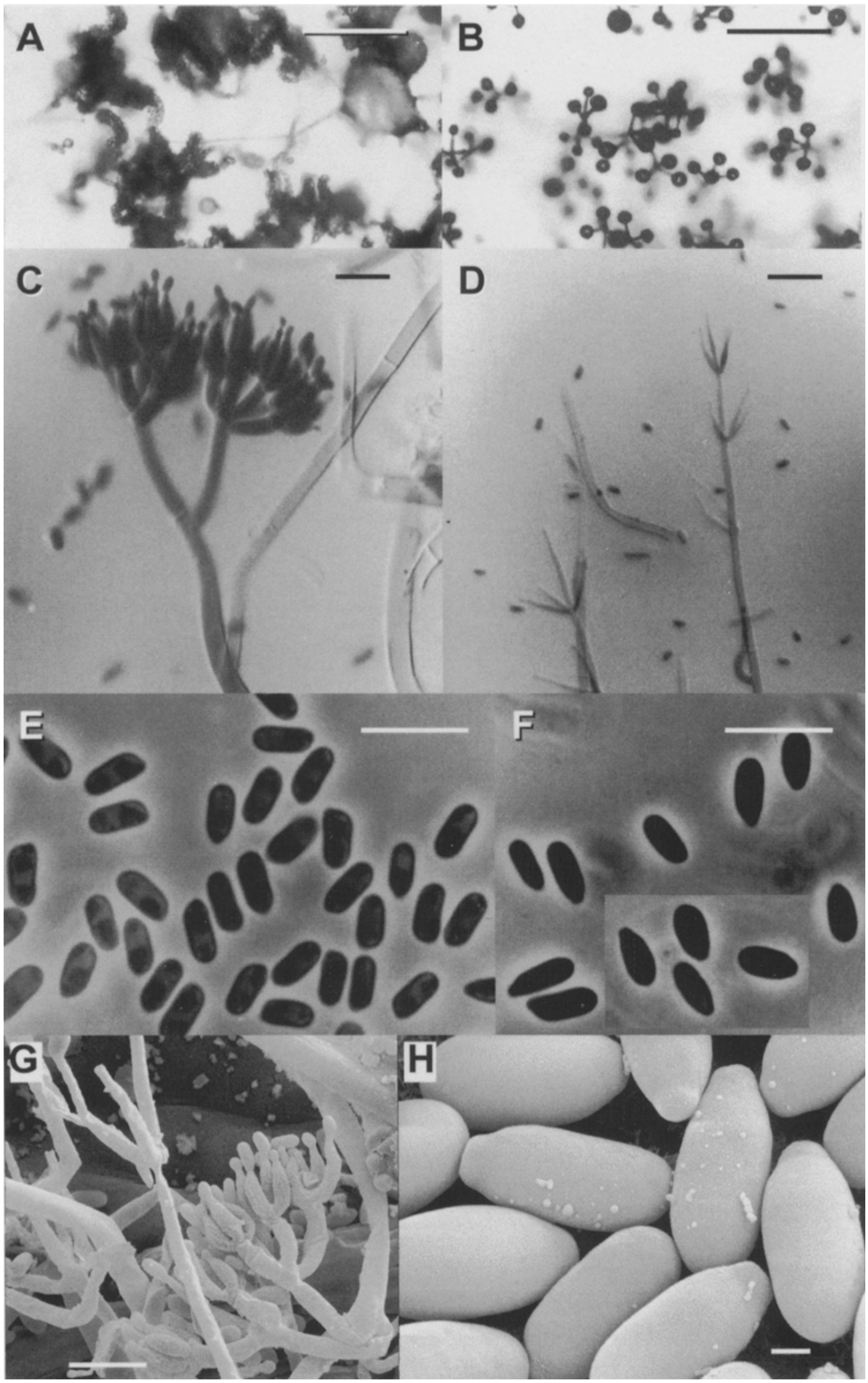

Fig. 4. Photomicrographs of *Gliocladium roseum* Group B, strain TC 1282. A, conidial chains from secondary conidiophores on OA, bar = 100 μm; B, conidial drops from primary conidiophores on LCA, bar = 100 μm; C, secondary conidiophore on OA, bar = 10 μm; D, primary conidiophores on OA, bar = 10 μm; E, conidia from secondary conidiophores on OA, bar = 10 μm; F, conidia from primary conidiophores on OA, bar = 10 μm; G, SEM of conidiophores LCA, bar = 10 μm; H, SEM of conidia on LCA, bar = 1 μm.

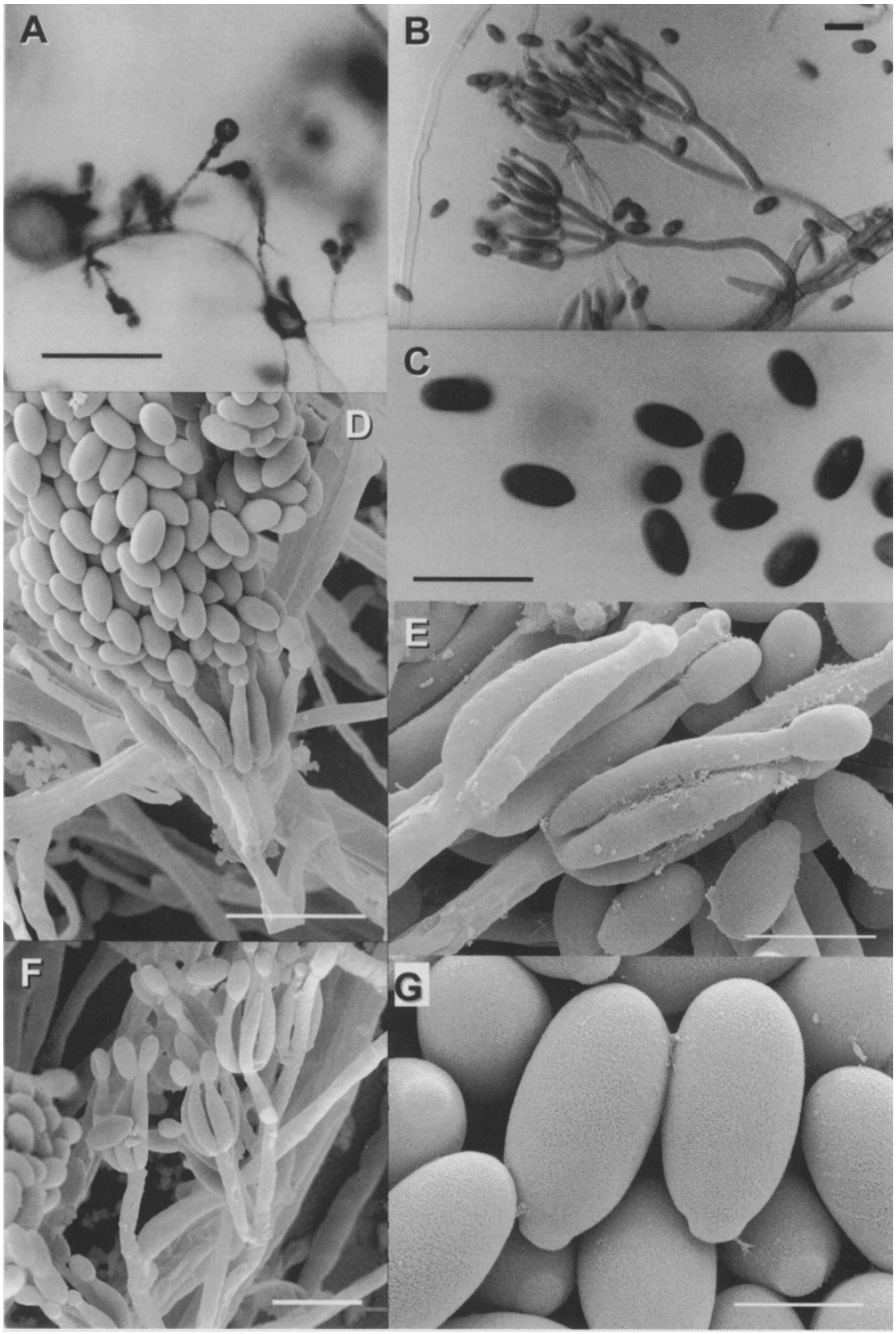

Fig. 5. Photomicrographs of *Gliocladium roseum* Group C, strain TC 1304. A, conidial drops from secondary conidiophores on OA, bar=100 μm; B, secondary conidiophores on OA, bar=10 μm; C, conidia on OA, bar=10 μm; D, SEM of conidial mass from a secondary conidiophore on LCA, bar=15 μm; E, SEM of phialides on LCA, bar=6 μm; F, SEM of secondary conidiophores on LCA, bar=10 μm; G, SEM of conidia on LCA, bar=3 μm.

The producing strains formed gray-green, pinkish, or off-white colonies. The strains forming a dark green conidial area, which were *Hypocrea*-derived "*Gliocladium*" or *Trichoderma*, did not produce these compounds. The strains assigned as *G. roseum* (12 strains) or *G. catenulatum* (4 strains) always produced TMC-151, TMC-154, or TMC-171.

Gliocladium catenulatum The four strains of *G. catenulatum* all produced TMC-151, but did not produce TMC-154 or TMC-171. These four strains were morphologically quite homogeneous. They grew rapidly, attaining a diameter of more than 40 mm after 7 d, and producing distinct funiculose surface with grayish green conidiation (Table 2). They developed both primary and secondary conidiophores. The secondary conidiophores characteristically produced oblique but relatively long conidial columns (Fig. 2; Table 3). Conidia were generally asymmetrical. The conidia from secondary conidiophores were consistent in shape and broadly ellipsoidal to navicular. The conidia from primary conidiophores were somewhat irregularly shaped or reniform with various sizes.

***Gliocladium roseum* and its related strains** The 12 strains of *G. roseum* were divided into three subgroups with regard to the production. The first group (*G. roseum* Group A=typical *Clonostachys rosea*) consisting of 10 strains produced TMC-151, but not TMC-154 or TMC-171. The second group (*G. roseum* Group B) consisting of 1 strain produced TMC-154 but not TMC-151 or TMC-171. The third group (*G. roseum* Group C), also consisting of 1 strain, produced TMC-171 but not TMC-151 or TMC-154. The *Clonostachys rosea* strains producing TMC-151 showed the following consistent cultural and morphological characteristics: colonies growing rapidly, attaining 40–50 mm in diam after 7 d at 25°C, more pinkish than the other groups, with funiculose to floccose conidial area (Table 2). The strains in this group were homogeneous in terms of morphology and cultural characteristics, as *G. catenulatum* strains were. However, formation of spore chains was dependent on strains: some strains formed distinctly oblique spore chains, while others formed wet spore masses or spherical spore balls (Table 3). Conidia were generally asymmetrical, but differed somewhat in shape depending on the type of conidiophore. The conidia from secondary conidiophores were more regularly cylindrical to navicular. Those from primary conidiophores were reniform or sometimes in irregularly shaped and of various sizes (Fig. 3; Table 3). *Gliocladium catenulatum* was morphologically close to *G. roseum* (Table 5). The main difference between *G. catenulatum* and *C. rosea* lies in the conidial mass color and circularity of their conidia. The average circularity of the conidia of *G. catenulatum* was 15.8–16.8, whereas that of *C. rosea* was larger, 16.4–19.3 (Tables 4, 5).

Compared with Group A, *G. roseum* species Group

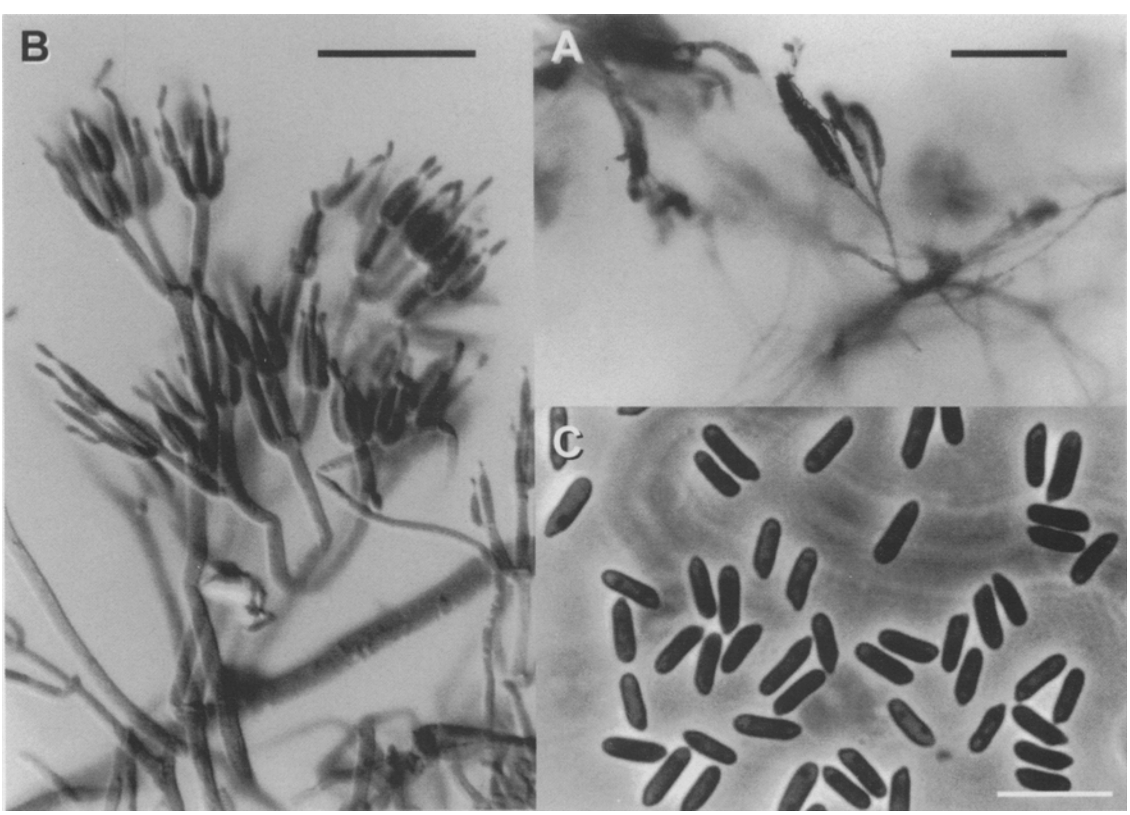

Fig. 6. Photomicrographs of *Clonostachys compactiuscula* TC 1292. A, conidial chains from secondary conidiophores, bar=100 μm; B, secondary conidiophores, bar=25 μm; C, conidia on OA, bar=10 μm.

B, TC 1282, was characterized by slower growth, attaining a diameter of 30–35 mm after 7 d, pale yellow colonies, secondary conidiophores bearing oblique conidial chains, and distinctly cylindrical or oblong conidia, not navicular (Figs. 1, 4; Table 3). The phialides were not straight but bent toward the main axis and shorter, 8.8–15.2×2.4–4.1 μm, L/W ratio 2.7–5.5. The conidia were more regularly cylindrical or oblong; the size was not significantly different from that of typical *G. roseum*, but the L/W ratio was slightly larger (1.9–2.3), as shown in Tables 3 and 5.

G. roseum Group C, TC 1304, had the following characteristics. This strain formed irregular masses of conidia from secondary conidiophores. According to the

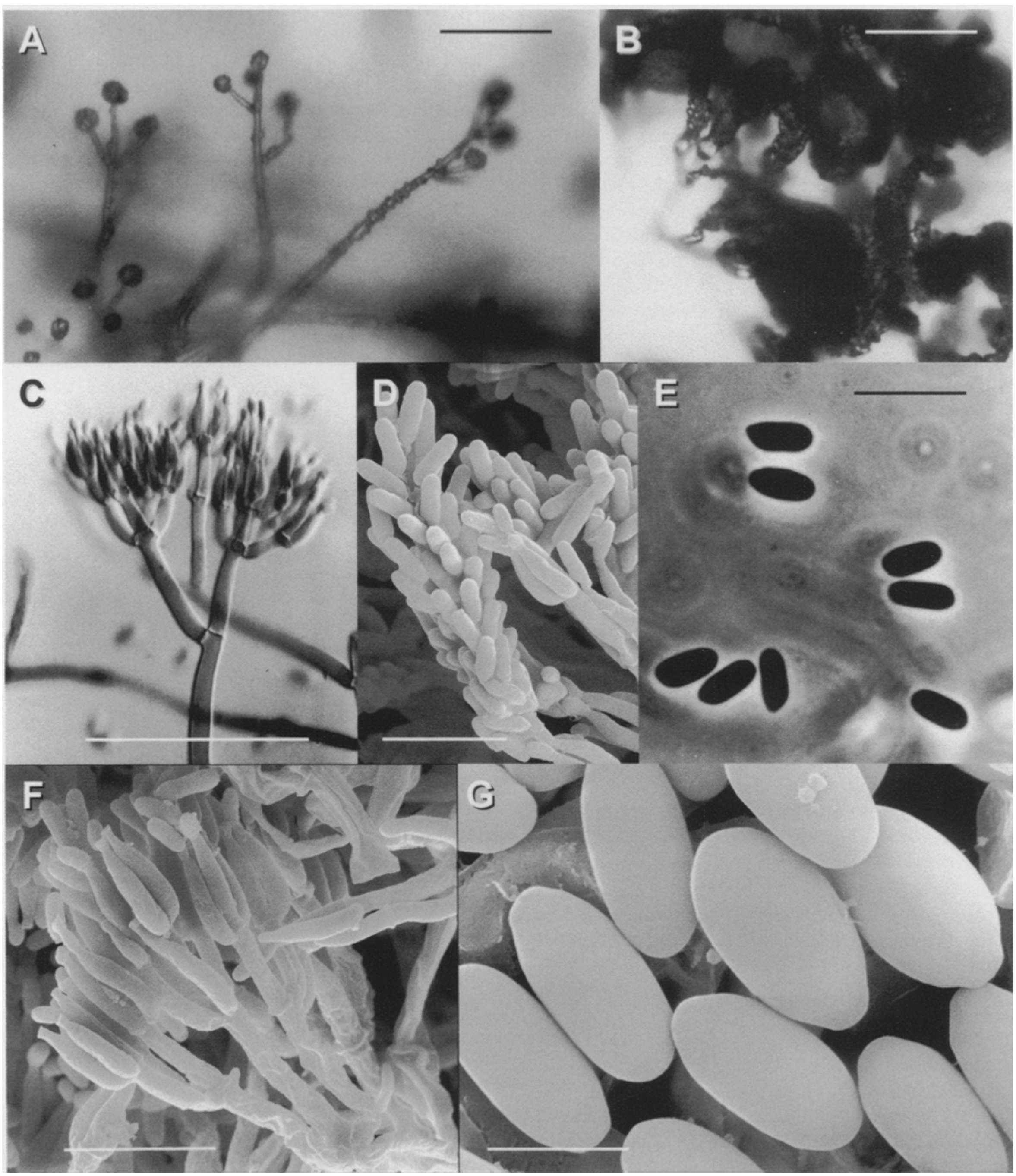

Fig. 7. Photomicrographs of *Clonostachys* sp. TC 1515. A, primary conidiophores on OA, bar=50 μm; B, conidial chains on secondary conidiophores on OA, bar=50 μm; C, secondary conidiophore on OA, bar=50 μm; D, conidial chains on secondary conidiophores on LCA, bar=12.5 μm; E, conidia on OA, bar=10 μm; F, secondary conidiophore on LCA, bar=12.5 μm; G, conidia on OA, bar=3 μm.

SEM observation (Fig. 5D), the conidial mass was reminiscent of conidial columns. This strain lacked *Verticillium*-like primary conidiophores, which was suggestive of *Clonostachys compactiuscula*. The phialides were more parallel compared to TMC 1282. The conidia were slightly larger (5.4–7.2×3.3–3.7 μm), symmetrical, and ellipsoidal (Fig. 5, Tables 3 & Table 4). The area and perimeter of the conidia were the largest of the three *G. roseum* groups (Tables 4, 5). *G. roseum* Groups B and C will possibly be reassessed in the future.

Chemotaxonomic consideration; comparison with *Clonostachys compactiuscula* Schroers (1998) reported that *G. roseum* is not distinct from *G. catenulatum* based on rDNA ITS-1 data. The morphology, ecology, and DNA sequence data further indicated that *G. roseum* should be separated from *Gliocladium* and classified as *Clonostachys rosea* (Link: Fr.) Schroers, Samuels, Seifert, & W. Gams, anamorph of *Bionectria ochroleuca* (Schw.) Schroers & Samuels of Bionectriaceae, while *Gliocladium*, or *Gliocladium* sensu stricto, should be restricted to the anamorph of *Sphaerostilbella* Henn. (Schroers et al., 1999; Rossman et al., 1999). In our experiments, both *C. rosea* (*G. roseum*) and *G. catenulatum* produced TMC-151 series compounds. This metabolic consistency supported the conclusion by Schroers (1998) that these two species are very closely related. Since Schroers et al. (1999) transferred *G. roseum* to the genus *Clonostachys*, we took this into account in examining the productivity of our metabolites from *C. compactiuscula* and *Clonostachys* sp. TC 1515 and 1516. None of the compounds was detected in six strains of the species tested. All except IFO 7066 grew slowly. They were less pinkish and off-white, and the conidia were cylindrical, similar to those of *G. roseum* Group B to some extent. However, the conidia of *C. compactiuscula* were much longer cylindrical with L/W ratio 2.4–3.0 and circularity of 19.5–25.8, which were the largest among the species compared (Fig. 6; Tables 3, 4). The area of *C. compactiuscula* conidia was the smallest of all: 7.1–8.4 μm^2 with an exception of IFO 7066 whose area was 15.5. This strain appeared to have deteriorated because it did not form secondary conidiophores, even though this is clearly mentioned in the original description by Tubaki (1963). Formation of the secondary conidiophores of *Clonostachys* tends to be easily lost during the successive transfer or long-term preservation. In fact, there is a report of an attempt to obtain strains with one type of conidiophores (Hosoya et al., 1995). The conidia from the secondary conidiophores were sometimes different from those from the primary conidiophores. Some strains formed more irregular-shaped conidia from the primary conidiophores. This is probably the reason for the exceptional value in IFO 7066. *Clonostachys* sp. TC 1515 and 1516 were somewhat closer to TC 1282, forming long oblique conidial chains, bent phialides, and distinctly cylindrical or oblong conidia (Fig. 7; Tables 3, 4). Their conidial area was paler than TC 1282 and almost white. The primary conidiophores were also present.

TMC-151, TMC-154, and TMC-171 are structurally close congeners: the former has a saturated bond at C14-15, whereas the latter two have an unsaturated bond at the same position. If these metabolites are biosynthesized via polyketide pathways, the double bond of TMC-154 and 171 is probably reduced by enoyl reductase to yield saturated TMC-151s. Since TMC-151 needs one more step for biosynthesis than TMC-154 and 171, the strains producing TMC-154 and 171 strains may lack enoyl reductase.

Limitation of *C. rosea* inferred from morphology and metabolite production We conclude that the metabolites in question are specific to *C. rosea*, its closely related strains, and *G. catenulatum*. The other related genera and species such as *G. penicillioides*, *G. sagariense* Saksena, *G. viride*, and *T. virens* lacked the ability to produce these metabolites. *Gliocladium catenulatum* and *C. rosea* (*G. roseum*) produced TMC-151 but not TMC-154 or 171. On the other hand, *G. roseum* Groups B and C produced respectively TMC-154 and TMC-171 but not TMC-151. The production spectrum of the *G. roseum* groups was well correlated with their cultural and morphological properties (Table 5). Our finding supported the conclusion by Schroers (1998) that *C. rosea* and *G. catenulatum* are conspecific. Furthermore, the producer strains were distinct from *Clonostachys compactiuscula* in terms of morphology and production of the metabolites.

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Literature cited

Domsch, K. H., Gams, W. and Anderson, T.-H. 1980. Compendium of soil fungi, vol. 1, pp. 368–377. Academic Press, London.

Fujimori, F. and Okuda, T. 1994. Application of the random amplified polymorphic DNA using polymerase chain reaction for efficient elimination of duplicate strains in microbial screening I. Fungi. J. Antibiot. **47**: 173–182.

Fujiwara, A., Okuda, T., Masuda, S., Shiomi, Y., Miyamoto, C., Sekine, Y., Tazoe, M. and Fujiwara, M. 1982. Fermentation, isolation and characterization of isonitrile antibiotics. Agric. Biol. Chem. **46**: 1803–1809.

Gams, W. and Meyer, W. 1998. What exactly is *Trichoderma harzianum*? Mycologia **90**: 904–915.

Hosoya, T., Matsuoka, T., Serizawa, N. and Furuya, K. 1995. Two morphological groups derived from *Clonostachys cylindrospora* and their relationship to trans-4-hydroxy-(L)-proline productivity. Mycoscience **36**: 193–197.

Kohno, J., Asai, Y., Nishio, M., Sakurai, M., Kawano, K., Kameda, N., Kishi, N., Okuda, T. and Komatsubara, S. 1999a. TMC-171A, B, C and TMC-154, novel polyketide antibiotics produced by *Gliocladium* sp. TC 1340 and TC 1282. J. Antibiot. (In press.)

Kohno, J., Nishio, M., Sakurai, M., Kawano, K., Hiramatsu, H., Kameda, N., Kishi, N., Yamashita, T., Okuda, T. and Komatsubara, S. 1999b. Isolation and structure determination of TMC-151s: novel polyketide antibiotics from *Gliocladium catenulatum* Gilman & Abbott TC 1280. Tetrahedron **55**: 7771–7786.

Matsushima, T. 1975. Icones microfungorum a Matsushima lectorum, pp. 73–74. Published by the author, Kobe, Japan.

Miura, K. and Kudo, Y. Y. 1970. An agar-medium for aquatic Hyphomycetes. Trans. Mycol. Soc. Japan **11**: 116–118. (In Japanese.)

Okuda, T., Fujiwara, A. and Fujiwara, M. 1982. Correlation between species of *Trichoderma* and production patterns of isonitrile antibiotics. Agric. Biol. Chem. **46**: 1811–1822.

Rossman, A. Y., Samuels, G. J., Rogerson, C. T. and Lowen, R. 1999. Genera of Bionectriaceae, Hypocreaceae and Nectriaceae (Hypocreales, Ascomycetes). Stud. Mycol. **42**: 1–248.

Schroers, H.-J. 1998. Variation in the mycoparasite *Bionectria ochroleuca*/*Gliocladium roseum* and closely related taxa. Inoculum **49**: 48. (Abstracts of annual meeting of the Mycological Society of America, June 11–16, 1998, San Juan, Puerto Rico.)

Schroers, H.-J., Samuels, G. J., Seifert, K. A. and Gams, W. 1999. Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. Mycologia **91**: 365–385.

Tubaki, K. 1963. Notes on the Japanese Hyphomycetes. I. *Chloridium, Clonostachys, Ishmospora, Pseudobotrytis, Stachybotrys* and *Stephanoma*. Trans. Mycol. Soc. Japan **4**: 83–90.