Morphological Mutant of *Rhizoctonia solani* and Its Cell Wall Component

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The perfect stage of *Rhizoctonia solani* Kühn R-44 (*Thanatephorus cucumeris*) was confirmed by microscopic observation of basidiospores after three successive single spore isolations. A morphological mutant, ML-19, was induced by UV-light irradiation of a mycelial-fragment suspension from a single basidiospore culture of the fourth generation, and characterized.

Different from in the cases of some kinds of morphological mutants of *Neurospora*, the morphology of this mutant was not responsible for conditional morphologicals, such as choline, inositol, linolenic acid and cAMP or hyphal extension factor.

The characterization of the cell wall by fractionation, acid hydrolysis, Smith degradation and methylation analysis revealed that fraction III (glycan fraction) was composed of 1,3-glucomannan containing small amounts of pentoses. The major difference in the fractions III from the original strain and the mutant was the change in the glucose/mannose ratio from 5/3 to 10/3.

The hyphal extension of *Rhizoctonia solani* was inhibited by validamycin (VM), leading to abnormal branching at the hyphal tips without growth inhibition.^{1,2)} The formation of hyphal extension factor (HE-factor), which is present in *R. solani* hyphae and stimulates the extension of VM-inhibited hyphae, was also inhibited by VM, and a restricted and compact colony was produced.^{3~5)}

On the other hand, *sparse*, *stumpy*, *fleecy*, *curly*, *rusty* and *ropy* mutants are known as morphological mutants of *R. solani*.^{6,7)} The relationship in morphology between a *fleecy* or *stumpy* mutant and the VM-inhibited hyphae of *R. solani* appeared to correspond to that between a colonial mutant (*frost*) and the sorbose-inhibited hyphae of *Neurospora*.^{8,9)}

It is interesting to isolate these morphological mutants of *R. solani* and to examine the biochemical determinants of the morphology or changes in cell wall components. Attempts to isolate *fleecy* or *stumpy* mutants were unsuccessful but *ropy*-type mutants were isolated. The morphological properties and cell wall

components of the *ropy*-type mutants are described in comparison with those of the original strain.

MATERIALS AND METHODS

Microorganism. *Rhizoctonia solani* Kühn R-44, which was kindly donated by the Kyoto Herbal Garden, Takeda Chemical Co., was used throughout this work. The strain was kept at 4°C after 5-days incubation at 27°C on potato sucrose agar.

Confirmation of the perfect stage. In order to confirm the perfect stage of *R. solani*, basidiospores were developed on the surface of soil by the method of McKenzie or Ogoshi.^{6,10)} The perfect stage of *R. solani* (*Thanatephorus cucumeris*) was confirmed on microscopic observation of basidiospores.

Induction of morphological mutants. Basidiospores, shed from fruiting bodies on the surface of soil were irradiated *in situ* in open Petri dishes under, a Hitachi 15-W germicidal lamp, at a distance of 40 cm. A mycelial fragment suspension was prepared by homogenizing the mycelia of a single basidiospore culture. The suspension was overlaid on a water-agar plate and irradiated with UV-light in a similar manner to as described above. After germination

of basidiospores or mycelial fragments, a slow growing colony or a colony showing morphological abnormalities at the hyphal tips was isolated.

Culturing of *R. solani*. *R. solani* was grown in a 200 ml Erlenmeyer flask containing 50 ml of enriched Czapek-Dox medium (3% sucrose, 0.2% NaNO_3 , 0.1% K_2HPO_4 , 0.05% KCl , 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% peptone, 0.5% corn steep liquor) on a rotary shaker at 27°C for 3 days. After 3 days culture, the wet mycelial mat was harvested by squeezing with cotton cloth. In order to prepare large quantities of mycelia, a 30-liter jar fermentation was carried out with the enriched Czapek-Dox medium described above.

Preparation of the cell wall. According to the method of Mahadevan and Tatum,¹¹⁾ the wet mycelial mat was washed with water and then lyophilized. The resultant mycelial mat was powdered with a mortar and pestle, and the dry mycelial powder (1 g) was treated with 100 ml of an aqueous solution of 1% sodium dodecylsulfate at 4°C with continuous stirring for 16 hr. The sedimentable cell wall preparation was washed with water several times until the absorbance at 280 nm of the cytoplasmic protein had disappeared. After washing, the cell wall preparation was washed with increasing concentrations of ethyl alcohol-absolute alcohol and then dried at 37°C until a constant weight was obtained.

Fractionation of the cell wall. Cell wall polymers were fractionated according to the method of Mahadevan and Tatum.¹¹⁾ This procedure is shown in detail in Fig. 4.

Analysis of cell wall fraction III.

Acid hydrolysis. Cell wall fraction III (10 mg) was hydrolyzed with 3.6 N H_2SO_4 (5 ml) for 16 hr at 100°C and then neutralized with BaCO_3 . After removal of BaSO_4 , the resulting hydrolyzate was reduced with sodium borohydride overnight at room temperature. After decomposition of excess sodium borohydride with Amberlite IR-120 B (H^+) resin, the residue was repeatedly evaporated with MeOH to remove boric acid. For GLC analysis, the reduced sugar mixture was acetylated with pyridine and acetic acid anhydride for 4 hr at 100°C. The reaction mixture was injected directly into the gas chromatograph.¹²⁾

Smith degradation. Cell wall fraction III (50 mg) was oxidized with 0.05 M periodate (50 mg) for 14 days at 4°C in the dark.¹³⁾ After the oxidation had been completed, the insoluble oxidation product was recovered by centrifugation. After washing with water, it was dissolved in 0.1 M sodium hydroxide (2 ml) and then reduced with sodium borohydride (10 mg) in the usual way. The excess borohydride was decomposed by the addition of acetic acid (final pH, 6.5), and the reaction mixture containing insoluble glycan polyalcohol was dialyzed overnight at 10°C, and then the non-dialyzable fraction was lyophi-

lized. A portion of the glycan polyalcohol was hydrolyzed with 3.6 N H_2SO_4 for 16 hr at 100°C. After neutralization with BaCO_3 , the hydrolyzate was converted to the corresponding alditol acetate and analyzed by GLC. The consumption of periodate was determined by the Fleury-Lange method.¹⁴⁾

Methylation analysis. Cell wall fraction III was methylated 4 times by the Hakomori method¹⁵⁾ as described by Lindberg.¹⁶⁾ The methylated fraction III was dissolved in 90% $\text{H} \cdot \text{COOH}$ and then the solution was heated for 2 hr at 100°C. The reaction mixture was concentrated to dryness and the residue was hydrolyzed with 0.5 N H_2SO_4 for 12 hr at 100°C. After neutralization with BaCO_3 , the hydrolyzate was converted to the corresponding acetyl derivative and analyzed by GLC.

Gas chromatography. Gas chromatography was carried out with a Hitachi 163 gas chromatograph, with a glass column packed with 3% ECNSS-M on gas chrom Q for the detection of alditol acetate. Nitrogen gas was used as the carrier gas at a flow rate of 60 ml/min.

RESULTS AND DISCUSSION

*Isolation and properties of morphological mutants of *R. solani**

Cultures of *Rhizoctonia solani* are known to be heterokaryotic with multinucleate cells.⁷⁾ Before the isolation of mutants, it was thought that the isolation of a homokaryotic culture from a single basidiospore was necessary. As shown in Fig. 1, the perfect stage of *R. solani* Kühn R-44 (*Thanatephorus cucumeris*) was confirmed by the method of McKenzie or Ogoshi.^{6,10)}

Selection was performed through three successive generations for a fertile homokaryotic single basidiospore culture that was the same in cultural appearance as the original

FIG. 1. Basidiospores of *R. solani* R-44.

TABLE I. CHARACTERISTICS OF *R. solani* R-44 AND ITS MUTANTS

Culture	Colony diameter (mm) ^a	Colony colour	Cultural appearance	Hyphal branching	Fructification
ML-19	47	White	Ropy, profuse branching along a few prominent faster growing runner hyphae	Irregular	(-)
ML-37	47	White	Same as ML-19	Irregular	(-)
R-44	90	White to light brown	Wild type	Regular	(+)

^a After 3-days incubation on potato-sucrose agar.

FIG. 2. Cultural Characteristics of *R. solani* R-44 and Mutant ML-19.

3-Days cultures on Czapek-Dox agar.

strain. The fourth generation of spores or the hyphal fragment suspension was mutated by means of UV-light irradiation as described under MATERIALS AND METHODS. Most of the isolates except mutants ML-19 and ML-37 were identical with the original strain in morphological features. The two mutants, which were isolated after irradiation of the mycelial fragment suspension, were slow-growing mutants. They were considered to be true mutants instead of segregants from the heterokaryon, because no change was observed in their morphological features after subculturing 5-times.

The leading hyphae of ML-19 or ML-37 usually grew out from the colony with a considerable distance between two hyphae.

Short side branches developed profusely from a leading hypha, having the appearance of rope-like strands. In general, development of hyphae was slow and consequently a relatively compact colony was produced. These

FIG. 3. Hyphal Tips of *R. solani* R-44 and Mutant ML-19.

3-Day cultures on Czapek-Dox agar ($\times 150$). Top, R-44; bottom, ML-19.

features resembled those of the *ropy* mutant described by McKenzie and Mishra.^{6,17} Some mycological properties of the original strain and morphological mutants ML-19 and ML-37, and morphological features of their hyphal tips are presented in Table I, and

previously. An outline of the fractionation procedure and the results are shown in Fig. 4 and Table II.

The percentage of cell wall of mutant ML-19 was much smaller than that of the original strain. Almost no differences between the original strain and the mutant were observed in fractions I, II and IV, respectively. From this, the cell wall of the mutant was considered to be a little thinner than that of the original strain.

Sugar components of fraction III (glycan fraction)

Preliminary experiments indicated that fractions I and III from both strains similarly contained xylose-like pentose, glucose or mannose and oligosaccharides, whereas fractions II and IV contained mainly glucose (mannose) and glucosamine, respectively, that is, corresponding fractions from the two strains were assumed to be composed of similar component sugars. The component sugar of fraction III, in which a quantitative difference was observed, was further investigated by acid hydrolysis, Smith degradation and methylation analysis.

1) *Acid hydrolysis.* As already described under MATERIALS AND METHODS, both fractions were hydrolyzed and the component sugars were examined. As shown in Table III, each fraction III contained glucose and mannose as major components, and xylose, ribose and arabinose as minor components, and hence it was assumed to be a kind of glucomannan. The major difference in the fractions III from the original strain and the mutant was the change in the glucose/mannose ratio from 5/3 to 10/3.

2) *Smith degradation.* Smith degradation of fraction III was carried out as described previously. The periodate consumption of the fractions from the original strain and the mutant was estimated to be 0.1 mol and 0.06 mol per sugar residues, respectively. As shown in Table IV, glucitol and mannitol were major degradation products, and small amounts of arabinose, erythritol and glycerol were also detected. Glycerol should have

TABLE III. SUGAR COMPONENTS OF FRACTION III

Alditol acetate	T^a	Molar ratios	
		R-44	ML-19
Ribitol	0.62	0.4	0.3
Arabitol	0.78	3.1	0.7
Xylitol	1.00	1.0	2.5
Mannitol	1.86	36.8	22.3
Glucitol	2.44	61.2	72.2

^a Relative to the retention time for the xylitol derivative.

TABLE IV. ANALYSIS OF THE SMITH DEGRADATION PRODUCTS OF F-III FROM *R. solani*

Alditol acetate	T^a	Molar ratios	
		R-44	ML-19
Glycerol	0.05	2.7	2.5
Erythritol	0.14	3.4	0.8
Ribitol	0.33	0.0	0.8
Arabitol	0.40	3.1	1.4
Mannitol	1.00	35.0	21.6
Glucitol	1.35	55.7	72.8

^a Relative to the retention time for the mannitol derivative.

arisen from the non-reducing terminal glucose, and glucitol, mannitol or arabitol from the 1,3-linked glucose, mannose or arabinose, respectively. The detection of erythritol indicated that 1,4-linked glucose is present in fraction III.

The differences detected in the Smith degradation products of the two fractions were a change in the glucose/mannose ratio and a change in the content of 1,3-linked arabinose or 1,4-linked glucose. Since glycerol or erythritol comes from non-reducing terminal glucose or 1,4-linked glucose residues, respectively, the total content of glucose was estimated to be 61.8 (55.7+3.4+2.7) percent. The ratio of glucose/mannose, 61.8/35.0, is almost the same as that of glucose/mannose, 61.2/36.8, in the case of the acid hydrolysis mentioned above.

3) *Methylation analysis.* Cell wall fractions III of the two strains were methylated and examined as described previously. The results

TABLE V. METHYLATION ANALYSIS OF F-111 FROM *R. solani*

Alditol acetate	Linkage indicated	T_g^a	Molar ratios	
			R-44	ML-19
2,3,5-Tri- <i>O</i> -methyl-D-xylitol	[Xyl] _r 1→ } →2 [Rib] _r 1→ }	0.70	0.6	0.7
3,5-Di- <i>O</i> -methyl-D-ribitol				
2,3,4,6-Tetra- <i>O</i> -methyl-D-glucitol	[Glc] _r 1→	1.00	2.9	1.7
2,4-Di- <i>O</i> -methyl-D-arabitol	→3 [Ara] _p 1→	1.46	3.9	2.2
2,3-Di- <i>O</i> -methyl-D-xylitol	→5 [Xyl] _r 1→	1.63	0.0	1.0
2,4,6-Tri- <i>O</i> -methyl-D-glucitol	→3 [Glc] _p 1→ } →3 [Man] _p 1→ }	1.89	87.0	93.0
2,4,6-Tri- <i>O</i> -methyl-D-mannitol				
2,3,6-Tri- <i>O</i> -methyl-D-glucitol	→4 [Glc] _p 1→	2.53	3.1	0.5
4,6-Di- <i>O</i> -methyl-D-mannitol	→3 [Man] _p 1→	3.00	3.0	1.0
	→2 [Man] _p 1→			
2,6-Di- <i>O</i> -methyl-D-glucitol	→3 [Glc] _p 1→	3.57	0.4	0.5
	→4 [Glc] _p 1→			

^a Relative to the 2,3,4,6-tetra-*O*-methyl-D-glucitol derivative.

showed the presence of a major peak corresponding to 2,4,6-tri-*O*-methyl-D-glucose and/or 2,4,6-tri-*O*-methyl-D-mannose. The component corresponding to this peak must be a mixture of these two methylated sugars, because the Smith degradation products contained 1,3-linked glucose and mannose, and the retention times of these methylated sugars were the same as in the case of the 3% ECNSS-M gas chrom Q column.

The results of methylation analyses of the two fractions III are shown in Table V. From the results, both fractions III were also assumed to be 1,3-linked glucomannan containing small amounts of pentoses (xylose, ribose and arabinose), and the differences between the cell wall fractions III of the original strain and the mutant were the change in the glucose/mannose ratio in 1,3-linked glucomannan and the change in the content of 1,4-linked glucose or 1,3-linked arabinose. These results were almost completely coincident with the results of acid hydrolysis and Smith degradation.

An increase in the level of cell wall fraction I (glucan) or a decrease in cell wall fraction III (β -1,3-glucan) was recognized in most of the morphological mutants of *Neurospora*.^{9,17)} In the case of morphological mutant ML-19 of *R. solani*, the cell wall itself was considered to be a

little thinner and a decrease in fraction III was also recognized as in *Neurospora*. The fraction III of *R. solani* was composed of glucomannan instead of the β -1,3-glucan in the case of that of *Neurospora*, and the change in the glucose/mannose ratio in the glucomannan moiety of the mutant was remarkable. A future problem to be solved is what kind of enzyme defect causes this change in the glucomannan moiety, accompanied by a morphological change.

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