Mutants of *Myrothecium roridum* with weak pathogenicity induced by irradiation with ultraviolet rays and toxin production ability

RITSUKO MURAKAMI and AKIRA SHIRATA
National Institute of Sericultural and Entomological Science
(Received Aug. 30, 1999)

Key words: *Myrothecium roridum*, mutant, pathogenicity, toxin, mulberry, ultraviolet rays

Myrothecium leaf spot of mulberry, caused by *Myrothecium roridum* was reported in 1994 (Takahashi et al., 1994). *M. roridum* produces toxins against mulberry (Murakami et al., 1995) and the toxins may be one of the pathogenic factors of the fungus (Murakami and Shirata, 1998). However, there is no clear evidence of the role of the toxins for the factor in pathogenicity. In this paper, the mutants with a weak pathogenicity were induced by irradiation with ultraviolet rays (UV) to clear the role of toxins in the pathogenicity of *M. roridum*, and discussed the relation between the toxins and pathogenicity.

**Materials and methods**

**Microorganisms and culture:** *M. roridum* (M9403) was isolated from infected mulberry leaves planted from mulberry field in Kagoshima Prefecture in 1994. *M. roridum* was incubated on potato sucrose agar medium (PSA: broth made of potato 200 g, sucrose 20 g, agar 18 g/1 liter) at 25°C and abundant spores were formed at 2 weeks after inoculation. The spores were washed with sterilized water 3 times and used.

**Mutagenesis procedure:** Spores were spread on 20 ml of PSA at the rate of 200 spores/petri dish and irradiated in a petri dish (9 cm diameter) with UV of short wave-length (GL-15, Toshiba) at a distance of 30 cm from the lamp.

**Selection of mutants with a weak pathogenicity:** The plates spread with *M. roridum* spores were incubated for 2-3 days, and colonies (ab. 1 mm diameter) were formed. Each colony was transferred with a sterilized toothpick into 5 ml of PS (PSA excepted for agar) and incubated at 25°C. After 5-7 days, 100 micro liters of each fungus culture was transferred to 5 ml of PSA in petri dishes (4 cm in diameter) and incubated for 1 week in dark. Small pieces of hyphae (ab. 3×3 mm) were cut off from the edge of the colony and inoculated onto mulberry leaves on the parts wounded with a bundle of 10 needles. The leaves were put in a plastic case with moisture condition and the strains formed small lesions were selected at 5 days after inoculation (pathogenicity easy detect method).

**Detection of toxin activity:** There are 2 methods. One method is toxin easy detect method. The strains were incubated for 2 weeks on PSA in petri dishes (4 cm diameter) at 25°C and the cultures were air-dried. Toxins were extracted from the dried culture with 10 ml methanol and 10 micro liters toxins extracts were spotted on wounded mulberry leaves. Then sizes of formed toxic lesions were compared with that of the control strain. Another was TLC-bioassay method. Each selected strain was incubated on
PSA for 2 weeks and the culture was air-dried. The dried culture was extracted with 20 ml of methanol and air-dried. The extract was dissolved again in 20 ml methanol and 10 micro liters was spotted on a thin-layer chromatography (TLC) plate (Art. 5554 DC-Alufolien Kieselgel 60 F254) according to the TLC-bioassay method (MURAKAMI and SHIRATA, 1998). The spotted plate was developed with diethyl ethel and PS containing spores of unidentified fungus (M9401), assay fungus for the toxins, was sprayed on the plate. Because susceptibility for the toxins of M9401 was high, M9401 had black hyphae and the TLC plate was white, clear white spots which the growth of M9401 was inhibited by the toxins were appeared at parts of existing the toxins. And because the growth of hyphae of M9401 was fast and M9401 produced many spores, M9401 used easy. The plate was incubated in a plastic case with moisture condition and then the white spots were appeared with in 2 days after incubation, which growth of M9401 was inhibited by the toxins. The relative quantities of the toxins were determined based on the area of white spots. Those tests were repeated 5 times.

Comparison of characteristics of obtained strains by irradiation with UV: Hyphae growth of the obtained strains was measured using a ruler, and spore formation, yellow pigment production, frequency of sectors and concentric rings in colony were checked by the naked eye.

Pathogenicity: There are 2 methods to examine the pathogenicity of the strains. One is the pathogenicity easy detect method as described in the previous section. Another is the spore suspension method. Each strain was incubated on PSA at 25°C, and formed spores were collected. Then the collected spores were washed by distilled water at 3 times, and the spore suspension (1×10⁷ spores/ml) and equal volume of PS were inoculated at the same time onto the mulberry leaves. The examinations were repeated at 5 times.

Results

The spores of *M. roridum* (M9403) were irradiated with UV for 0, 0.5, 1, 2, 3 and 5 minutes for determination the optimum conditions of mutant induction. When the irradiation time increased, the number of colonies formed decreased and no colonies were formed when the irradiation time exceeded 3 min. (Fig. 1). The objective was the mutants which changed of only toxin production were gotten in the test of UV for 0.5 min. irradiation. When 3,000 spores were irradiated with UV for 0.5 min., 337 spores (17.9 %) were survived and formed colonies. Characteristics of the survived strains were compared with a strain non-irradiated with UV (control). Major characteristics were hyphae growth, spore formation, yellow pigment production, variability of colony color which were appeared as sector, concentric rings in colony, toxin production and pathogenicity were checked. The toxin production was compared by the toxin easy detect method and the pathogenicity was compared by the pathogenicity easy detect method. The 337 obtained strains were classified with 41 groups by these characteristics and the groups were classi-
Table 1. Characteristics of strains obtained strains not changed growth of hyphae by irradiation with ultraviolet rays.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Yellow pigment production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appearance of sectors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Appearance of concentric rings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxins production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each characteristic was compared with the control strain. Three thousands of *M. roridum* spores were irradiated with ultraviolet rays (UV) for 0.5 min. In obtained strains, those showing growth of hyphae not changed was shown. a) +: When the characteristics were more pronounced than those of the control, no signs similar to control, −: less pronounced than the control. b) Results acquired in obtained strains are taken as 100%. No. 20 group belonging column were named A group.

fied with 2 groups on a large scale. One group strains showed hyphae growth was similar to control (20 groups), and another group strains showed hyphae growth and toxin production were lower than control (21 groups). There were not groups strains showed hyphae growth was faster than control. In this examination, because only one character and pathogenicity changed mutants were acquired was purpose, the group hyphae that growth was similar was shown in Table 1. In obtained strains, there were many strains obtained group showed low spore formation (No. 3: 20.1%) and high yellow pigment production (No. 6: 4.9%). The percentage of the strains having the same characteristics as control was 17.3%. The induced mutants were inoculated onto mulberry leaves to find mutants with weak pathogenicity, and 2 strains (No. 20, named group A: A-1 and A-2) were selected (Fig. 2). Strains of group A showed the same characteristics as control strain except for toxin production. Hyphae growth of the 2 strains was not significant from control strain (at ANOVA p>0.20) and other characteristics were not different from that of control strain by checking with the naked eye. The toxin production by TLC-bioassay method differed significant between A-1, 2 strains and the control strain (p<0.001) under the same conditions (Fig. 3).

Control strain produced 4 kinds of toxins (Rf 0.18, 0.24, 0.33, 0.39), but strains of group A did not produce or few produced the toxins (Fig. 4). As compared with the pathogenicity by pathogenicity easy detect method, each strains of group A formed smaller lesion than that by control.
Fig. 3. Characteristics of mutants A-1 and A-2 strains. Characteristics and pathogenicity of A-1 and A-2 strains were compared with control strain (%). Characteristics and pathogenicity of control were shown 100%: 1) a: It was not significant at ANOVA (at p>0.2), b and c: It was significant (b: 0.01<p<0.05, c: p<0.001), 2) A: selected mutant strains of group A, C: control strain, 3) Characteristics of group A strains were measured by hyphae growth using ruler and toxin production by TLC-bioassay method, 4) Pathogenicity was investigated by inoculation of hyphae (pathogenicity easy detect method) and spores with PS (spore suspension method) to compare with control strain.

Fig. 4. Toxins production by mutants with weak pathogenicity. A-1 and A-2: selected mutant strains induced by irradiation with ultraviolet rays (UV) for 0.5 min., C: control strain. (at 0.01<p<0.05) and the lesion formed by the spore of each that by control strain by the spore suspension method (at 0.01<p<0.05).

Discussion

Mutants of *M. roridum* with deficient toxin production were induced by UV irradiation. The group A strains, the various characteristics of the colonies on PSA were same as that of the control strain except for toxin production were gained. In figure 4, it is seemed that the toxins were not produced by the group A strains, however, when the extracts of the group A strains were spotted at a high concentration on TLC plate, little toxins were detected at Rf as in the control. Therefore, group A strains produced few toxins than the control. So, the group A strains were selected as the fungi that produced few toxins.

On the other hand, the lesions formed on mulberry leaves by the group A strains were smaller than those by the control strain. The results suggested that ability of toxin production may be related to the pathogenicity of *M. roridum*. When *M. roridum* was isolated from mulberry field, a strain with weak pathogenicity was obtained. The strain also showed weak ability of toxin production (MURAKAMI and SHIRATA, 1998).

There are many reports of mutants induced by irradiation UV in many lives. In fungus, toxin, cytocharasin E deficient mutants were isolated by irradiation with UV in *Rosellinia nectrix*, recently (KANEMATSU et al., 1997). In *R. nectrix*, toxin deficient mutants were obtained by UV irradiation method, but the mutants showed same pathogenicity as control for seeds and seedlings (KANEMATSU et al., 1997). However, in *M. roridum*, mutants which produce deficient toxins showed weaker pathogenicity than control strain for cut leaves. The difference may cause by species of fungi and inoculated methods. It is necessary to do eliminate or insert study of the genes for toxins production. To determine clearly the role of the toxins in the pathogenicity of *M. roridum*, the results did not enough to elucidate the relationship between toxin production and the
pathogenicity of *M. roridum*. However, the results in this paper may offer an evidence that toxin production is related to the pathogenicity.

**References**


