

Fusarium oxysporum f.sp.cucumerinumのオレンジ色突然変異株の耐久生存と病原性

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Survival and Pathogenicity of the Orange Mutant of *Fusarium oxysporum* f. sp. *cucumerinum*

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Abstract

An orange mutant of *Fusarium oxysporum* (Schlecht.) emend. Snyder et Hansen, f. sp. *cucumerinum* Owen CH11200 (Foc) was obtained by ultraviolet irradiation. Pathogenicity, morphological features and some other properties of this mutant (T107) and its 15 single spore isolates relating to their survival in soil were compared with those of Foc. Microconidia of T107 were formed on the phialide. Productivity of microconidia of T107 and its 15 isolates on PSA was almost the same as that of Foc. The effect of temperature on growth of both Foc and T107 seemed to be similar. T107 survived in soil as chlamydospore and caused wilt of cucumber seedlings. It was proved that T107 had the same morphological and biological features and behaviors in soil as Foc. This orange mutant is considered to be suitable for ecological study on *Fusarium oxysporum* f. sp. *cucumerinum*.

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Key words: *Fusarium oxysporum*, mutant, ultraviolet irradiation, pathogenicity, chlamydospore.

INTRODUCTION

From the point of ecological view on *Fusarium*-suppressive soil, behavior of *Fusarium* species in soil have been interested⁸⁾. In naturally infested soil the role of nonpathogenic *Fusarium* is one of the important factors to control diseases^{6,9,11)}. For detecting the *Fusarium* colonies selective media were developed³⁾. These media have been progressed in sensitivity for *Fusarium* species, however there was still a problem with distinguishing the pathogenic *Fusarium* from nonpathogenic ones⁵⁾. It is difficult to recognize which form a specialis each colony may belong in. Determination of true nonpathogenic *Fusarium* species is much time-consuming in practical procedures. In order to solve this problem, addition of genetic marker is required. Auxotrophic, morphological and color mutant can be induced from the standard method of mutagenesis. Color mutant is considered to be more effective *in situ* than the others. Using the orange mutants of *Fusarium oxysporum* f. sp. *apii* in the study with suppressive soil, parasitic competition with non-pathogenic *Fusarium* on the celery roots was clearly showed¹¹⁾. Recently gene engineering method was applied in phytopathogenic bacteria and these bacterial colonies showed various intensities of blue color on a specific media¹⁾. This idea, using both a mutant and a selective medium, would also be beneficial to the detection of target fungal pathogen. Another recent current for the detection of pathogen was immunoassays and has been tried in *F. oxysporum*²⁾. This method facilitated recognition of an individual pathogen and its behavior.

In the present study survival in soil, pathogenicity and some morphological and biological properties of the orange mutant of *Fusarium oxysporum* f. sp. *cucumerinum* were investigated for ecological study.

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MATERIALS AND METHODS

Mutagenesis and selection of mutants. *Fusarium oxysporum* (Schlecht.) emend. Snyder et Hans. f. sp. *cucumerinum* Owen CH11200 (Foc), was grown on potato sucrose agar (PSA) for 10 days at 27 C under dark condition. This fungus presented the morphological characters of white aerial hyphae and densely violet basal hyphae on PSA. Conidia were scraped from colonies on PSA and suspended in sterile distilled water. The suspension was filtrated through a glass filter (20–30 μ m of pore size) aseptically. The conidial suspension (0.1 ml) of Foc was spread on the plates of PSA with a sterile glass rod. These plates were subjected to mutagenic ultraviolet light (Toshiba GL15, 254 nm) and uncovered during irradiation. After irradiation all dishes were packed in a carton box and incubated at 27 C for 7 days under dark condition. Any colony which presented lightly either orange or violet pigment was isolated on PSA and was serially transferred at 5-day intervals. Growth and color of the colonies was observed for selecting mutant. As appeared colonies on irradiated plates did not necessarily originate from a single spore, single spore isolation was tried to know stability of the mutant selected. Fifteen microconidia of a mutant were randomly isolated and replicated on Komada's medium.

The orange mutants were compared with Foc concerning the colony growth and the number of microconidia produced on PSA. Colony growth of Foc and the original isolate of T107 were measured at 10, 15, 20, 25, 30 and 35 C, respectively. As 25 C was the optimal temperature, colony growth of 15 isolates of T107 were compared only at 25 C. Fifteen-day-old culture on PSA was scratched and then suspended in a definite amount of sterile distilled water. Number of microconidia was counted with Thoma's haemocytometer. Mean numbers were averaged on 3 or 5 replicates.

Pathogenicity and survival in soil of orange mutants. Microconidia collected from the colony which was grown on PSA for 15 days were added to steam-sterilized soil (Andosol, Chiba Univ.) for artificial infestation. Seventeen experimental plots were separately infested with Foc, T107 and its 15 single spore isolates. Cucumber seeds (*Cucumis sativus* L. 'Ootone-Ichi-Go'), previously disinfested for 5 min in 5% sodium hypochloride solution and washed in running tap water, were pregerminated at 27 C for 24 hr on moist filter paper in a large petri dish. Germinated seeds were sown and cultured in each infested soil for 40 days. This experiment was repeated twice (from July 8 and from September 12). Plants were observed every day and number of wilted plants was recorded. At the end of the experiment, about 10 mm long of hypocotyl of all standings were sampled and sterilized for 5 min with 5% sodium hypochloride solution, then washed three times in sterile distilled water. These segments were put on water agar (WA) and incubated for 3 days and observed on microconidia formation in false heads. They were also examined for the appearance of *Fusarium oxysporum* on Komada's medium. Population density was determined with the dilution plating method using Komada's medium 60 and 120 days after infestation. Every incubation was done as described above.

Chlamydospore formation in the infested soil was also examined. Existence of chlamydospore was detected by fluorescent staining technique. Solution of 0.1% glucose and 0.1% sodium asparaginate was added to 0.3 g of soil samples which were collected 180 days after the infestation. After 18 hr incubation at 25 C under dark condition, soil samples were stained with 0.3% Calcofluor White M2R New (Sigma Chemical Co.) and observed with fluorescent microscope^{10,13}.

To know how the orange mutant can be distinguished on the dilution plates of natural soil samples, five different Andosol, Gunma (Sawa, Tsumagoi), Chiba (Chiba, Matsudo), and Kanagawa (Miura) were artificially infested with the orange mutant. Appearance of orange mutant from these soils was examined with dilution plating method using Komada's medium.

RESULTS AND DISCUSSION

Induction and isolation of orange mutants

Effect of irradiation on Foc is shown in Fig. 1. Two colonies which showed lightly orange ones (T105 and T106) in basal hyphae on PSA were obtained. They arose under 4,200 erg/mm² and 14,400 erg/mm², respectively. They were transferred on PSA after 5 days incubation at 27 C. After first replication on PSA, both aerial and basal hyphae showed only lightly violet pigment. Therefore microconidia of T105 were irradiated again in the same manner, and a clear orange colony (T107) was obtained on PSA. The character of mutant T107 was orange aerial hyphae and marbling basal ones with orange and lightly violet pigment after mycelial replication on PSA, even though T107 was recognized as clear orange aerial and basal hyphae on Komada's medium. No revertant was found.

Characters of orange mutants

Effect of temperature on colony growth was almost the same between Foc and T107 (Table 1) and among 15 isolates of T107. Microconidia productivity showed little difference among the mutants (Table 2). Observed conidia of T107 were morphologically identical to Foc. Microconidia of T107 were formed on the phialides as well as those of Foc. Exceeding presence of macroconidia was not observed on T107.

Survival and pathogenicity of orange mutants in soil

In regards of changes in population density in sterilized soil the manner of dynamics of Foc and the mutants was examined (Fig. 2). Population density at 60 days was more diversified than that at 120 days. Maximal difference of population density was 10,000 cfu/g of dry soil at 60 days. However the population density of the mutants were distributed around that of Foc at 120 days. Effect of roots and its exudates on the inocula should be considered as pop-

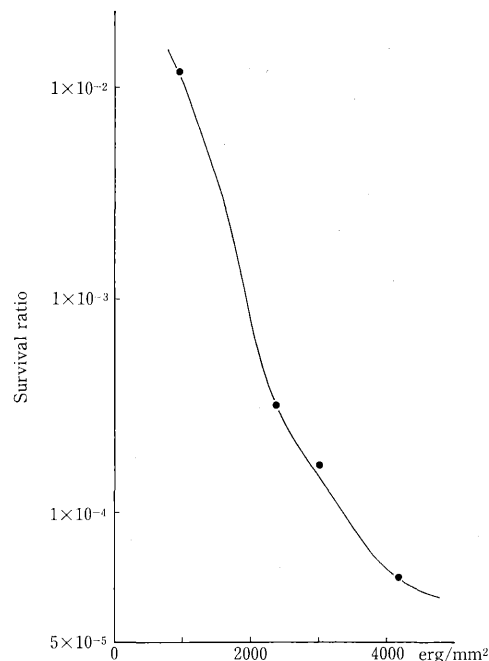


Fig. 1. Effect of ultraviolet intensity on the survival of microconidia of *Fusarium oxysporum* f. sp. *cucumerinum*.

Table 1. Effect of temperature on mycelial growth of *Fusarium oxysporum* f. sp. *cucumerinum* and its orange mutant (T107) on PSA ^{a)}

Temperature (C)	Days after inoculation				
	2	3	4	6	7
10	3.7 ^{b)}	5.8	9.4	14.6	18.0
	4.0	7.2	10.8	16.0	19.0
15	12.8	19.0	25.2	34.6	40.2
	14.0	19.6	25.8	34.4	39.6
20	15.8	23.6	30.8	44.6	51.0
	15.6	24.2	31.2	44.8	52.0
25	19.6	29.6	40.6	60.2	71.2
	20.8	31.0	41.4	59.0	68.8
30	21.8	30.6	40.9	59.2	69.8
	21.7	31.3	41.2	57.6	68.4
35	2.2	3.8	5.2	6.9	8.2
	3.6	4.3	5.8	7.9	9.1

a) Average of 5 replicates.

b) Upper column: colony diameter (mm) of Foc, lower column: diameter of T107.

Table 2. Comparison of colony diameter and number of microconidia between *Fusarium oxysporum* f. sp. *cucumerinum* and its orange mutant (T107) at 25 C on PSA ^{a)}

Isolate	Colony diameter ^{b)} (mm)	No. of microconidia/ml ^{c)}
Foc ^{d)}	73.7	2.4×10^7
T107	71.0	1.8×10^7
T107-1 ^{e)}	73.3	2.0×10^7
T107-2	73.3	1.9×10^7
T107-3	72.7	2.1×10^7
T107-4	71.7	2.0×10^7
T107-5	69.0	1.7×10^7
T107-6	77.0	2.5×10^7
T107-7	77.0	3.5×10^7
T107-8	79.0	2.5×10^7
T107-9	68.0	1.9×10^7
T107-10	72.3	2.0×10^7
T107-11	77.0	1.5×10^7
T107-12	71.7	2.3×10^7
T107-13	70.3	2.2×10^7
T107-14	69.3	1.9×10^7
T107-15	69.0	1.9×10^7

a) Average of 3 replicates.

b) Measured 7 days after inoculation.

c) Counted 15 days after inoculation.

d) *Fusarium oxysporum* f. sp. *cucumerinum*.

e) 15 single spore isolates of T107.

ulation density was examined at the end of the culture of cucumber. The residues of plant roots were considerably left at the end of second experiment of cucumber. This condition may be favorable for the distribution of inocula even if the number of exogenous microbes were infected as nutrient competitors. Thus inocula of both Foc and the mutants at 120 days would be more randomly distributed than those at 60 days. Feature of colonies were clearly repre-

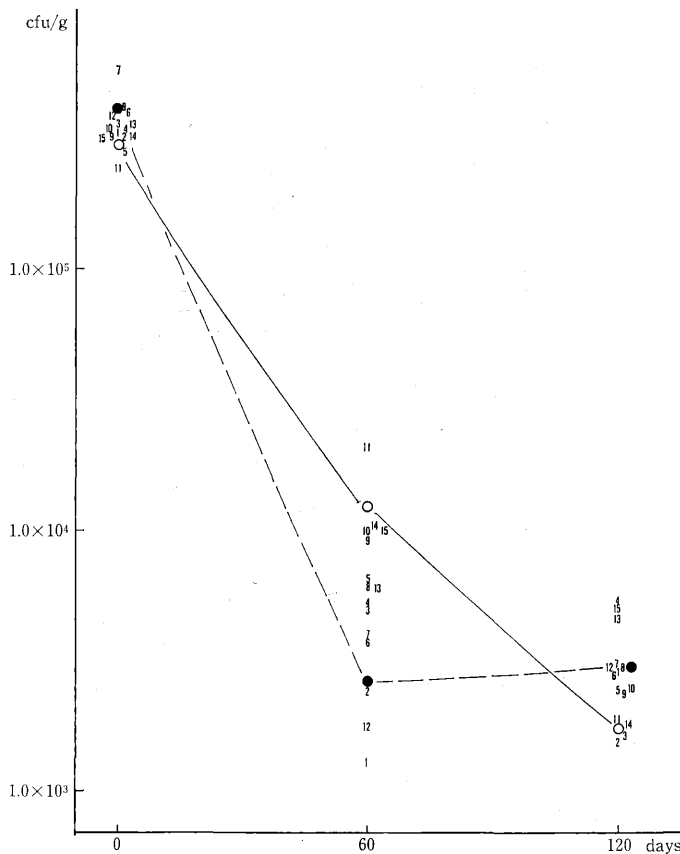


Fig. 2. Population dynamics of *Fusarium oxysporum* f. sp. *cucumerinum* (Foc) and its orange mutant (T107) inoculated in sterilized soil. ●: Foc, ○: T107. Numbers represent the isolates of T107.

sented on the dilution plate using Komada's medium. These mutants showed the phenotype with both orange aerial and basal hyphae and in addition orange color was evoked whether under illuminated or not, which was contrasted the critical feature of *Fusarium oxysporum* on Komada's medium (Plate I-1); i.e. this medium exerted the selectiveness incubating at room temperature under continuous fluorescent light for at least 1 week⁵⁾. This would facilitate to distinguish the infested inoculum in natural field soil. For instance the soil infested with T107 was mixed with five natural field soils and examined how the orange mutants could be distinguished. As the result, the mutants can easily be found by only 3 day incubation in dark at 27 C (Plate I-2). However colonies of some fungi (*Trichoderma* spp., etc.) and/or some bacteria could affect the coloring of this mutant and made them reddish (Plate II-3) as it was observed even in native *Fusarium oxysporum*. This red pigment seemed to be a bikaverin as it appeared under antagonistic conditions⁹⁾. In another way this orange pigment was exerted in the mycelia of colonies on Komada's medium and in the growing mycelia on segments of cucumber put on WA. It suggested that the production of orange pigment was affected by the composition of media. While it was reported that the production of bikaverin was affected by the nitrogen sources (especially asparagic acid)⁴⁾, the relationship between the bikaverin and this orange pigment was not considered in this study. Several color mutants have been selected for *F. oxysporum* as indicators. Both yellow and white mutants were arisen from the orange mutants

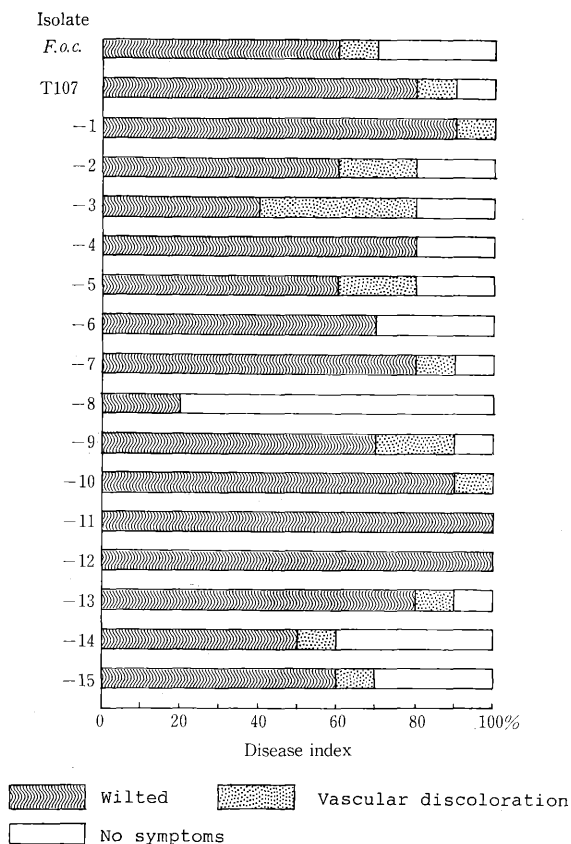


Fig. 3. Pathogenicity of *Fusarium oxysporum* f. sp. *cucumerinum* and its orange mutant (T107) to cucumber plants.

of *F. oxysporum* f. sp. *apii* by repetitionary ultraviolet treatment⁷⁾. While the effect of ultraviolet intensity and the sensitivity of mutagenized isolates should be considered, these variable coloration suggested a cascade reaction of pigment production induced by the mutagenesis. The nature of orange pigments and their pathway of production will be examined in the future.

Pathogenicity of T107 and its 15 isolates were comparable to Foc (Plate II-2). Although there are more differences in virulence among the isolates, they showed a tendency in successive plantings. So those differences may be inherent in the isolates (Fig. 3). It was reported that morphology of the mutants of *Fusarium solani* f. sp. *pisi* did not affect pathogenicity¹⁴⁾. The results described above supported the independence between the colony morphology and the pathogenicity.

As these mutants could survive in soil it was examined whether chlamydospore was formed as a resting structure or not. In the soil infested with Foc or T107 germinated spherical bodies were observed, respectively, after 18 hr incubation (Plate II-1A, 1B). These bodies looked like germinated chlamydospores reported previously⁶⁾. These bodies are seemed to be chlamydospores of inoculated fusarium. Abnormality on germination was not observed for the mutant T107 compared with that of Foc.

Orange mutants in *F. oxysporum* f. sp. *apii* have been checked for the reversion with several points, for example, culture on diluted PSA, isolation from infected plants which were grown and inoculated *in vitro*¹²⁾. The mutants T107 and its 15 isolates were also observed and checked

Plate I

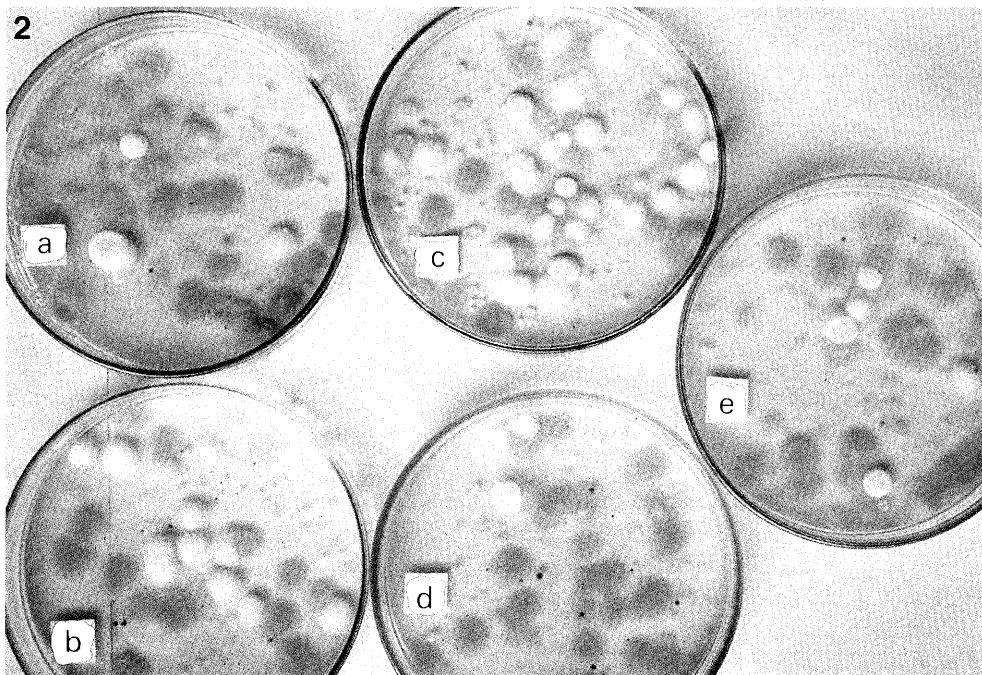
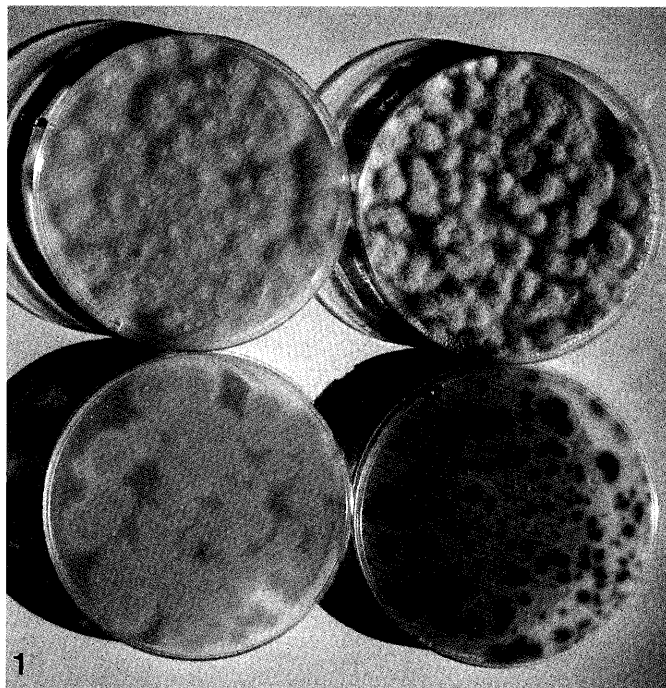
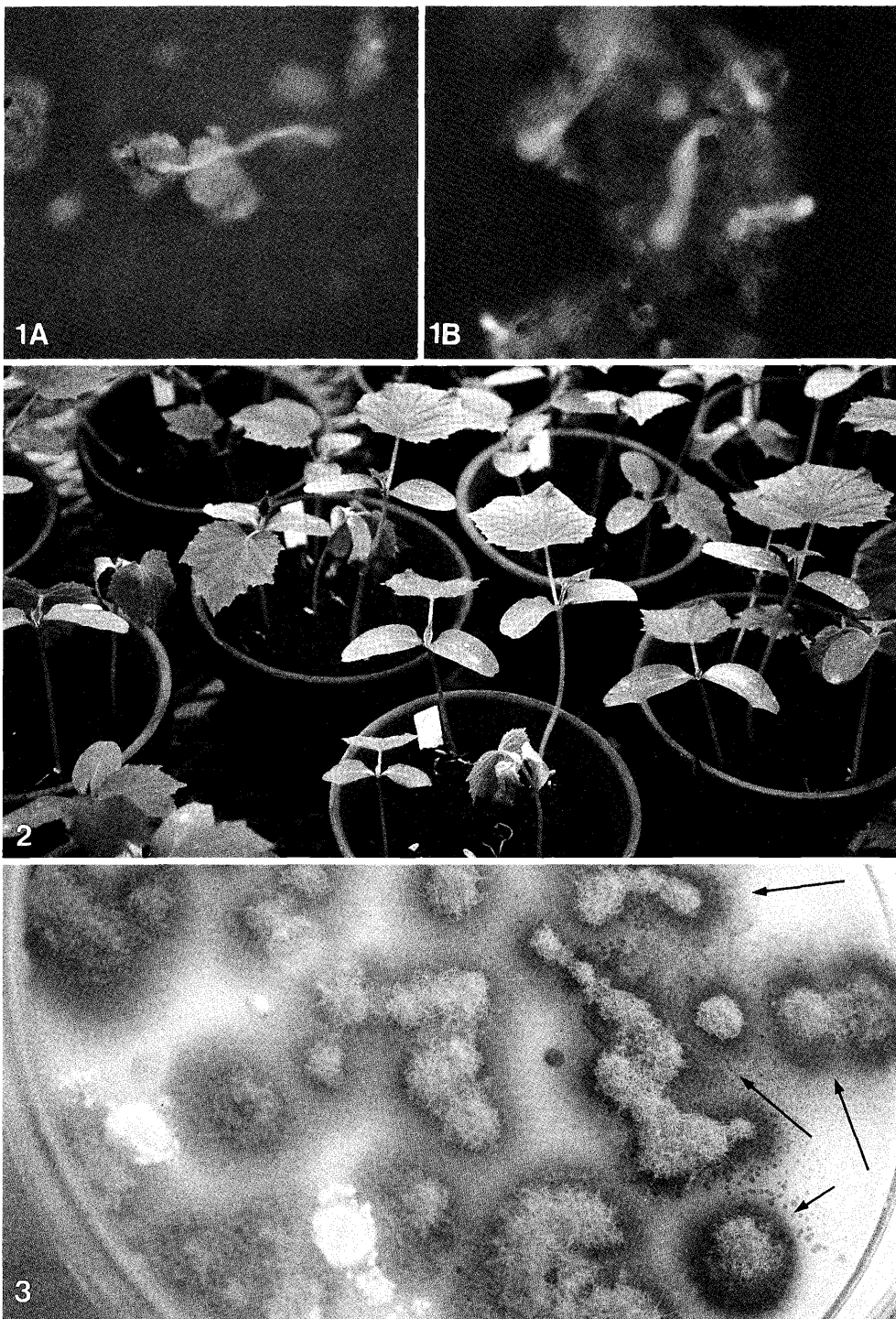


Plate II



carefully in several points and they did not revert. For pathogenicity test, only the orange colonies were found from hypocotyl segments of infected cucumber plants which were surface sterilized and put on Komada's medium or WA.

It is evident that this orange mutants retain the features and behaviors of Foc. It will provide a favorable information in the ecological studies on *Fusarium oxysporum* in soil.

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和 文 摘 要

長尾英幸・平野和弥: *Fusarium oxysporum* f. sp. *cucumerinum* のオレンジ色突然変異株の耐久生存と病原性

紫外線照射により *Fusarium oxysporum* f. sp. *cucumerinum* (Foc) のコロニーがオレンジ色を呈する突然変異株を得た。この突然変異株 (T107) とその単胞子分離によって得られた 15 株を土中での生存, 病原性および他の形態的な特徴について Foc と比較検討した。その結果, T107 とその分離菌株は, 土中で厚膜胞子として耐久生存でき, キュウリ幼苗につる割病を発生させることができた。また, 形態的な特徴に関しては, いずれも小型分生胞子をフィアライド上に形成し, PSA 上での小型分生胞子数も Foc と T107 の分離 15 菌株間ではほとんど同じであった。Foc と T107 のコロニー生育に対する温度の影響はほぼ同じと考えられた。以上のことから, これらのオレンジ色突然変異株は, Foc と同じような特徴と行動を示すことが明らかにされた。本菌は, *Fusarium oxysporum* f. sp. *cucumerinum* の生態学的研究に利用しうると考えられる。

Explanation of plates

Plate I

1. Differential characters of *Fusarium oxysporum* f. sp. *cucumerinum* (Foc) and its orange mutant (T107) on Komada's medium. Right: Foc, left: T107. Appearance of reverse side of colonies in lower column, respectively.
2. Colonies of orange mutant in 5 different natural soils, 3 days after incubation under dark condition at 27 C. Soils from Gunma (Sawa): a, Gunma (Tsumagoi): b, Chiba (Chiba): c, Chiba (Matsudo): d, and Kanagawa (Miura): e.

Plate II

1. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *cucumerinum* (A) and its orange mutant (B) stained with Calcofluor White.
2. Wilted cucumber plants caused by orange mutant (T107).
3. Pigmentation affected by some kinds of bacteria.