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Taxonomic Consideration on Two Closely Related Species, *Pythium paddicum* and *P. polypapillatum*

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Abstract

Seventy-two isolates were tentatively identified as *Pythium paddicum* and several of them were compared for the morphological variation of projection on the oogonium. This fungus produced four types of projections on various culture media with different isolates and incubation temperatures, unlike the original description by Hirane[1]. *P. paddicum* having various types of projection resembled the original description of *P. polypapillatum*. In November, 1985, two isolates of *Pythium* sp. which resembled *P. polypapillatum* were newly isolated from damp ground adjacent to the pond where *P. polypapillatum* had been originally isolated 50 years ago. *P. paddicum* seems to be identical with *P. polypapillatum*.

Introduction

Pythium paddicum was first described as a causal fungus of snow rot of wheat and barley in Japan[1]. Although characterized by short, blunt projections on the oogonium[1], this fungus frequently produced oogonia with long branched projections on V-8 juice agar, indicating similarities to *P. polypapillatum*.

On the other hand, *P. polypapillatum* has also been found in Japan[2] and recently in U.S.S.R.[3]. It had been isolated from pond water and was weakly parasitic to rice seedlings on inoculation[4]. Since neither dried material nor a living culture of this fungus is available[3], the morphology of *P. paddicum* and *P. Polypapillatum* can not be compared directly.

This study deals with the difference between the two species, especially in the morphology of projections on oogonium of the former. Both species were also isolated from different locations, including the pond where *P. polypapillatum* had been originally isolated. Part of this work has already been reported elsewhere[5].

Materials and Methods

Isolates Seventy-two isolates obtained from Fukui and Niigata Prefectures and tentatively identified as *P. paddicum* were employed for the present experiment. The isolates were maintained by subculturing every 6-12 months on cornmeal agar (CMA; cornmeal 20 g, agar 15 g, distilled water 1,000 ml) at 20°C.

Media Eleven media used were as follows: Sachs's agar (SA)–KNO_3 1 g, NaCl 0.5 g, Ca $(NO_3)_2$ 0.5 g, Ca_3 $(PO_4)_2$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeCl_2$ trace and agar powder 10 g were added in this order to 1,000 ml distilled water (DW); Bacto-cornmeal

agar (DF-CMA); Bacto-lima bean agar (DF-LBA); Bacto-oatmeal agar (DF-OMA); potato carrot agar (PCA)[3]; concentrated potato carrot agar (Conc. PCA)– carrot root 227.5 g, potato tuber 500 g, glucose 10 g and agar powder 15 g in DW 1,000 ml; rice leaf extract agar (RLEA)– above-ground parts of rice plant 33.3 g and agar powder 1.5 g in DW 100 ml, sterilized by millipore filtration (pore size: 0.22 μm); rice root agar (RRA)– rice root 6 g and agar powder 9 g in DW 600 ml; rice root extract agar (RREA)– rice root 21.7 g and agar powder 1.5 g in DW 100 ml, sterilized by millipore filtration (pore size: 0.22 μm); turf leaf agar (TLA)– turfgrass blades 30 g (dry weight) and agar powder 15 g in DW 1,000 ml; V-8 juice agar supplemented with wheat germ oil (Japan Impex Co., Ltd., Tokyo) at 500-5,000 ppm (V8A). All the media were adjusted to pH 6-7 with 0.1 N NaOH.

Grouping of the oogonia of P. paddicum After observing *P. paddicum* on V8A at 15°C, oogonia were divided into following 4 groups according to the morphology of their projections: Type I – typical oogonium with only short, unbranched projections (Fig. 1); Type II – typical oogonium with one or more short (less than the diameter of the oogonium), branched projections (Fig. 2); Type III – atypical oogonium with one or more long (more than the diameter of the oogonium) projections (Fig. 3); Type IV – oogonium-like structures not belonging to any above types (Fig. 4).

Observations of various types of oogonium Three isolates (PP15, PP16 and PP23) were cultured on V8A at 15°C. PP23 was then cultured on 7 media (SA, DF-CMA, DF-LBA, DF-OMA, Conc. PCA, RLEA and RREA) at 15°C, and on V8A at 5, 15 and 25°C. Oogonia were observed in the 40-day-old culture. The morphology of the oogonium was examined concerning frequency of observation, proportion of mature oospores and dimensions of projections.

Isolate PP23 was incubated on SA and PCA at room temperature and the oogonia were successively observed and microphotographed to describe the formation of 4 types of oogonium during incubation, after they had been differentiated.

Analysis of factors controlling different types of oogonium Four isolates (PP15, PP16, PP32 and PP66) randomly selected from 72 isolates were inoculated on 7 different media (SA, CMA supplemented with wheat germ oil at 500 ppm, DF-OMA diluted 10 times with DW, PCA, RRA, TLA and V8A). After incubating for 30 days at 15°C, the frequency of different oogonium types was examined for 200 individuals. A two-way analysis of variance was carried out on each oogonium type using data transformed by arcsin square root. A broad sense heritability (H) of the ability of oogonial formation was estimated by the following equations with components of variance.

$$V_E = (SS_E + SS_{AxB}) / (\phi_E + \phi_{AxB}),$$

$$H = \frac{\sigma^2_A}{\sigma^2_A + \sigma^2_E} = \frac{V_A - V_E}{V_A + 69V_E},$$

where SS_E, SS_{AxB}, ϕ_E, ϕ_{AxB}, σ^2_A, σ^2_E, V_A and V_E are error sum of square, interaction sum of square, error degree of freedom, interaction degree of freedom, genetical component of variance, environmental component of variance, genetical mean square and environmental mean square, respectively[6].

Isolation of P. paddicum or P. polypapillatum from soil The isolation was seasonally performed 5 times in 1985; once each in spring and summer, and 3 times in autumn. Samples of water and soil were collected from different points in Kyoto, Sakai and Sennan Cities.

Baiting method: Three-mm apple pericarp slices, hulled seed of rice (cv. Tozan No.38) boiled for 10 min. and dead blowflies (*Aldrichina grahami*) were used for baiting materials. Six pieces of apple and rice surface sterilized in 70 % ethanol were put together into 200-250 ml water samples or 200 ml sterile water mixed with 2 g soil samples, and incubated at 20 C for 2 days. Separate drops of 10,000 ppm streptomycin sulfate (Meiji, Tokyo) was placed on symmetrical points along the edge of DF-CMA plate. The baits were taken out, washed in sterile water, excess water removed by filter paper and placed onto the points where streptomycin had been applied. Three dead blowflies soaked in the samples were cut into 2 or 3 pieces and then similarly plated onto DF-CMA.

Another baiting method was performed using essentially the same method as already described[7]. The bait was a 2 cm-long leaf piece cut from 1 cm below the tip of the first leaf of a 9-day-old wheat (cv. Norin No.61) seedling. Six pieces were put into each 300 ml Erlenmyer flask containing 100 ml of water samples or 100 ml sterile water mixed with 1 g soil samples. After incubating for 40-42 days at 1-7 °C, the leaf pieces were taken out, washed in running tap water, dried on filter paper, then placed onto selective DF-CMA supplemented with 100 ppm vancomycin (Sigma, St. Louis), 10 ppm thiophanate-methyl (Nihon-Soda Co., Ltd., Tokyo) and 40 ppm pentachloronitrobenzene (Nissan Kagaku Kogyo Co., Ltd., Tokyo).

Direct inoculation method: Five-ml water samples were directly plated on P10VP mediun[8]. Small amounts of soil were also directly seeded on P10VP and DF-CMA.

In all cases, the isolation was conducted at 15 and/or 20 °C. After 3-7 days' incubation, hyphal tips were isolated and then subcultured on CMA. Taxonomic studies were performed using autoclaved grass blades[9].

Results

Morphological variation in projections on the oogonia of P. paddicum The frequency of observation of different oogonial types and the proportions of mature oospores in each type varied with isolate, medium and temperature, while the dimentions of the projections tended to be invariable in the same oogonium type (Tables 1–3). Types I and II were commonly observed with high rates of oospore maturity. Many oogonia of type III were found on such media as SA, Conc. PCA, RLEA and RREA. SA was the only medium on which many type III oogonia contained mature oospores. Few oogonia of type IV was found except on RLEA and RREA.

Successive observations on oogonial formation Figures 5–28 are successive microphotographs of oogonium types I–IV, respectively. An oogonial initial was formed as a terminal swelling of a hypha after incubation for at least 48 hr. Large amounts of protoplasm passed into the swelling from the hypha. When the swelling reached full size, the young oogonium became polygonal in shape (Figs. 5, 11, 17, 23). Then projections on the oogonial wall extended (Figs. 6–8, 12–16, 18–21, 24–28), sometimes branching dichotomously (Figs. 27, 28). If the antheridial hypha came in contact with the oogonium, the projections stopped elongating and began to digest their protoplasm (Figs. 9, 15, 21). The whole processes of sexual reproduction was finally completed, resulting in oogonium type I or II (Figs. 10, 16), containing round, thick-walled oospore. If antheridial development was delayed, the oogonium continued to extend projections filled with protoplasm. When the oogonium came in contact with the antheridium, the specific shape of type III was formed (Fig. 22). The young oogonium of type IV continued to extend complex projections, probably without encountering an antheridium (Fig. 28).

Table 1. Morphological variation in oogonia of three isolates of *P. paddicum* on V8A.[1)]

Oogonium type	Characteristics observed	PP15	PP16	PP23
I	Frequency (%)[2)]	36.3	86.8	75.8
	Mature oospores (%)[3)]	96.0	100.0	95.0
	Dimensions of projections (μm)[4)]	5.6 X 8.2	4.7 X 6.3	5.2 X 9.3
II	Frequency (%)	62.0	13.0	11.3
	Mature oospores (%)	96.0	100.0	100.0
	Dimensions of projections (μm)	6.6 X 7.3	7.8 X 4.7	6.3 X 6.4
III[5)]	Frequency (%)	1.7	0.2	12.9
	Mature oospores (%)	84.6	– [6)]	71.7
	Dimensions of projections (μm)	5.1 X 19.0	– [6)]	5.0 X 25.9
IV	Frequency (%)	0.0	0.0	0.0

1) Results are the average of 2 replications.
2) The frequency (%) was determined by examining 200 oogonia per 2 mycelial agar disks cut from a 30-day-old culture.
3, 4) Mature oospores (%) and the dimensions (width X length) of the largest projection on each oogonium were determined with 50 oogonia per disk cut from a 40-day-old culture.
5) Since there were few oogonia type III in a mycelial disk, the results of isolates PP15 and PP23 were obtained from 13 and 43 oogonia, respectively.
6) The percentage and the dimensions of type III were not determined because the frequency was too low.

Table 2. Morphological variation in oogonia of *P. paddicum* (PP23) on various media.[1)]

Oogonium type	Characteristics observed	SA	DF-CMA	DF-LBA	DF-OMA	Conc.PCA	RLEA	RREA
I	Frequency (%)[2)]	10.0	63.0	0.0	53.8	52.0	15.8	3.3
	Mature oospores (%)[3)]	86.2	0.0	–[5)]	98.0	29.0	0.0	38.5
	Dimension of projections (μm)[4)]	5.3X9.8	5.1X8.4	–	4.4X6.2	5.1X8.5	5.4X10.7	4.8X12.2
II	Frequency (%)	20.8	26.3	0.0	46.2	13.8	7.8	5.5
	Mature oospores (%)	87.0	0.0	–	99.0	50.0	0.0	21.2
	Dimension of projections (μm)	6.9X11.0	6.2X5.8	–	5.4X5.4	6.3X7.1	5.8X7.4	6.2X9.9
III	Frequency (%)	68.5	10.7	0.0	0.0	34.3	20.0	48.5
	Mature oospores (%)	82.0	0.0	–	–	4.0	0.0	12.0
	Dimension of projections (μm)	5.2X21.7	5.2X18.4	–	–	4.8X28.8	5.0X22.1	5.0X27.7
IV	Frequency (%)	0.8	0.0	0.0	0.0	0.0	56.4	42.7

1) Results are the average of 2 replications.
2) The frequency (%) was determined by examining 200 oogonia per 2 mycelial disks cut from a 30-day-old culture.
3, 4) Mature oospores (%) and the dimensions (width X length) of the largest projection on each oogonium were determined for 50 oogonia per disk cut from a 40-day-old culture.
5) As in 6) on Table 1.

Table 3. Morphological variation in oogonia of *P. paddicum* (PP23) on V8A under different incubation temperatures.[1)]

Oogonium type	Characteristics observed	5°C	15°C	25°C
I	Frequency (%)[2)]	55.8	61.5	82.5
	Mature oospores (%)[3)]	99.0	100.0	72.0
	Dimensions of projections (μm)[4)]	5.3 X 7.5	5.4 X 6.6	5.2 X 5.7
II	Frequency (%)	44.3	37.5	12.0
	Mature oospores (%)	100.0	98.0	72.5
	Dimensions of projections (μm)	6.7 X 6.4	6.8 X 6.1	5.6 X 8.7
III	Frequency (%)	0.0	1.0	5.5
	Mature oospores (%)	– [5)]	63.0	25.8
	Dimensions of projections (μm)	–	5.2 X 21.2	5.2 X 32.8
IV	Frequency (%)	0.0	0.0	0.0

1) Results are the average of 2 replications.
2) The frequency (%) was determined by examining 200 oogonia per 2 mycelial disks cut from a 30-day-old culture.
3, 4) Mature oospores (%) and the dimensions (width X length) of the largest projection on each oogonium were determined fro 50 oogonia per disk cut from a 40-day-old culture.
5) As in 6) on Table 1.

Table 4. Development of four types of oogonium of *P. paddicum* in different periods of time after inoculation

Incubation period (hr)	No. of various types of oogonia differentiated in the same microscopical field during incubation									
	At 5 mm distance from the inoculum					At 20 mm distance from the inoculum				
	I	II	III	IV	Total	I	II	III	IV	Total
43 – 50	0(0)[1)]	0(0)	0(0)	0	0	0(0)	0(0)	0(0)	0	0
50 – 58	0(0)	4(0)	6(1)	10	21	0(0)	0(0)	0(0)	0	0
58 – 66	0(0)	2(0)	2(0)	3	7	0(0)	0(0)	0(0)	0	0
66 – 74	0(0)	0(0)	3(0)	6	9	0(0)	0(0)	0(0)	0	0
74 – 82	0(0)	0(0)	1(0)	5	7	0(0)	2(1)	7(2)	1	13
82 – 90	1(0)	4(0)	1(0)	0	6	1(0)	6(0)	0(0)	0	7
90 – 102	4(0)	0(0)	0(0)	1	5	0(0)	9(1)	0(1)	0	11
102 – 168	4(0)	13(0)	2(0)	0	19	1(0)	11(1)	1(0)	0	14
Total	9(0)	23(0)	15(2)	25	74	2(0)	38(3)	8(3)	1	45

1) The number in the parenthesis indicates abortive oogonia.

Some of the type IV oogonia possessed secondary oogonia at the tips of their projections and others had projections extending further, like mycelium. In any cases, all the oogonium types had the same origin as a hyphal swelling.

Oogonium types III and IV tended to emerge faster than types I or II (Table 4).

Factors controlling the formation of various types of oogonium The results shown in Table 5 are the means of 10 replications. The typical oogonium of type I or II was commonly produced on all the media, whereas many atypical oogonia of type III or IV were found on the specific media such as SA, PCA and TLA. Analysis of variance in-

Table 5. The mean proportion of each oogonial type in four isolates of *P. paddicum* cultured on various media for 30 days.

Medium	Percentages/oogonial type/isolate															
	PP15				PP16				PP32				PP66			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
SA	6.5	4.0	74.5	15.2	16.2	2.8	61.8	19.2	8.9	13.1	57.4	20.6	9.1	16.5	71.5	3.0
CMA	47.0	48.5	4.3	0.4	88.6	11.2	0.3	0.0	69.4	30.5	0.1	0.0	31.1	68.6	0.3	0.0
DF-OMA	42.0	58.0	0.0	0.0	88.0	12.0	0.0	0.0	34.6	63.4	1.1	1.0	45.1	59.9	0.0	0.0
PCA	8.0	2.7	48.3	41.0	74.9	2.9	16.3	5.9	—[1]	—	—	—	58.1	32.7	9.0	0.2
RRA	94.9	3.0	1.6	0.6	96.4	0.4	2.5	0.7	87.3	10.2	2.1	0.5	99.3	9,1	0.6	0.1
TLA	72.4	1.8	25.1	0.7	74.8	0.5	22.6	2.2	81.7	0.3	15.4	2.7	92.7	0.7	5.7	1.1
V8A	53.6	44.5	1.9	0.0	97.5	2.6	0.0	0.0	70.7	28.8	0.5	0.1	40.2	59.9	0.0	0.0

1) No oogonia were formed.

Table 6. The analysis of variance on the frequency of four oogonial types of *P. paddicum* when cultured on various media.

Type	Source of variation	SS[1]	Df[2]	V[3]	F_0[4]
I	Total	147519	279		
	Between isolates	20817.6	3	6939.2	260.387***[5]
	Between media	88785.9	6	14797.6	8.537***
	Interaction (isolates × media)	31200.1	18	1733.3	65.042***
	Error	6715.7	252	26.6	
II	Total	92629.5	279		
	Between isolates	19079.1	3	6359.7	401.339***
	Between media	54540.1	6	9090.0	10.896***
	Interaction (isolates × media)	15017.0	18	834.3	52.648***
	Error	3993.3	252	15.8	
III	Total	106385	279		
	Between isolates	4251.5	3	1417.2	83.872***
	Between media	88329.8	6	14721.6	27.761***
	Interaction (isolates × media)	9545.3	18	530.3	31.384***
	Error	4251.0	252	16.9	
IV	Total	30770.2	279		
	Between isolates	2119.1	3	706.4	48.430***
	Between media	14714.7	6	2452.5	4.302**
	Interaction (isolates × media)	10260.9	18	570.0	39.083***
	Error	3675.5	252	14.6	

1) Sums of square.
2) Degrees of freedom.
3) Variances.
4) F values.
5) Probabilities.
* = $0.01 < P \leqq 0.05$,
** = $0.001 < P \leqq 0.01$,
*** = $P \leqq 0.001$.

Table 7. The mean dimensions of sexual and asexual structures of four isolates of *Pythium* sp. obtained from soils of relatively warmer region where neither wheat cultivation nor deep snow is found.

Isolate No.	City collected	Mean dimension (μm)[1]			
		Sporangium[2]	Oogonium[3]	Projection[4]	Oospore[5]
S 22-8	Sennan	—[6]	19.4	8.7 × 5.1	17.7
S 61-2	Sakai	—	22.3	7.1 × 4.3	19.4
247-51	Kyoto	20.5 × 17.1	19.3	8.5 × 5.1	16.8
247-52	Kyoto	19.9 × 15.5	18.4	7.5 × 5.0	16.4

1) Mean of 500 individuals on autoclaved turfgrass-blades.
2) Diameter of sporangium (long diam. × short diam.).
3) Diameter of oogonium.
4) Dimension of projection of oogonium (length × width).
5) Diameter of oospore.
6) Not observed.

dicated that the formation of each oogonium type was significantly affected by isolate, medium and their interaction ($P < 0.01$) under those experimental conditions (Table 6). The broad sense heritabilities of types I–IV were estimated as 40.9 %, 56.1 %, 27.6 % and 15.3 %, respectively.

Isolation of Pythium sp. from samples collected in Kyoto, Sakai and Sennan Cities

Four isolates of *Pythium* sp. with short, blunt projections on their oogonia were obtained in autumn (Oct.–Nov., 1985) by the method used for isolating snow rot fungi. Two isolates were from a damp ground soil in the Botanical Garden, Kyoto University, where *P. polypapillatum* had been originally isolated*. The other two were from paddy field soil in Sennan City, and from soybean field soil where rice plants had been grown until previous year at the University Farm, University of Osaka Prefecture, Sakai City. The mean dimensions of sporangia, oogonia, projections of oogonia and oospores in each isolate are shown in Table 7. All isolates agree with *P. paddicum* in shape and size of sexual structures.

Discussion

The isolates of *P. paddicum* used in this experiment produced 4 types of oogonium in various proportions. Type I was a typical oogonium and agreed with the original description of this fungus[1]. The type III oogonium commonly observed on SA was unusual and morphologically similar to the oogonium of *P. polypapillatum*[2]. The four types of oogonium, however, developed from an individual oogonial initial. An oogonial initial of any type differentiated as a terminal hyphal swelling, formed a young oogonium, then began to extend projections. The elongation of projections, sometimes with branching, seemed to be regulated by meeting of the host oogonium with an antheridium. It is probable that the developing oogonia of various types retain their different external

* *Pythium* sp. which resembled *P. paddicum* was periodically tried to isolate from the Botanical Garden since then and forty-five isolates of this fungus were obtained during one year period ending November 28, 1986.

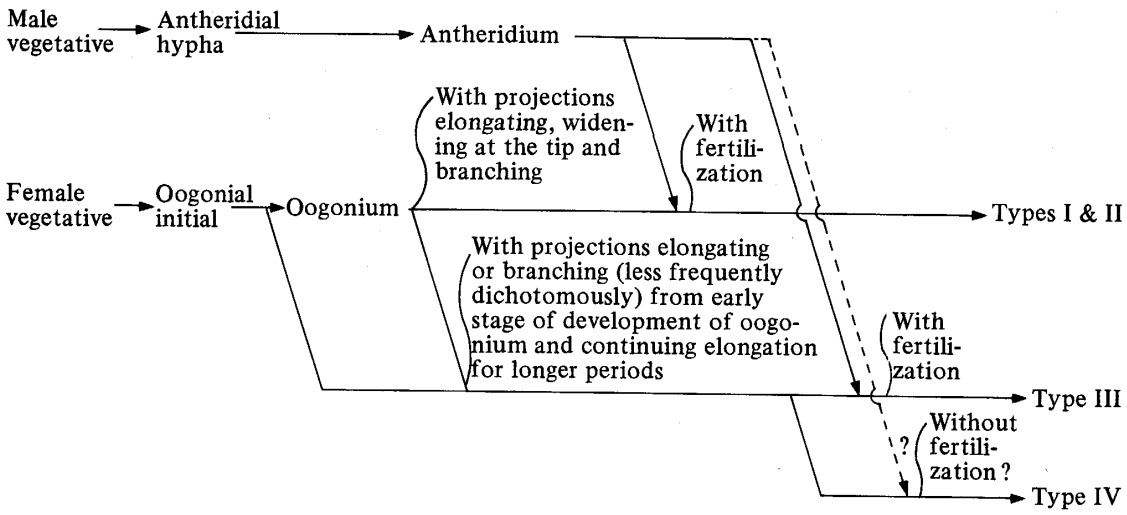

Fig. 29. Possible stages of the development in various types of oogonium of *P. paddicum* from vegetative hyphae.

forms, depending upon the time when they encounter an antheridium (Fig. 29). However, it is unknown what kind of factors control the branching and elongation of projections, the time of differentiation of sexual structures, and their contact. Further studies are needed using modified SA in order to analyze the mechanism of oogonial formation.

A two-way analysis of variance was carried out on each oogonium type using data transformed by arcsin square root and a broad sense heritability of the ability of oogonial formation was estimated in the present experiment. However, further statistical analyses are needed to be done, using more isolates over several years, before final conclusion can be drawn.

According to the above discussion, the isolates of *P. paddicum* used in this experiment, however, have considerably different oogonial characteristics from those found in the original description[1]. *P. polypapillatum* produced the oogonium types I–III, designated here, as described by T. Ito[2]. All the isolates of *P. paddicum* used, therefore, are considered to be identical to *P. polypapillatum.*

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Explanation of Plates

Fig. 1–4 Four types of oogonium of *P. paddicum*.
(1) Type I (Typical). (2) Type II (Typical). (3) Type III (Atypical). (4) Type IV (Atypical). This structure does not belonging to types I–III because of considerably complicated projections (arrows) on the oogonium-like body.

Figs. 5–10 Successive observation of type I oogonial formation. An oogonium was observed in a field 27 mm from the inoculum during incubation on PCA at 16.4-21.4°C and microphotographed at 81 hr 30 min (5), 84 hr 30 min (6), 86 hr (7), 90 hr 30 min (8), 96 hr 30 min (9) and 103 hr 30 min (10) after inoculation.

Figs. 11–16 Successive observation of type II oogonial formation. An oogonium 22 mm from the inoculum on PCA at 15.4–20.2°C was microphotographed at 80 hr (11), 84 hr 30 min (12), 86 hr (13), 87 hr 30 min (14), 92 hr 30 min (15) and 103 hr 30 min (16).

Fig. 17–22 Successive observation of type III oogonial formation. An oogonium 22 mm from the inoculum on PCA at 16.4–21.4°C was microphotographed at 80 hr (17), 83 hr (18), 84 hr 30 min (19), 89 hr (20), 96 hr 30 min (21) and 125 hr (22).

Figs. 23–28 Successive observation of type IV oogonial formation. An oogonium 4 mm from the inoculum on PCA at 17.0–21.3°C was microphotographed at 55 hr (23), 58 hr (24), 62 hr (25), 67 (26), 80 hr (27) and 111 hr (28).

All bars represent 20 μm. Bars on the figs. 10, 16, 22 and 28 are applicable to the Figs. 5–9, 11–15, 17–21 and 23–27, respectively.

Plate I

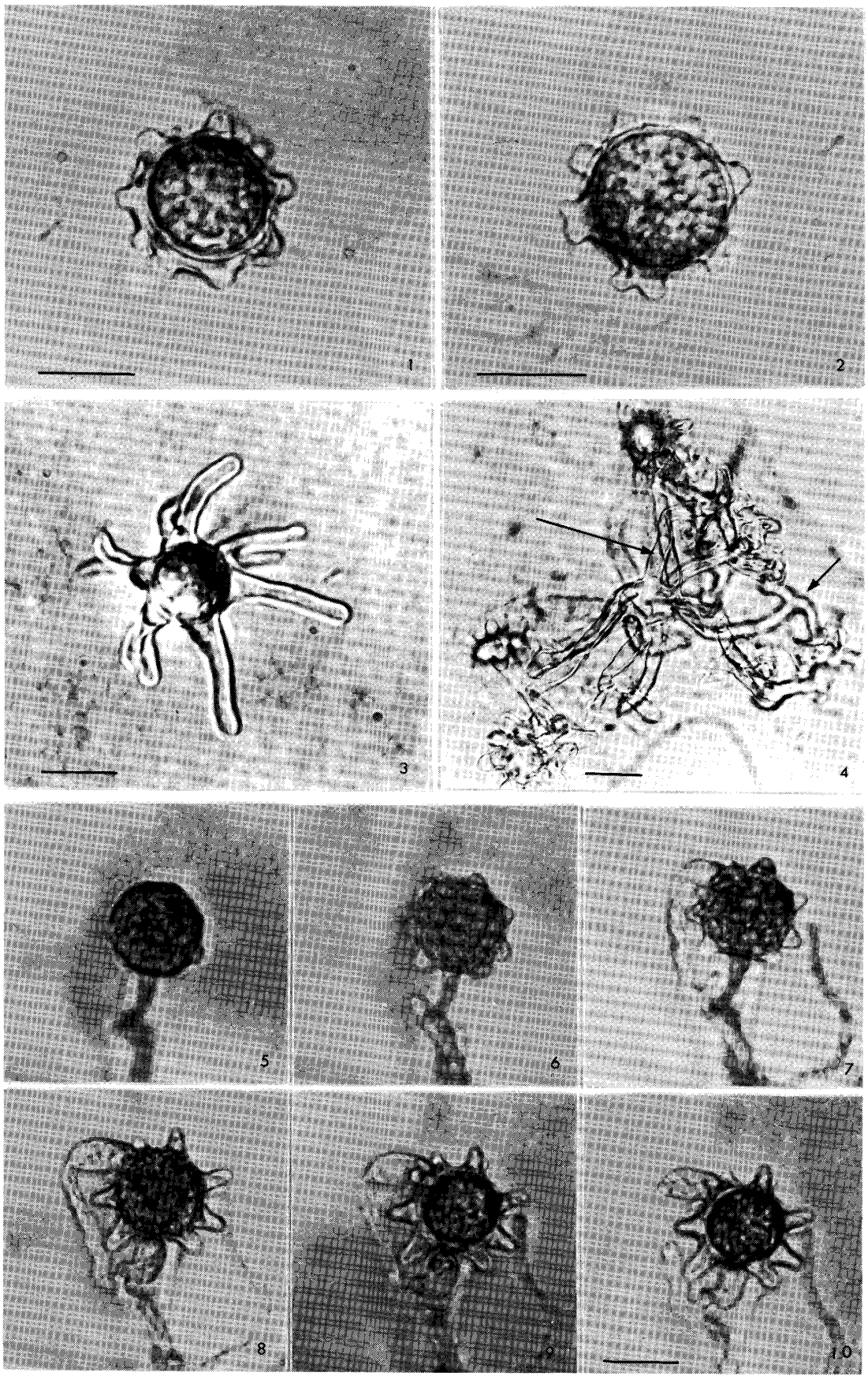

Plate II