Heterothallism in *Peronospora effusa*

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ABSTRACT

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Nineteen isolates of the spinach downy mildew pathogen, *Peronospora effusa*, were collected from spinach seedlings infected by seedborne oospores. The isolates were subcultured individually in spinach cotyledons for 6–15 mo, and no oospores were detected. Although inoculation with single isolates did not result in oospore production, oospores were observed in cotyledons inoculated with certain specific pairs of isolates. The isolates were grouped into two mating types; crosses within each group were sterile, whereas those between the groups were fertile. Seventeen isolates belonged to the mating type P1 and two isolates to the mating type P2. Antheridia were declinous, paragynous, and single. These results clearly demonstrate the existence of heterothallism in *P. effusa*.

Additional key words: *Spinacia oleracea*.

Downy mildew caused in spinach (*Spinacia oleracea* L.) by *Peronospora effusa* (Grev. ex Desm.) Ces. (syn. *P. spinaciae* Laub. and *P. farinosa* Fr.) (11,13) is one of the most devastating diseases of spinach in many countries (4). Oospore production by the spinach downy mildew fungus has been documented and the epidemiological importance of oospores has been demonstrated (9,12). In Japan, however, oospores of the fungus are not always detected in infected leaves collected in the field (2). Moreover, neither the environmental factors and host conditions affecting oospore formation by *P. effusa*, nor the sexual system of the fungus have been elucidated.

Heterothallism has been demonstrated in the downy mildew fungi *Bremia lactucae*, *Peronospora parasitica*, and *Sclerospora graminicola*, with oospore formation requiring the presence of two sexual compatibility (or mating) types (1,5,8). At present, however, little is known of the nature of the sexual systems and the factors controlling sexual reproduction in the downy mildew fungi.

The purpose of the studies reported here was to examine the sexual system of *P. effusa*.

MATERIALS AND METHODS

Collection of isolates. Commercial seed lots of three spinach cultivars, Akagi, Kurobi, and Maruryu Münster, in which the numbers of oospores detected by the seed-washing method was high (3), were used to obtain infected seedlings. A mixture of garden soil and manure (2:1, v/v) in unglazed pots (18-cm diameter) was steam sterilized at 1.2 kg/cm^2 pressure for 3 hr. Seeds were soaked in distilled water for 1 day at 15 C, then drained and incubated for 3 days at 15 C to promote germination. Germinating seeds were sown (100 per pot), and the pots were placed in a growth chamber set at 15 C and located outdoors to receive natural light through the glass sides and roof. After 21 days, at the cotyledon stage, the potted seedlings were placed in a moist chamber for 20 hr at 20 C to induce sporulation. The conidia were collected from one cotyledon with profuse sporulation for a given isolate by vigorously shaking the diseased cotyledon in distilled water. One isolate (A1) was collected from the cultivar Akagi, 17 isolates (K1-K17) from the cultivar Kurobi, and one isolate (M1) from the cultivar Maruryu Münster.

Maintenance of isolates. A commercial seed lot cultivar Popeye, in which oospores could not be detected by the seed-washing method, and from which no infected seedlings could be observed in the seed transmission test (3), was used for maintenance of isolates. The germinated seeds were sown in pots (15-cm diameter) filled with sterilized soil (autoclaved for 3 hr at 1.2 kg/cm^2 pressure), at a rate of 80–100 seeds per pot, and grown at 20–23 C in a greenhouse. Each isolate was subcultured at 8-day intervals as follows: 11- to 14-day-old seedlings, at the cotyledon stage, were sprayed with conidia suspended in distilled water containing 0.01% Tween-20, incubated in a moist chamber for 20 hr at 20 C to obtain infection, and then in an outdoor growth chamber at 15 C; on the 8th day after inoculation, the potted seedlings were placed in a moist chamber for 20 hr at 20 C to induce sporulation, and the conidia were harvested from cotyledons by shaking them in distilled water.

Tests for heterothallism. The cultivar Popeye was grown and inoculated as described above. Two pots were employed for each isolate and isolate combination. Seedlings were inoculated with conidia of each isolate or mixtures of conidia from pairs of isolates and placed in a growth chamber at 20 C outdoors. On the 7th day after inoculation, infected cotyledons were cut into sections 5–6 cm long, and stained with aniline blue according to the method of Shipton and Brown (10) for microscopic observation of oospores in plant tissue. All the experiments were repeated twice, and 200–400 cotyledons were observed for each isolate and isolate combination.

To quantify oospore production, counts of oospores were made in 20 stained cotyledons of each cross 7 days after inoculation. Each cotyledon was mechanically ground in a glass mortar containing 1 ml of distilled water, and the number of oospores was determined microscopically by counting those contained in 10 μl of the resulting suspension.

RESULTS

In the first experiment, isolates K1, K2, M1, and A1 were employed, and the cotyledons were inoculated with conidial suspension at the concentration of 2×10^4 conidia per milliliter, either alone or in paired combinations (Table 1). In the paired combinations, each conidial suspension was mixed in equal volume before inoculation. The four isolates did not produce oospores when cultured alone. Two paired combinations of isolates K1 and K2, and isolates M1 and A1, did not produce oospores. However, oospores were produced when the isolates K1 or K2 were paired with isolates M1 or A1. Therefore, these isolates could be grouped into two mating types, designated P1 (isolates K1 and K2) and P2 (isolates M1 and A1).

To determine the mating type of the other 15 isolates (K3-K17), each conidial suspension (2×10^4 conidia per milliliter) was mixed with the conidial suspension of either isolate K1 (mating type P1) or isolate M1 (mating type P2) in equal volume (also at 2×10^4 conidia

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per milliliter) before inoculation. In each test a total of 200 cotyledons was observed and the experiments were repeated twice. All 15 isolates were designated as mating type P1, because oospores were produced when any of them was paired with isolate M1 (mating type P2), and no oospores were produced when any of them was combined with isolate K1 (mating type P1).

In the second experiment, frequencies of cotyledons containing oospores and numbers of oospores formed in cotyledons following inoculations with the mixture of conidial suspension of two mating types at three different final concentrations were analyzed. Two paired combinations consisting of isolates K1 (mating type P1) and M1 (mating type P2), and isolates K2 (mating type P1) and A1 (mating type P2), respectively, were evaluated. An equal volume of conidial suspension of isolates of paired combinations at three different concentrations, 2×10^2, 2×10^3, and 2×10^4 conidia per milliliter, was mixed before inoculation. Percentages of cotyledons containing oospores and numbers of oospores formed in cotyledons were highest at the highest inoculum concentration and decreased at lower concentrations (Fig. 1). Oospores were round and smooth. The mean diameters, based on measurements of 200 oospores, were 30.7 μm for isolates K1 and M1, and 30.6 μm for isolates K2 and A1.

The heterothallism of *P. effusa* was further confirmed in a third experiment. In the paired combination of two mating types, the conidial suspension was mixed at different ratios at the concentration of 2×10^4 conidia per milliliter before inoculation. Two paired combinations of isolates K1 (mating type P1) and M1 (mating type P2), and isolates K2 (mating type P1) and A1 (mating type P2) were evaluated at the mixing ratios of 1:0, 100:1, 10:1, 1:1, 1:10, 1:100, 0:1 (v/v), respectively. Seven days after inoculation, percentages of cotyledons containing oospores were determined. Maximum oospore production for each pair of isolates occurred when conidia of the two mating types were applied in equal proportions (Fig. 2). Equal concentrations of inoculum, but with the predominance of conidia of one mating type, resulted in less oospore production.

Interactions between the two mating types were also examined in excised cotyledons. Cotyledons cut 7–8 cm long 11 days after sowing were placed on a glass slide in a petri dish with high moisture. Two 5-μl droplets, each containing 3×10^5 conidia per milliliter, of members of mating pairs, isolates K1 (mating type P1) and M1 (mating type P2), were placed on the cotyledon 1 cm apart. The petri dishes were placed in a chamber at 15 C under light at 20,000 lux supplied by a Mitsubishi model D-SDL fluorescent lamp. After 4, 5, and 6 days, 10 inoculated cotyledons were stained

TABLE 1. The production of oospores in spinach cotyledons following inoculation with conidia of four isolates of *Peronospora effusa* singly and in all paired combinations[a]

Isolate	Presence or absence of oospores with isolate or isolate combination[b]			
	K1	K2	M1	A1
K1	−			
K2	−	−		
M1	+	+	−	
A1	+	+	−	−

[a] Conidial concentration of 2×10^4 conidia per milliliter; in the paired combinations, the conidial suspensions were mixed in equal volume before inoculation.

[b] Oospores were examined 7 days after inoculation. Symbols: + = oospores detected in 98–100% of inoculated cotyledons, − = no oospores detected. The experiment was repeated twice, and the total number of cotyledons observed in each case was 250.

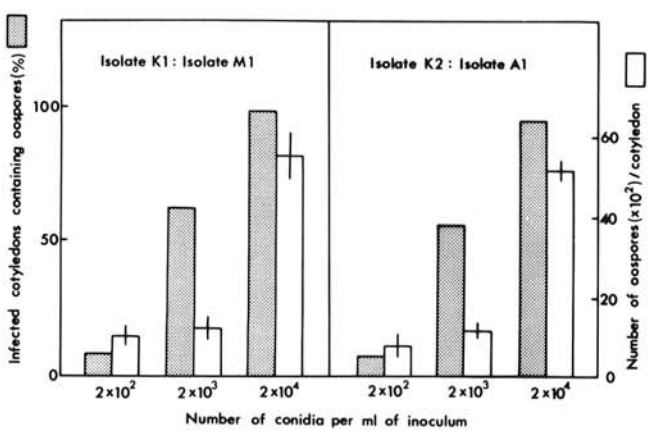

Fig. 1. Inoculum concentration effects on the percentage of spinach cotyledons containing oospores (▦) and numbers of oospores formed per cotyledon (□) 7 days following inoculation with a 1:1 mixture of conidia of isolates of two mating types of *Peronospora effusa*, at three different final concentrations. Data on percent cotyledons containing oospores represent the mean of counts from two experiments (300 cotyledons for each concentration); data on the number of oospores represent the means of two experiments (20 cotyledons for each concentration). The vertical bar at the top of each column indicates the standard error.

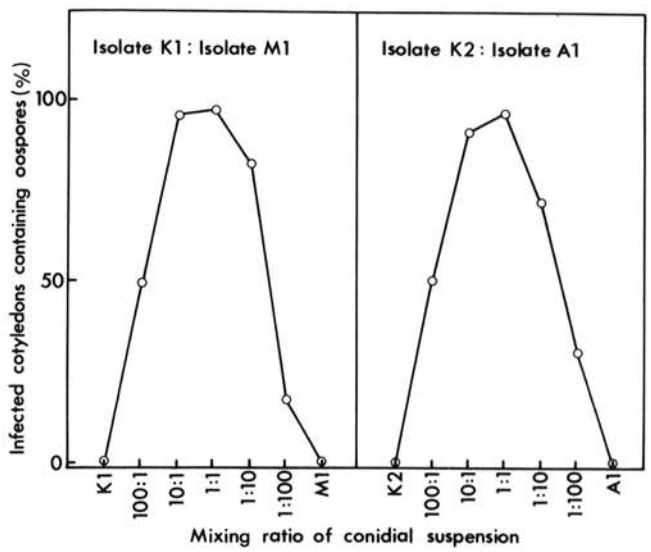

Fig. 2. Percentage of spinach cotyledons containing oospores following inoculation with mixtures of conidia of two mating types of *Peronospora effusa*, in different ratios. Conidial suspensions of the two types, both at 2×10^4 conidia per milliliter, were mixed in the following ratios before inoculation: 1:0, 100:1, 10:1, 1:1, 1:10, 1:100, 0:1 (v/v). Cotyledons were observed for oospore production 7 days after inoculation. The experiment was repeated twice, and percentages of cotyledons containing oospores were based on inspection of 400 cotyledons for each mating type ratio.

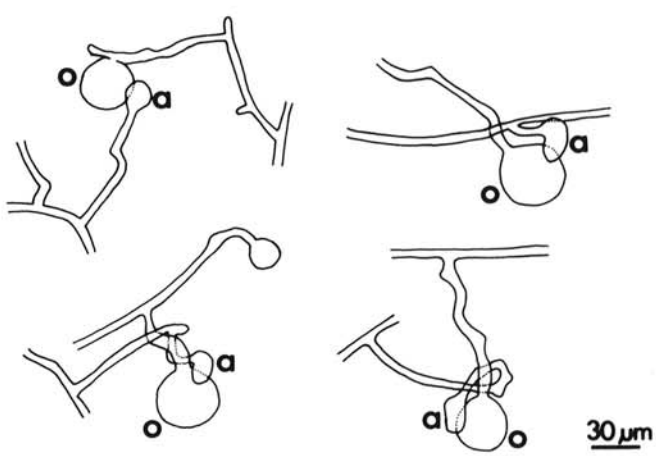

Fig. 3. Reproductive structures of *Peronospora effusa* in spinach cotyledon 5 days after inoculation with mating types. Symbols: a = antheridium and o = oogonium.

as already described, and oogonia and oospores in the cotyledons were observed via Nomarski interference microscopy (×400). Oogonia and oospores formed between inoculation sites, where the vegetative mycelia of the two mating types came into contact. Reproductive structures formed in cotyledons collected 5 days after inoculation were investigated. Antheridia were diclinous, paragynous, and single (Fig. 3). It is concluded from this experiment that contact between mycelia of opposite mating types is necessary for sexual reproduction.

DISCUSSION

The results obtained clearly demonstrate that these isolates of *P. effusa* exhibit characteristics of a heterothallic mating system. The isolates could be grouped into two mating types; crosses within each group of isolates were sterile, whereas those between the groups were fertile. Among the 19 isolates, 17 isolates belonged to the mating type P1, and two isolates to the mating type P2. These observations are similar to those reported for *B. lactucae, P. parasitica*, and *S. graminicola* (1,5,8). Therefore, it is possible that heterothallism may be common among members of the downy mildew fungi.

In *B. lactucae, P. parasitica*, and *S. graminicola*, some homothallic isolates were also detected (1,5,7,8). However, no homothallic isolates were detected among the 19 isolates used in the present study, as no oospores were observed in the cotyledons infected with single isolates during subculture for 6–15 mo. It was deduced that all isolates were pure with regard to the sexual system of *P. effusa*. More isolates of *P. effusa* from diverse locations would be required to determine whether more than two mating types exist.

In Japan, Sakano (9) observed oospores in infected leaves collected in the field, but this is not always the case (2). When oospores were not found, the inoculum in the field may not have contained the appropriate mixture of mating types, the levels of infection may have been too low to allow sexual reproduction to take place, or one mating type may have predominated in spite of the presence of appropriate mating types in the population. Our second experiment demonstrates the importance of using large numbers of conidia to ensure high levels of oospore formation, and the third experiment shows that a predominance of one mating type results in lower levels of oospore production.

Observations on the origin of the gametangia of *P. effusa* suggest that the antheridia and oogonia arise from different hyphae. These observations agree with those made in *B. lactucae* (6). However, this evidence does not prove that isolates of *P. effusa* are unisexual. As it was impossible to determine in the crosses whether either mating type formed only oogonia or antheridia, the sexual characteristics of the groups remain to be elucidated.

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