

Formation and Pathogenicity Variation of Oospores of *Phytophthora capsici* Infecting Black Pepper

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Phytophthora capsici Leonian is the causal agent of foot rot disease of black pepper (*Piper nigrum* L.). Foot rot disease is the most destructive disease which can cause significant economic losses of black pepper. Two mating types of *P. capsici* were found in black pepper plantations in Lampung. This research was aimed at examining the effects of temperature, light, and polycarbonate membrane on oospore formation. Also the effect of light on germination and determining both mating types of 30 randomly selected progenies. The results showed that oospores were produced through either hyphal contact or without any contact. Oospores were produced abundantly on V8 agar in the dark at 16-24 °C. Oospores germinated after 3-weeks incubation in the dark followed by 1-week incubation under TL fluorescent light. All progeny were heterothallic, and consisted of 16 isolates of A1 mating type and 14 isolates of A2 mating type. There was no correlation between mating type categories and their pathogenicities on black pepper leaves. Twenty-six progeny isolates may infect unwounded pepper leaves, whereas another four isolates may only infect wounded pepper leaves. Pathogenicities of all progenies were lower than those of their parents.

Key words: *Phytophthora capsici*, oospore, black pepper, Lampung

Phytophthora capsici is a pathogenic causal agent of foot rot disease on black pepper (*Piper nigrum* L.). The attack of *P. capsici* on the root and collar region causes sudden plant death. Foot rot disease on black pepper was found for the first time in Lampung in 1885 (Muller 1936). At present this disease has spread to all black pepper plantations in Indonesia and is the main constraint in black pepper production.

P. capsici is a heterothallic fungus. Sexual reproduction can occur when there are two compatible mating types (A1 and A2) for oospore formation. Two mating types of *P. capsici* from black pepper plants were reported to have been found in Lampung (Manohara and Sato 1992). Ko (1980) used polycarbonate membrane to prove that oospore formation of *P. cinnamomi*, *P. parasitica*, and *P. palmivora* could take place without contact hyphal of the two compatible mating types.

The formation of oospores of *Phytophthora* is affected by several environmental factors such as temperature and light. Harnish (1965) and Brasier (1969) stated that light inhibited the formation of oospores but stimulated sporangia formation. Optimum temperature for the formation of oospores of *Phytophthora* differed, depending on the species.

Oospore germination is affected by light, oospore maturity (age), temperature, enzyme treatment and chemical treatment (Ribeiro 1983). In general, the quantity of germinating oospores will increase in line with the increase of an oospore's age. According to Hord and Ristaino (1991), the germination rate of oospores of *P. capsici* originating from chili pepper plants improved by 3% at the age of 14 days, and became 20% at the age of 60 days. The light and temperature requirement during the oospore germination process varied, depended on the species of *Phytophthora*.

Oospores are very important in epidemiology and etiology of *Phytophthora* because they are able to survive

in extreme environmental conditions. The oospore is also the stage where genetic recombination occurs which can create a new individual more pathogenic than its parents (Hausbeck and Lamour 2004). The objectives of this research are to study: (i) the effects of temperature and light on oospore formation, (ii) oospore formation with polycarbonate membrane, (iii) oospore germination, and (iv) mating types and pathogenicities of progeny isolates.

MATERIALS AND METHODS

Fungal Material. *P. capsici* isolates were obtained from diseased black pepper plants in North Lampung, i.e. isolate N2 (A1 mating type), and in South Lampung, i.e. isolate N4 (A2 mating type). The two isolates are assumed to have the same ploidy i.e. diploid. These isolates were grown and maintained in V8 agar (200 ml V8, 1.0 g CaCO₃, 20 g agar, and 800 ml aquadest).

The Effect of Temperature on Oospore Production. Culture disks of each of the isolates, N2 and N4 (diameter 0.5 cm), were placed in pairs facing each other on V8 agar with 5-cm distance from each other, and then incubated at 16, 20, 24, 28, and 32 °C. Every treatment was replicated three times. Colony growth was observed every day until both isolates made contact. After the culture reached the age of 14 days, the number of oospores formed was counted by taking two samples of media disks (diameter 0.5 cm) from each Petri dish. The sample was placed on object glass, stained using lactophenol trypan blue with simultaneous heating, and then covered with a cover glass. The number of oospores was counted employing light microscope at 200 x magnification. Every disk was examined at three randomly chosen places.

The Effect of Light on Oospore Production. Culture disks of isolates N2 and N4 (diameter 0.5 cm) were placed facing

each other on V8 agar at a 5-cm distance, and then incubated under several light treatments using a TL fluorescent lamp with 600 lux intensity and placed at a 40-cm distance from the Petri dish lid. The durations of light exposure are 0, 6, 12, 18, and 24 h respectively. Temperature inside the incubation room ranged between 26-28 °C. Each treatment was replicated three times. The determination of oospore number was carried out as given above.

Oospore Production with Polycarbonate Membrane.

Isolates N2 and N4 were grown on V8 agar and incubated for 3 days in the dark. Culture disks of isolate N2 (diameter 1.0 cm) were obtained from the edge of a growing colony then placed in the middle of a sterile Petri dish, covered with a polycarbonate membrane (CPR, 0.2 µm, 90 mm diameter; Nucleopore Corporation, Pleasanton, California, USA). On top of this was placed another culture disk of isolate N4. For comparison, an opposite treatment was conducted using the same isolates (Ko 1980). Incubation was carried out in the dark at 20 °C. After 7 days, the number of oospores formed during incubation was observed.

Oospore Germination. Oospores were released from agar media by crushing in a blender at 5000 rpm for 5 min. The oospore suspension was then centrifuged at 2000 rpm for 5 min. The sediment was filtered employing a gradual sieving filter with pore diameter of 75, 38, and 20 µm respectively. The remaining oospores left in 20 µm filter were then separated and washed with 0.5% (wt/vol) KMNO₄ for 20 min. The oospore suspension was then immersed in warm water (36-38 °C) for 2.5 h (Wahyuno *et al.* 1995). The oospore suspension was then diluted to obtain about 20 oospores per inoculating loop tip, and then streaked on basalt media (20 mg glucose, 100 mg lecithin, and 10 ml basalt solution). The incubation treatment consisted of 8 lighting combinations (Table 1). The percentage of oospore germination was estimated visually.

Mating Types and Pathogenicities of Progeny Isolates on Black Pepper Leaves. Thirty progeny isolates were obtained from randomly selected germinating oospores (single spore isolation), isolated on V8 agar. The mating types of progeny isolates were determined using pairing method by pairing the isolates with known-tester isolates (A1 or A2 mating type) on V8 agar. The presence of oospores was observed. Pathogenicities of progeny isolates were tested by inoculating them on black pepper leaves. These progeny isolates were grown on V8 agar and incubated for 7 days under continuous light. An inoculum disk (diameter 0.5 cm) was placed on the lower side of unwounded black pepper leaf surface and then one drop of sterile water was added. These leaves were placed inside a plastic container whose base had been covered by moist tissue paper. Measurement of leaves spots formed was conducted after 4-day incubation at room temperature.

RESULTS

Optimum Temperature for Oospore Formation. Oospore formed within the temperature range of 16-32 °C (Table 2). When incubated at 16-24 °C, sexual reproduction structures began to appear one day after both colonies made contact. However at 28 and 32 °C, the structures were formed 4 days

after both colonies made contact. The antheridia were amphigynous and colorless (hyaline). Oogonia had spherical or subspherical form and were colorless (hyaline). Oospores were colorless (hyaline) at the young stage and became brownish when mature (Fig 1a,b). Oospores were generally globose in shape with thick walls (Fig 1c). The optimal temperature for oospore formation was 20 °C (Table 2).

The Role of Light in Oospore Production. Oospore production was very sensitive to light. The contact of both colonies occurred 4 days after incubation, and on the fifth day, several reproduction structures had been formed

Table 1 Incubation treatment for the germination of oospores of *P. capsici*

Incubation in the dark (20 °C)	Incubation under the light (26-28 °C)
2 weeks	1 week
2 weeks	2 weeks
2 weeks	3 weeks
2 weeks	4 weeks
3 weeks	1 week
3 weeks	2 weeks
3 weeks	3 weeks
3 weeks	4 weeks

Table 2 The effect of temperature on the formation of oospores of *P. capsici*

Incubation temperature (°C)	Colony contact (day)	Number of oospores ^a
16	4	44.16
20	3	85.67
24	3	46.67
28	3	14.33
32	4	1.00

^athe average of 18 microscope view area with 200x magnification.

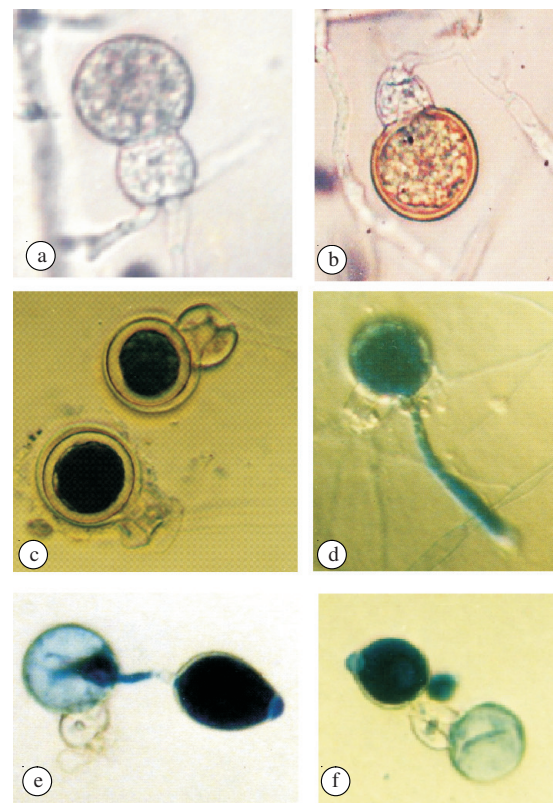


Fig 1 The oospores of *P. capsici* and their germination. a. young oospore, b immature oospore, c. mature oospore stained with lactophenol trypan blue, d. hypha formation of germinating oospores, e and f. sporangium was formed by germinating oospores.

(in the dark). The treatment of a 6-hour exposure to light inhibited oospore formation, while exposure to light for more than 6 hours caused no oospore formation (Table 3).

The Effect of Polycarbonate Membrane. This is impenetrable by the hyphae of *P. capsici*. This can be proven by inserting the membrane between culture disks of *P. capsici* and fresh V8 media. It turned out that oospores continued to be formed on isolate disk N2 and N4 even though polycarbonate membrane had been inserted between them (Table 4). This result revealed that oospores formation took place without direct contact between the hyphae of two compatible mating types. Oospore formation seemed to be induced by the presence of compounds that could penetrate the membrane. Compared with isolate N4, the isolate N2 always produced the most oospores regardless of the position of pairing (Table 4).

Oospore Germination. Oospore germinated when they were first incubated for 3 weeks in the dark, followed by exposure to continuous light for 1 week or more (Table 5). Oospores germinated to form germ tubes that penetrated oospore walls or passed through antheridia. The number of germ tubes produced could be more than one. The germ tubes grew longer and became hypha, or created sporangium at the tip. (Fig 1d, e, f).

The treatment of incubation in the continuous dark caused no oospore germination (data are not shown). A longer light treatment increased the ability of oospore to germinate. The number of germinating oospores increased in line with the duration of light exposure for up to the 3 weeks (Table 5). Four-weeks of exposure to light resulted in

Table 3 The effect of the duration of light exposure on the formation of oospores of *P. capsici*

Light exposure (h)	Number of oospores ^a
0	51.00
6	3.33
12	0.00
18	0.00
24	0.00

^athe average of 18 microscope view area with 200x magnification

Table 4 Formation of oospores of *P. capsici* with polycarbonate membrane

Combination ^a			Number of oospores ^b	
Top	x	Bottom	Top	Bottom
N2		N4	89.5	52.4
N4		N2	58.5	86.4
N2		N2	0	0
N4		N4	0	0

^aPolycarbonate membrane was inserted between the two isolates, ^bthe average of 18 microscope view area with 200x magnification.

Table 5 Oospore germination of *P. capsici*

Incubation treatment		Oospore germination (%)
In the dark (20 °C)	Under the light (25-27 °C)	
2 weeks	1 weeks	0.00
2 weeks	2 weeks	0.00
2 weeks	3 weeks	0.00
2 weeks	4 weeks	0.00
3 weeks	1 weeks	29.02
3 weeks	2 weeks	39.27
3 weeks	3 weeks	52.10
3 weeks	4 weeks	20.56

a lower number of germinating oospores compared to 3-week light exposure (Table 5). Further assessment found that this treatment produced many empty oospores, probably resulting from lysis.

Mating Types and Pathogenicities of Progeny Isolates.

All progeny isolates were heterothallic; they formed oospores with their compatible tester. Sixteen progeny isolates belonging to the A1 mating type formed oospores with isolate N4, while the remaining 14 isolates belong to the A2 mating type, formed oospores with isolate N2 (Table 6).

The symptom of infection on black pepper leaves could be seen as small spots which were brown-blackish color and which appeared 24 h after inoculation with parental isolates (N2 or N4). On the 4th day, the leaves spot areas are 17.45 cm² and 15.87 cm² respectively. Eight progenies (Hy 9, Hy 11, Hy 14, Hy 18, Hy 26, Hy 27, Hy 29, and Hy 30) caused symptoms similar to their parents on the 2nd day after inoculation. On the 4th day, the leaves spot area ranged between 7.53-10.45 cm². Four progenies (Hy 10, Hy 13, Hy 16, and Hy 19) did not show any symptoms. However, if the leaves were wounded before inoculation, these four isolates were then able to infect and produce small spots after 4-days incubation. From the 30 progenies tested, it was evident that none of the isolates had the same or more pathogenicity compared to their parental isolates. Most of them (18 progenies) produced symptom less than 3.00 cm² (Table 6).

DISCUSSION

The formation of oospores of *P. capsici* in vitro is clearly affected by temperature and light. Light was a constraining factor for oospore formation. In the dark, oospores could be produced within the temperature range of 16-32 °C. The optimum temperature for oospore formation was 20 °C. This temperature was lower than the optimum temperature for vegetative growth, i.e. 24-28 °C (Manohara 1988). This findings are similar to Brasier's (1969) results which revealed that the optimum temperature for vegetative growth of

Table 6 The variety of 30 progeny isolates of *P. capsici* and their pathogenicities on black pepper leaves

Progeny isolates (F1)	Mating type	Leaf spot (cm ²) ^a	Progeny isolates (F1)	Mating type	Leaf spot (cm ²) ^a
Hy 1	A1	2.10abc	Hy 17	A2	0.93ab
Hy 2	A2	6.63def	Hy 18	A2	7.85efg
Hy 3	A1	1.48ab	Hy 19	A1	0.00a
Hy 4	A1	5.66de	Hy 20	A1	0.27a
Hy 5	A2	1.20ab	Hy 21	A2	0.53a
Hy 6	A2	0.78a	Hy 22	A1	1.15ab
Hy 7	A2	0.52a	Hy 23	A2	0.77a
Hy 8	A1	0.39a	Hy 24	A1	0.11a
Hy 9	A1	9.31fg	Hy 25	A1	0.10a
Hy 10	A2	0.00a	Hy 26	A2	7.53efg
Hy 11	A1	9.32fg	Hy 27	A1	9.62fg
Hy 12	A1	2.82abc	Hy 28	A2	3.90bcd
Hy 13	A2	0.00a	Hy 29	A2	8.25efg
Hy 14	A2	9.44fg	Hy 30	A1	10.45g
Hy 15	A1	4.53cd	N2 (parent)	A1	17.45h
Hy 16	A1	0.00a	N4 (parent)	A2	15.87h

Number followed by the same letters are not significantly different at 5% DMRT, ^aunwound inoculated

P. palmivora was 27.5-30 °C, while sexual reproduction occurred in the range of 16-20 °C.

Oospore formation could occur without hyphal contact between the two mating types. It was probable that the two isolates produced compounds that induced each other to form gametangium and then oospores. This compound was then declared to be a sexual hormone. The A1 mating type produced α 1 sexual hormone whereas the A2 mating type produced α 2 sexual hormone (Ko 1980). Furthermore Shen *et al.* (1983) reported that this sexual hormone was not specific. For example, *P. infestans* with an A1 mating type will produce oospores if paired with an A2 mating type such as *P. parasitica*, *P. capsici*, and *P. palmivora* besides with *P. infestans* itself. Jee *et al.* (2002) found that phytol was highly stimulatory to oospore formation of *P. cactorum* and *P. parasitica*. Elliot (1983) suspected that the role of the sexual hormone was in the inhibition of steroid synthesis which controls sexual reproduction. Production of sexual hormone was affected by temperature and light. Yu *et al.* (1981) reported that the optimum temperature for *P. colocasiae* to produce the hormone was 25 °C, at which temperature it would be able to stimulate *P. parasitica* to form oospores in large quantity.

My research reported in this paper showed that oospores of *P. capsici* from black pepper plants would be mature after 3-weeks incubation in the dark, but that germination would require light. One-week exposure to light resulted in 29.02% germination. Increasing the duration of the exposure to up to 3 weeks would increase the percentage to 52.10%. Germinating oospores formed germ tubes that would develop into hyphae and mycelia or sporangia which would in turn form zoospores. According to Banihashemi (2004), oospore germination is determined by the level of maturity of oospores as the internal factor, and an external factor such as exposure to light, whose duration depended on the species of *Phytophthora*.

Observation by Banihashemi (2004) proved that *P. cactorum* needed dark conditions during the formation and maturation of oospores. The germination would then occur if incubated under continuous fluorescent illumination, but would not occur without the exposure to light. The germination of oospores of *P. cactorum* was only 3% if incubated in the dark, while-light exposure would increase it to 38%. Oospores of *P. infestans* germinated up to 70% under cool white fluorescent light (Chang and Ko 1991). The opposite happened in the germination process of oospores of *P. capsici* obtained from chili pepper plants. Germinating oospores would be abundant if light treatment was applied during both formation and germination process (Hord and Ristaino 1991).

My work reported here, shows that the pathogenicities of progenies seemed to have no correlation with mating types. Pathogenicities of all progenies were lower than those of their parents. The same result was described by Mayton *et al.* (2000) who did the mating with *P. infestans*. From 53 progenies, 52 isolates had lower pathogenicities compared to their parents. Abu-El Samen *et al.* (2003) found a genotypic variability among the asexual progenies of *P. infestans*.

Although oospores as the sexual reproduction have been considered as the important resting spore, the stage where genetic recombination occurs and the primary source of inoculum in the field, but little information is reported about the influence of some factors which stimulate the formation, germination and the pathogenicity of their progenies. Thus it is an important precedent for research in *P. capsici* especially on black pepper.

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