Effect of Metalaxyl and N-nitro-N-nitrosomethylurea on Mating Type of *Phytophthora infestans*

Larissa V. Savenkova & Marina I. Cherepennikova-Anikina

Department of Mycology, Moscow State University, Vorob'evi gori, Moscow, Russia, 119899
E-mail: mianikina@yahoo.com
fax: (095)-939-39-70

ABSTRACT

It is demonstrated that isolates of *Phytophthora infestans* (Al and A2 mating types) have the ability to change mating type as result of treatment by N-nitro-N-nitrosomethylurea solution and/or metalaxyl. After treatment of the strain K29 (Al), only self-fertile isolates were obtained. Meanwhile, when the strain K22 (A2) was treated with N-nitro-N-nitrosomethylurea and/or metalaxyl, isolates of Al, A2, AO and A1A2 were obtained in addition to the initial mating type. Isolates of Al, A2, and AO mating types were reversible, switching to Al A2, Al and occasionally to A2 mating types in zoospore generations. Influence of N-nitro-N-nitrosomethylurea solution and the fungicide metalaxyl on oospore formation of *P. infestans* was observed.

Key words: Phytophthora infestans, mating type, oospore, metalaxyl, N-nitro-N-nitrosomethylurea.

Abbreviations: NMU, N-nitro-N-nitrosomethylurea.

Савенкова Л.В. и Черепенникова-Аникина М.И. Влияние металаксила и N-нитро-N-нитрозометилмочевины на тип спаривания *Phytophthora infestans*.

Продемонстрировано, что изоляты *Phytophthora infestans* (Al и A2 типов спаривания) способны к изменению типа спаривания после обработки раствором N-нитро-N-нитрозометилмочевины и/или металаксила. После обработки штамма K29 (Al) были полученны только самофертильные изоляты. При обработке штамма K22 (A2) раствором N-нитро-N-нитрозометилмочевины и/или металаксила в дополнение к исходному типу спаривания были полученны изоляты Al, A2, AO и A1A2 типов спаривания. В зооспоровых генерациях наблюдали, что изоляты Al, A2, и AO типов спаривания были способны к переключению на Al A2, Al типы спаривания и особенно к исходному A2 типу спаривания. Так же рассмотренно влияние раствора N-нитро-N-нитрозометилмочевины и фунгицида металаксила на формирование ооспор *P. infestans*.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary is a heterothallic fungus having two mating types, Al and A2 (Savage et al, 1968). All isolates are potentially bisexual. The ability to produce mainly oogonia or antheridia (or both but at a definite ratio) depends on the sexual capacity of partners and their physiological condition. Oospore formation in self-sterile isolates is possible in the presence of an isolate belonging to the opposite mating type (Brassier & Hansen, 1992). In addition to the hybridization, there is possible self-fertilization for one or both parental isolates (Elliot, 1983). Noon & Hickman (1974) were the first to observe oospore formation in a heterothallic species, *P. capsici,* cultivated on an agar medium containing chloroneb, the isolates being of the type A2 but not Al. Afterwards, conversion of a mating type (from Al to A2 but not the reverse) was demonstrated also in cultures treated with chloroneb, ethazol or metalaxyl (Ann & Ko, 1988; Ko & Chang, 1990). Ko and Chang (1990) showed the possibility of switching the mating type Al over to A2 by the systemic fungicide metalaxyl. Nevertheless, all these works did not deal with the influence of chemical compounds on the oospore formation in *Phytophthora* species.

The present work is to demonstrate the influence of fungicide metalaxyl and N-nitro-N-nitrosomethylurea on the mating type and oospore formation in *P. infestans.*

MATERIALS AND METHODS

Strains and Growth Conditions

In this work the following strains of *P. infestans* were used: K22-metalaxyl resistant (lethal concentration higher than 100 μg ml^{-1}), mating type A2; and K29 — metalaxyl sensitive (lethal concentration 1 μg ml^{-1}), mating type Al. Isolates B5 (Al) and 1S1 (A2) were used as testers. All strains were isolated from potato of Moscow area, Russia. The strains were cultivated and maintained

on oatmeal agar (130 g of oatmeal, 15 g agar, 1 L distilled water).

Determination of mating type

The strains were paired with tester strains in plates with oatmeal agar, in two replicas. The plates were incubated for 14 days in darkness at 18°C. If abundant oospores were revealed, the isolate tested was considered to the opposite mating type. The strains were also examined for presence of oospores in the single culture. If oospores in the single culture were revealed, the strain was identified as self-fertile isolate.

Zoospore Production

To obtain a fungal zoospore suspension, zoosporangia were washed off the surface of the 8–10-days old culture with sterile distilled water. The suspension obtained was incubated at +4 °C for 30 to 40 minutes, thus stimulating zoospore formation and release.

Mutagenesis

A 5 μg ml^{-1} N-nitro-N-nitrosomethylurea (NMU) was used as a mutagene. Zoospores were treated with the mutagene solution for 18 to 20 hours in darkness at 4°C. Effective mutagene concentrations were taken off by means of threefold centrifugation in sterile distilled water at 3000 rpm for 15 minutes. The supernatant was poured out, and sterile distilled water was added to the residue. The suspension of zoospores treated with the mutagene was sown out in oat agar with metalaxyl at a concentration of 600 μg ml^{-1}. Single zoospore colonies were isolated and their characteristics were studied.

RESULTS

The influence of metalaxyl

For the purpose of studying the influence of metalaxyl on the mating type, metalaxyl was added to the agar medium at a concentration of 1–5 μg ml^{-1} for the metalaxyl-susceptible strain K29, and 150 μg ml^{-1} for the metalaxyl resistant strain K22. The strains were cultivated in plates with oatmeal agar containing metalaxyl and incubated during 30 days. Then agar pieces from the colony margins were transferred into tubes with oatmeal agar slopes. The isolates obtained were sown in oat media with increasing concentrations of metalaxyl (2; 10; 20 μg ml^{-1} for K29 and 200; 250; 300 μg ml^{-1} for K22), and analysis of single zoospores was carried out. Containing metalaxyl at a concentration of 1–5 μg ml^{-1}, only one of the isolates obtained (K29-4) changed its initial Al mating type: it became self-fertile (A1A2). Unlike the initial isolate K29, the colony morphology in K29-4 changed: it was characterised by prostrate growth and total lysis of aerial mycelium in 24 days after inoculation. When K29-4 was subsequently treated with increasing concentrations of metalaxyl (from 2 to 20 μg ml^{-1}), no change of mating type was observed, i.e. the strain K29-4 remained self-fertile. When analysis of single zoospores was performed, only 14 of 65 clones of the strain K29-4 returned to the initial mating type Al, other clones being self-fertile. A strain, K29-2, which retained the initial mating type Al after cultivation in a medium containing 1–5 μg ml^{-1} metalaxyl, became self-fertile (K29-2/20) after cultivation in a medium containing 20 μg ml^{-1} metalaxyl. Synchronously, a change in colony morphology occurred (analogous to that in K29-4). Treatment with more elevated concentrations of metalaxyl (25 and 30 μg ml^{-1}) did not result in a change in the mating type. In an analysis of single zoospores of a self-fertile isolate, K29-2/20, 6 of 17 isolates possessed the initial Al mating type while others were self-fertile (Fig. la). In some self-fertile clones obtained during from single zoospores of strains K29-4 and K29-2/20, disturbance in oospore formation was observed. Many oospores were formed insufficiently: they were 2 to 3 times smaller than normal ones and had thick-deformed colored walls with little content (Fig. 2 a-d). Mating of these clones with each other and with natural isolates could result in either formation of mainly oospores or oospore-like bodies and oospores or mainly oospore-like bodies (it depended on the mating combination). Joining of stra-

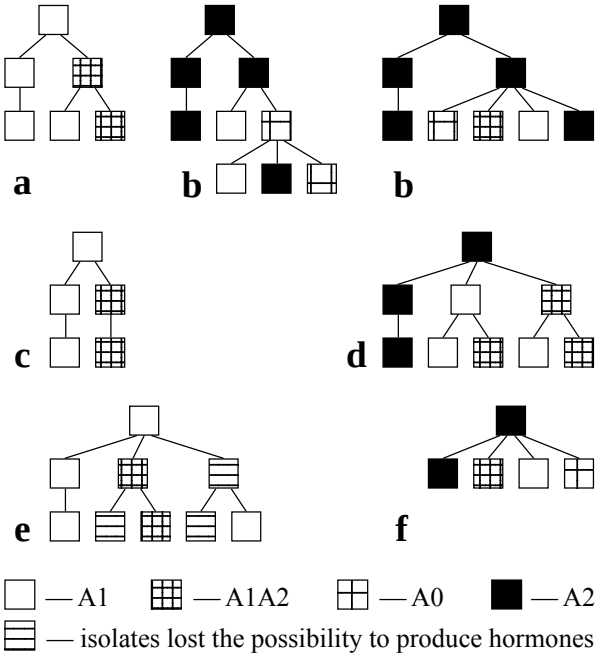

Fig. 1. Zoospore segregation in *P. infestans*

a — zoospore segregation of the strain K29 after growth in a medium containing metalaxyl; *b* — zoospore segregation of the strain K22 after growth in a medium containing metalaxyl; *c* — zoospore segregation of the strain K29 after treatment with NMU; *d* — zoospore segregation of the strain K22 after treatment with NMU; *e* — zoospore segregation of the strain K29 after treatment with NMU and growth on a medium containing metalaxyl; f — zoospore segregation of the strain K22 after treatment with NMU and growth in a medium containing metalaxyl.

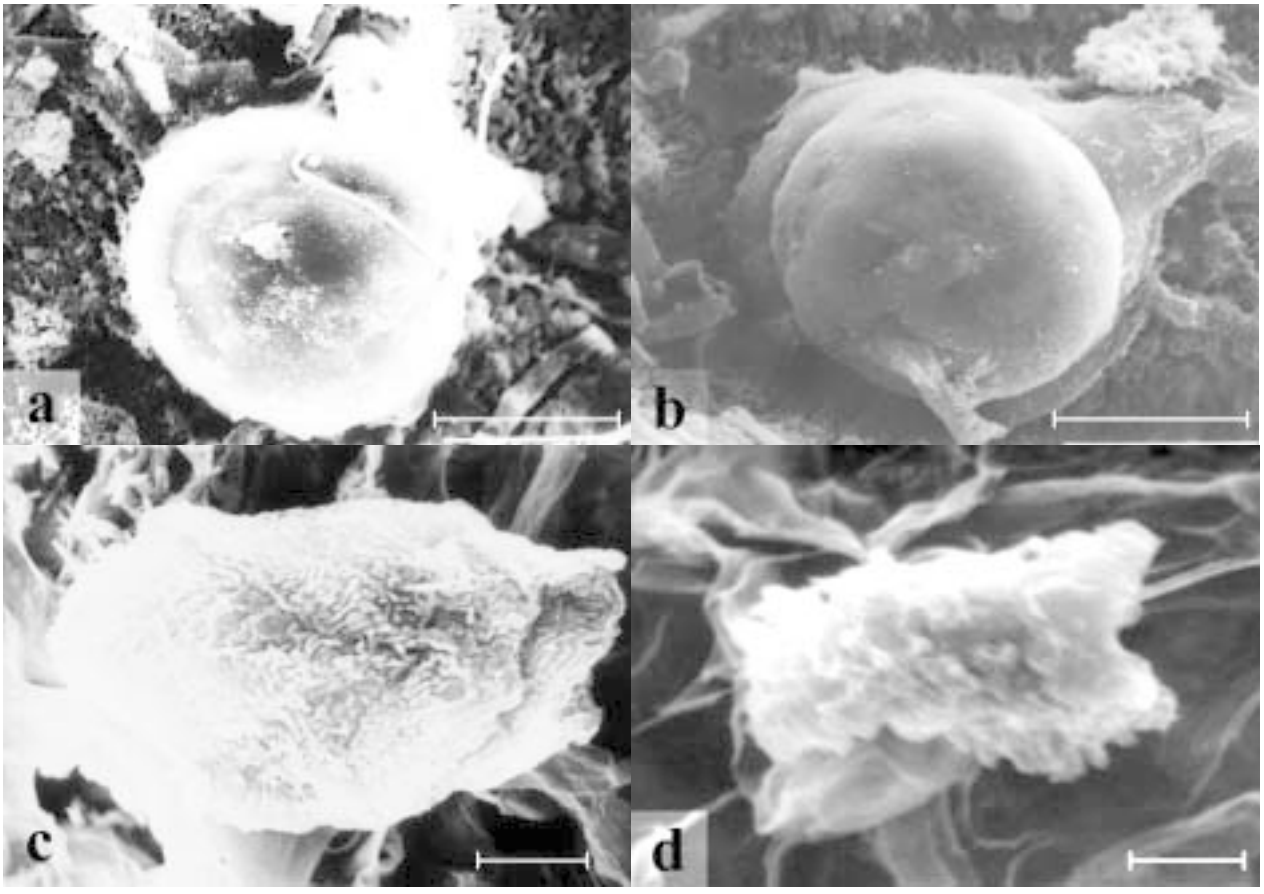

Fig. 2. Scanning electron micrograph of oospores and oospore-like bodies
a, b — normal oospores, bars, 20 μm; c, d — oospore-like body, bars, l0 μm.

ins K29-4 and K29-2/20 with testers 1S1 and B5 separated by a polycarbonate filter (Ko, 1978) showed that oospore-like bodies form only from the side of strains K29-4 and K29-2/20. Experiments involving separating of strains by a polycarbonate filter permit us to assume that the ratio of oospore-like bodies in mating depends on natural strains differing in their sexuality (Galindo, Gallegly, 1960). Mating of natural isolates possessing weak tendency to produce oogonia with isolates obtained after treatment with metalaxyl may thus result in predominance of oospore-like bodies, while in the case of using natural isolates with a strong female tendency, a minute quantity of oospore-like bodies is to be expected. No influence of metalaxyl on vegetative structures is revealed.

When the strain K22 was grown in medium containing metalaxyl at a concentration of 200 μg ml^{-1}, the isolated clone K22-4 retained its initial mating type A2. Then we sowed the isolate in media with metalaxyl added at concentrations of 300 or 400 μg ml^{-1}. Clones isolated from these media retained their initial mating type A2, but mycelial growth in isolates K22-4/300 and K22-4/400 was 4 times slower than in the initial strain K22 (the colony diameter was 2 cm 8 days after inoculation). The isolate K22-4/300 was replaced 6 times in a medium to which metalaxyl at a concentration 300 μg ml^{-1} was added. Isolates recovered from the last passage of K22-4/300-1, K22-4/300-2, K22-4/300-3, and K22-4/300-4 formed colonies with a diameter of 6 to 7 cm in 8 days. They all retained the initial mating type A2. Analogous data were obtained with isolates recovered from the medium to which metalaxyl at a concentration of 400 μg ml^{-1} had been added. Three of 13 recovered isolates changed their initial mating type A2 to Al, while the other 10 isolates produced oospores neither with any tester (1S1, B5) nor in single culture, i.e. they became AO (isolates belonging to this mating type group are known as deviating types for heterothallic *Phytophthora* species devoid of sexual competence (Ko, 1980). When one isolate of AO mating type was exposed to an analysis of single zoospores, splitting occurred: one part of the clones became the Al mating type, the other part was restored to the initial A2 mating type. When another isolate of the AO mating type was exposed to an analysis of single zoospores, 2 of 9 clones had the AO mating type while the other 7 had the Al mating type (Fig. 1b). The recovered isolate K22-4/400, which grew well after cultivating in a medium with metalaxyl added at a concentration of 400 μg ml^{-1} (see above), was inoculated in a medium with metalaxyl at a concentration of 600 μg ml^{-1}. Three of 11 isolates recovered from this medium were self-fertile, 2 isolates did not form oospores either with any tester or in a single culture (AO), 5 isolates changed their initial mating type from A2 to Al (Fig. 1b). No changes in oospore formation were observed.

Influence of N-nitro-N-nitrosomethylurea

Performing a study of the mating type in isolates obtained from strains K29 and K22 after treatment with 5 μg ml^{-1} NMU, we found that the mutagene provoked change in the mating type. Of 24 isolates obtained after the treatment of the strain K29 with NMU, 5 isolates became self-fertile, and the others retained their initial mating type.

In an analysis of a single zoospore of self-fertile isolates, all clones were self-fertile. No changes in oospore formation were observed (Fig. 1c).

After treatment of the strain K22 with 5 μg ml^{-1} NMU solution, only 6 of 38 isolates retained their initial A2 mating type, 24 isolates became self-fertile, and 8 isolates changed their initial A2 type to Al. Isolates that changed the initial A2 type to Al were tested repeatedly. Three isolates (K22-N15, K22-N21, and K22-N40) of 24 self-fertile ones changed their mating type to A2, and four (K22-N2, K22-N5, K22-N38, and K22-N39) of 8 isolates belonging to the Al mating type became self-fertile. In the analysis of single zoospores of isolates K22-N15, K22-N21, and K22-N40, 7 of 21 single zoospore clones had the Al mating type, the others were self-fertile. In the analysis of single zoospores of the isolate K22-N39, only 2 of 8 clones had the Al mating type; the 6 other isolates were self-fertile. Meanwhile, in the analysis of single zoospores of the strain K22-N5, all 9 clones were self-fertile (Fig. 1d). No changes in oospore formation were observed.

Influence of N-nitro-N-nitrosomethylurea solution and metalaxyl

After treatment with 5 μg ml^{-1} NMU solution, the isolate K29 was inoculated in a medium to which metalaxyl at a concentration of 600 μg ml^{-1} was added. Twenty colonies were obtained from this medium; 16 of them became self-fertile, 1 retained its initial Al mating type, and 2 isolates (K29-NM2-8 and K29-NM3-2) formed oospores with both testers but did not produce oospores in a single culture. Hence, the isolates K29-NM2-8 and K29-NM-3-2 began, after chemical treatment, either to produce hormones (thus stimulating testers 1S1 and B5 to form oospores) or to apprehend hormones from both testers. To prove this, the isolate K29-NM2-8 was grown in separate Petri dishes with testers: K29-NM2-8 x B5 and K29-NM2-8 x 1S1. Strains were separated by a porous polycarbonate membrane (Ko, 1978). The dishes were microscopied after 14 days of incubation. In all Petri dishes, oospores were found only where the mutants K29-NM2-8 and K29-NM3-2 grew, while where the testers 1S1 and B5 grew oospores were not found in any of the dishes. When analysis of single zoospores of 15 clones of the isolate K29-NM2-8 was performed, 5 clones had the initial Al mating type, while 10 clones formed oospores with both testers. None of the 15 clones formed oospores in single culture. In repeated testing after 2 months, K29-NM3-2 became self-fertile, and K29-NM2-8 formed oospores with both testers, but not in single culture (Fig. 1e). It was also noted that the isolates K29-NM3-1 and K29-NM3-7 (which two months earlier were self-fertile) formed oospores with both testers, but not in single culture. Testing of the isolates K29- NM3-1 and K29- NM3-7 through a porous polycarbonate membrane with the testers resulted in presence of oospores only where mutants grew. Hence, they had lost the possibility to produce hormones. When the isolate K29 was treated with 5 μg ml^{-1} NMU solution and then plated on oatmeal agar containing the fungicide metalaxyl (600 μg ml^{-1}), some clones (K29- NM2-8, K29-NM3-3, and K29-NM3-8) were found to produce oogonia with several (2 to 8) oospores (Fig. 3 a–g). Staining with DAPI (4,6-diamidino-2-phenylindol) (Whittaker et al., 1991) revealed one nucleus in each oospore. It is possible that this single pleiotripic mutation was induced by NMU and the phenotype was selected on metalaxyl. When analysis 19 of single zoospore colonies of isolates of K29-NM2-8 was performed, 4 isolates had oogonia with several oospores. All 4 clones were able to form oospores with both testers but did not form oospores in single culture. The ability to form oogonia with multiple oospores was observed at field isolates of *P. infestans* (Anikina et al., 1999) and *P. sojae* (Cherepennikova-Anikina, unpublished).

After treatment of the isolate K22 with 5 μg ml^{-1} NMU solution and its inoculation in oatmeal agar to which metalaxyl at a concentration of 600 μg ml^{-1}, 5 clones were isolated. They all had the initial A2 mating type. Analysis of single zoospores of 12 clones of the strain K22-NM3-2 resulted in the following pattern: 8 clones had the Al mating type, 1 clone became self-fertile, 1 clone did non form oospores both with two testers and in single culture, 2 clones retained the initial A2 mating type. No changes in oospore formation were observed (Fig. 1f).

DISCUSSION

The results obtained demonstrate that the ability to switch with the influence of NMU and/or metalaxyl is characteristic of *P. infestans* isolates belonging both to Al and A2 mating types. After treatment of the strain K29 (Al), only self-fertile isolates were obtained. Meanwhile, when the strain K22 (A2) was treated with NMU and/or metalaxyl, isolates of Al, A2, AO and A1A2 were obtained in addition to the initial mating type. Isolates of Al, A2, and AO mating types were reversible, switching to A1A2, Al and occasionally to A2 mating types in zoospore generations. Ko (1981) suggested that the genetic control of *Phytophthora* sexual reproduction is fulfilled by a pair of diallele coupled loci with incomplete dominance: P1 — hormone α-1 synthesis, P2 — hormone α-2 synthesis, Rl — α-1 recognition, R2 — α-2 recognition. Transcription of these loci is controlled by a repressor, in one conformation inhibiting α-1 R2 (Al mating type) and in the second one inhibiting α-2Rl (A2 mating

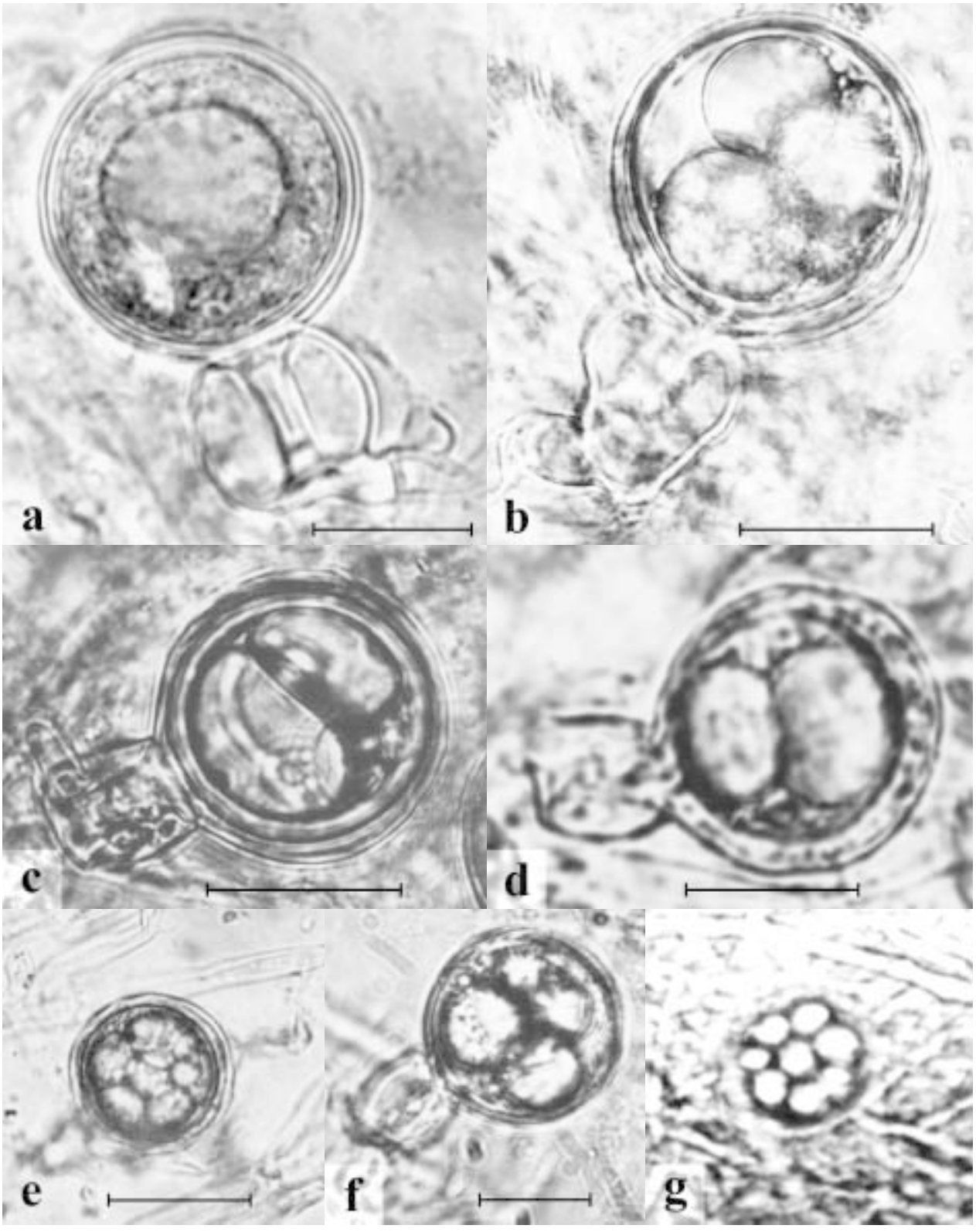

Fig. 3. Oogonia *of P. infestans* with one or multiple oospores.
a — Oogonium with one oospore, bars, 20 μm; b — oogonium with three oospores, bars, 26 μm; c — oogonium with two oospores, bars, 30 μm; d — oogonium with two oospores, bars, 44 μm; e — oogonium with six oospores, bars, 46 μm; f — oogonium with three oospores, bars, 35 μm; g — oogonia with eight oospores.

type). Ko succeeded in demonstrating that α-hormone is able to pass through a polycarbonate membrane and stimulate self-fertilization. Results of this work permit us to suggest that metalaxyl and NMU may change the mating type by reversing the repressor function. The insutiable nature of isolates A1A2 and AO of *P. infestans* obtained owing to treatment with chemical substances is similar to that of isolates A1A2 of *P. parasitica* (Ko, 1981).

Thus, influence of NMU solution and the systemic fungicide metalaxyl on the mating type and oospore formation in *P. infestans* depends on the combination of chemical substances used.

ACKNOWLEDGEMENTS

This study is supported by RFFI grant.

LITERATURE CITED

Anikina, M.I., Savenkova, L.V., Dyakov, Yu.T. & Shaw, D.S. (1999) Oogonia with multiple oospheres in *Phytophthora infestans. Mycol. Res.* **103:** 1332–1334.

Ann, P. J. & Ko, W. H. (1988) Hormonal heterotallism in *Phytophthora parasitica*, a novel mode of sexual reproduction. *J. General Microbiol.* **134:** 2985–2992.

Brassier, C. M. & Hansen, H. N. (1992) Genetics *of Phytophthora. Ann. Rev. Phytopathol.* **30:** 153–200.

Elliott, C. G. (1983) Physiology of sexual reproduction in *Phytophthora.* In: *Phytophthora: its Biology, Taxonomy, Ecology and Pathology.* D.C. Erwin, S. Bartinicki-Garcia & P.H. Tsao (Eds.) Amer. Phytopathol. Soc. Press, St. Paul, MN, P. 71–80.

Galindo, J. & Gallegly, M.E. (1960) The nature of sexuality in *Phytophthora infestans. Phytopathology* **51:** 123–128.

Ko, W. H. (1978) Heterothallic *Phytophthora*: Evidence for hormonal regulation of sexual reproduction. *J. Gen. Microbiol.* **107:** 15–18.

Ko, W.H. (1980) Gormonal regulation of sexual reproduction in *Phytophthora. J. Gen. Microbiol.* **116:** 459–463.

Ko, W. H. (1981) Reversible change of mating type in *Phytophthora parasitica. J. Gen. Microbiol.* **125:** 451–454.

Ko, W.H. & Chang, T.T. (1990) Effect of metalaxyl on mating type *of Phytophthora infestans* and *P. parasitica. Ann. Phytopathol. Soc. Japan* **56:** 194–198.

Noon, J.P. & Hickman, C.J. (1974) Oospore production by single isolate of *Phytophthora capsici* in the presence of cloroneb. *Can. J. Botan.* **52:** 1591–1595.

Savage, E.J., Clayton, C.W., Hunter, J.Y., Brenneman, J.A., Laviola, C. & Gallegly, M. E. (1968) Homothallism, heterothallism and interspecific hybridization in the genus *Phytophthora. Phytopathology* **58:** 1004–1021.

Whittaker, S. L., Shattock, R. C. & Shaw, D. S. (1991) Variation in DNA content of nuclei of *Phytophthora infestans* as measured by a microfluorimetric method using the fluorochrome DAPI. *Mycol. Res.* **95:** 602–610.