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Phytophthora multivesiculata, a new species causing rot in *Cymbidium*

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Abstract

A *Phytophthora* species was isolated from blackened leaves and stems of infected *Cymbidium* plants. Cultural characters did not fit descriptions of any known *Phytophthora* species. It was concluded that a new *Phytophthora* species, described here as *Ph. multivesiculata* is the causal agent. Isozyme data support this claim.

Introduction

Different *Phytophthora* spp. are known to attack orchids. Burnett (1974) reported *Ph. cactorum* together with *Pythium ultimum* as the two most destructive fungi of *Cattleya*-type orchids, causing black rot. In Taiwan death of orchid plants often is caused by *Ph. palmivora* and *Ph. nicotianae* (Chen and Hsieh, 1978). Orchid genera *Vanda* and *Cymbidium* have been reported to be resistant to the latter (Chen and Hsieh, 1978; Wey, 1988). In addition to *Ph. nicotianae*, *Ph. erythroseptica* var. *erythroseptica* has been observed on orchids in Australia (Hall, 1989; Duff, 1993). Four *Phytophthora* spp. have been found on orchids in Hawaii: *Ph. palmivora*, *Ph. nicotianae* and, although less frequent, *Ph. cactorum* and *Ph. cinnamomi* (Uchida, 1994). Since 1991, the Dutch Plant Protection Service has received samples of diseased *Cymbidium* plants from different growers from which *Phytophthora* isolates were obtained that could not be assigned unambiguously to one of the known species of the genus, although they vaguely resembled *Ph. porri* and *Ph. megasperma*. This paper presents the results of studies on morphology, pathogenicity, disease symptoms and isozyme patterns of *Phytophthora multivesiculata* ex *Cymbidium* and supposedly related species.

Materials and methods

Isolation

Ph. multivesiculata was isolated from the base of rotted stems and leaves of *Cymbidium* plants as well as from leaf tissue 1 cm above the necrotic area on cherry-decoction agar (CA) (Gams et al., 1987) and water agar (WA). Emerging colonies of *Phytophthora* were subcultured and stored on slants of V8 juice agar. The *Phytophthora* isolates studied here are listed in Table 1.

Morphology

Colony morphology and growth rate were compared on cornmeal agar (CMA), V8 juice agar (V8), cherry-decoction agar (CA), potato dextrose agar (PDA) and oatmeal agar (OA). Pieces of mycelial agar culture of the same size (5mm⌀) were used as inoculum; they were taken from actively growing colony margins of young cultures (three days old), in order to avoid delay in growth start and placed in the centre of the dish. Isolates were incubated at 20 °C in the dark and colony diameter was measured after 3 days along two perpendicular diameters. Sporangium formation and morphology were studied on solid media and in water cultures (Ilieva et al., 1995). The production and morphology of sexual structures were studied on different agar media at 20 °C. For all characteristics studied, at least 25 measurements were made for each isolate.

Table 1. Isolates of *Phytophthora* spp. used in isozyme comparison

Phytophthora spp.	no	host	ET[3]
Ph. megasperma var. *megasperma*	PD 94/59	*Daucus*	3
Ph. megasperma var. *megasperma*	PD 94/118	*Rubus ideaus*	3
Ph. megasperma var. *megasperma*	PD 94/687	*Malus*	3
Ph. megasperma var. *sojae*	PD 88/421	*Asparagus*	2
Ph. megasperma var. *sojae*	PD 94/570	*Asparagus*	2
Ph. megasperma var. *sojae*	PD 94/615	*Asparagus*	2
Ph. multivesiculata	PD 91/1973	*Cymbidium*	1
Ph. multivesiculata	PD 93/1296	*Cymbidium*	1
Ph. multivesiculata	PD 94/830	*Cymbidium*	1
Ph. multivesiculata	PD 95/4744	*Cymbidium*	1
Ph. multivesiculata	PD 95/8679[1)]	*Cymbidium*	1
Ph. multivesiculata	PD 95/8679R[2)]	*Cymbidium*	1
Ph. multivesiculata	PD 95/8680	*Cymbidium*	1
Ph. porri	PD 92/214	*Allium porrum*	5
Ph. porri	PD 95/683	*Allium porrum*	4
Ph. porri	PD 94/64	*Brassica chinensis*	6
Ph. porri	PD 94/166	*Brassica oleracea*	4

All isolates originate from the Netherlands.
1) This isolate is deposited as holotype at the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands under number CBS 545.96.
2) Reisolation from *Cymbidium* infected with PD 95/8679.
3) ET: Electrophoretic type based on isozyme analysis.

Pathogenicity

Pathogenicity of the isolates was tested by inoculation at 20 °C. Healthy *Cymbidium* leaves (25 cm long, measured from the top) were cut and, after washing under running tap water, placed with the injured end submerged into Erlenmeyer jars, each containing 25 ml of sterile distilled water and 8 discs (5 mm ⊘) from a 7 days old culture on V8. For inoculation 4 *Ph. multivesiculata* isolates (PD 91/1973, PD 95/4744, PD 95/8679 and PD 95/8679R) were used. The final record of disease symptoms was made 10 days after inoculation.

Isozyme analysis

Ph. multivesiculata and *Ph. megasperma* were cultured in 50 ml Tryptone Soy Broth medium in 250 ml erlenmeyer flasks on a rotary shaker (40 rpm) in the dark at 23 °C for seven days. *Ph. porri* was grown for three weeks in liquid V8 medium (Gams et al., 1987) in Petri dishes at 18 °C in the dark. The mycelia were collected by sieving, then dried by pressing between filter paper and stored overnight at −20 °C before extraction.

Frozen mycelium, (about 0.5 g) thawed at 4 °C for three h, was ground in a mortar, with silversand and 70 μl of extraction medium, chilled on ice. The extraction medium consisted of 0.1 m Tris-HCl, pH= 7.0, 1mM Dithiothreitol, 50 mM Ethylene Diamine Tetraacetic Acid, 10% Poly Vinyl Pyrrolidon w/v (soluble), 50 μg/ml soybean trypsin inhibitor, 0.1 mM Phenyl Methyl Sulfonyl Fluoride and 5% glycerol v/v. All mycelia were ground three times for one min with intervals of one min. The homogenate was transfered to Eppendorf tubes and centrifuged for ten min at 14,000 rpm (4 °C). The supernatant (40–80 μl) was collected and stored at −70 °C.

Electrophoresis was carried out at 4 °C on automated PhastSystem (Pharmacia). Samples were mixed with glycine to a final concentration of 0.22 M. Per assay 0.8 μl of each sample was applied to a 12.5% homogeneous native polyacrylamide gel (MDHP), or to a 8–25% gradient native polyacrylamide gel (MDH and IDH). Extracts assayed for MDH had to be diluted five to forty times, to avoid smearing.

Three enzymes were analysed, namely malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (MDHP, EC 1.1.1.40) and isocitrate dehydrogenase (IDH, EC 1.1.1.42).

The staining solutions for each enzyme were as follows:

MDH: 25 ml 0.2 m Tris-HCl pH=8.0, 440 mg L-malic acid disodium salt, 12.5 mg Nicotinamide-Adenine-Dinucleotide, 7.5 mg Nitro Blue Tetrazolium (NBT) (Sigma N6876), 1 mg Phenazine Methosulfate PMS (N9625).

MDHP: 25 ml 0.2 m Tris-HCl pH=8.0, 440 mg L-malic acid disodium salt, 12.5 mg Nicotinamide-Adenine-Dinucleotide Phosphate (NADP), 7.5 mg NBT, 1 mg PMS.

IDH: 25 ml 0.1 m Tris-HCl pH=8.0, 12.5 mg DL-isocitric acid trisodium salt, 100 mg $mgCl_2$, 10 mg NADP, 7.5 mg NBT, 1 mg PMS.

Gels were immersed in freshly prepared staining solutions in the dark at 37 °C for 5 min (MDH, IDH) or 20 min (MDHP).

Alleles were scored according to the method of Oudemans and Coffey, 1991a. Two zones of activity were assigned to two putative loci, the slowest locus 1 and the fastest locus 2. Each band was interpreted as an allele of a specific locus; bands were labelled alphabetically from the slowest to the fastest. Since the genus *Phytophthora* is diploid, two identical letters were assigned to one band. A three banded pattern was interpreted as a product of two alleles, coding for a dimeric enzyme (Richardson et al., 1986). Different enzyme profiles were grouped in electrophoretic types (ET's).

Figure 1. Symptoms of *Phytophthora multivesiculata* on *Cymbidium* leaves.

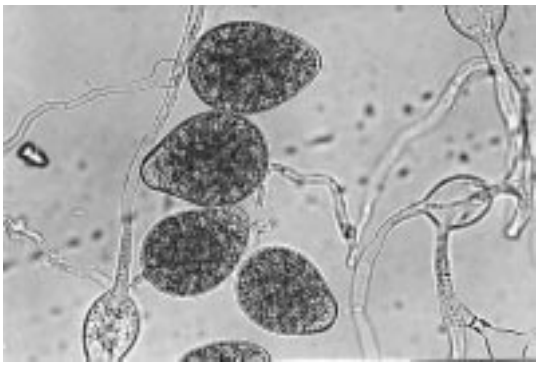
Results

Naturally infected *Cymbidium* plants showed dry rot of leaves (with a somewhat waxy looking surface) with a change of colour to brown with typical horizontal zebra-like stripes, about 0.5 cm wide with lighter discolouration in the middle and a dark brown to black margin (Figure 1). The base of the *Cymbidium* bulbs showed wet brown-black discoloured tissue. On artificially inoculated *Cymbidium* leaves, symptons became visible after 6 days. Dry brown rot was observed, but the typical zebra-like stripes were less conspicuous. After re-isolation, the fungus proved to be identical (morphologically as well as isozymatically) to the original pathogen.

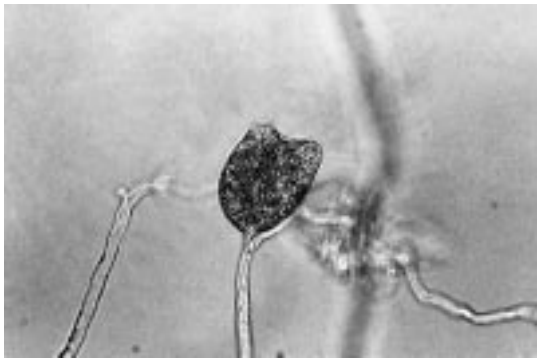
Characteristics of Phytophthora multivesiculata

All isolates readily formed sporangia on solid as well as in liquid media, although more abundant in the latter. Sporangia were mainly ovoid (Figure 2a) and occasionally obpyriform (Figure 2c), non-papillate and semi-papillate; rarely two apices were noticed (Figure 2b). In many sporangia, residual globules and 'papilla like' prolongations were observed . These 'papillate' sporangia usually germinated with a germ tube, forming new sporangia. Most sporangia had a rounded base (Figure 2a, 2c), occasionally a tapered base, although not as often as in the *Ph. porri* isolates used for comparison. Laterally attached sporangia were also present. Sporangia dimensions varied considerably within one isolate but there were no significant differences between isolates. Summarized data of all isolate dimensions are: 30–60 $\times$ 20–41μm (av. 45 $\times$ 33μm) with length:breadth ratio 1.10–1.78 (av. 1.43); exit pore 8.0–14.0 μm (av. 10μm).

Sporangia usually were produced singly on long, slender, mainly twisted sporangiophores (Figure 4), but sympodial arrangement of sporangia up to 3 was common on both solid and liquid media. Internal proliferation of sporangia was observed in water cultures



a



b

c

Figure 2. Ph. multivesiculata sporangia shapes in water culture: a. ovoid; b. two apices; c. obpyriform.

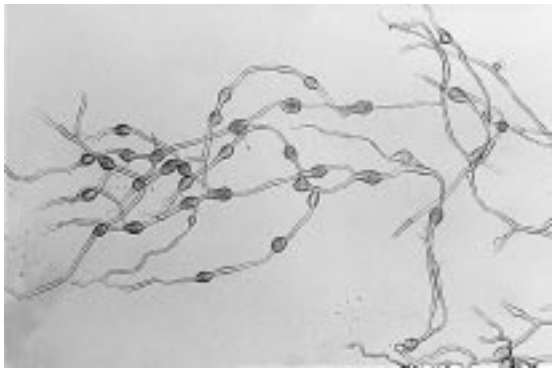


Figure 3. Ph. multivesiculata - catenulate hyphal swellings.

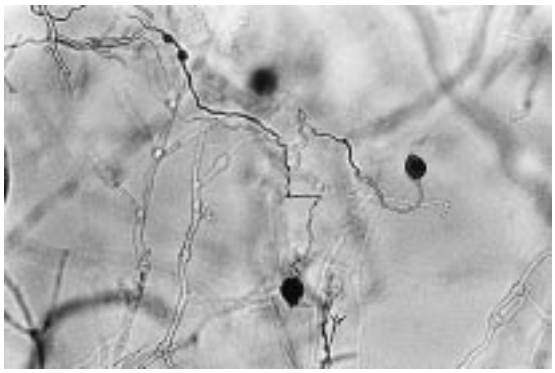


Figure 4. Ph. multivesiculata - slender twisted sporangiophores.

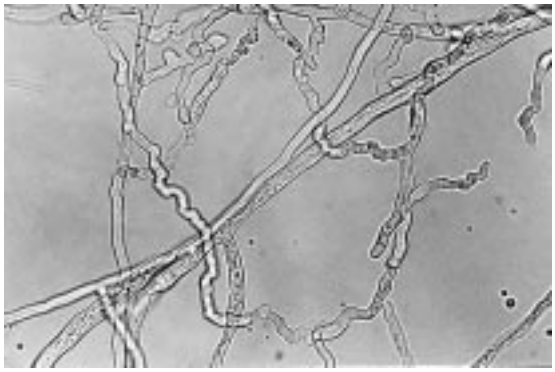


Figure 5. Ph. multivesiculata - coiled and twisted hyphae in water culture.

in all isolates, more readily in isolate PD 94/830. Sometimes hyphal swellings were observed beneath the sporangium (Figure 2a).

Ph. porri isolates sparsely formed sporangia on solid media (de Cock et al., 1992, reported that some *Ph. porri* isolates showed abundant sporangia formation on agar media), often with a tapered base and never with internal proliferation. Sporangia formation of *Ph. megasperma* isolates was observed only in water culture whereas internal proliferation was more common than in *Ph. multivesiculata*.

Sexual structures of *Ph. multivesiculata* were produced on both agar and in liquid media as well as in plant tissues, where they were formed in groups. Antheridia were irregularly spherical or ellipsoidal (Figure 6b), sometimes prolongation of hyphae was present. Antheridia were mostly amphigynous (95%) and some diclinous (Figure 6a). In *Ph. porri* and *Ph. megasperma* isolates mostly paragynous antheridia were observed.

Oogonia were spherical, smooth-walled; dimensions on V8 28.0–50.0 μm (av. 41 μm). Oospores mainly aplerotic (Figure 6b); dimensions 24.0–42.0

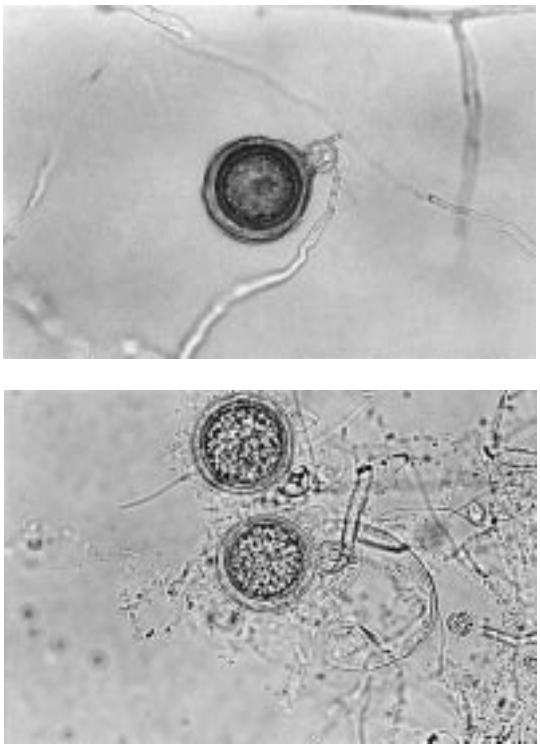


Figure 6. Ph. multivesiculata oogonia and antheridia: a. amphigynous diclinous antheridium; b. amphigynous antheridium; aplerotic oospore.

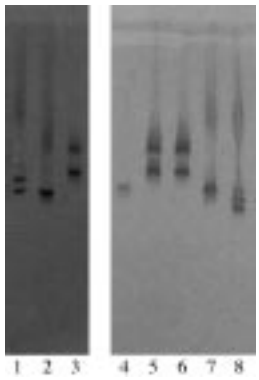


Figure 7. Banding pattern of MDH generated in *Ph. megasperma* var. *megasperma* (lane 1), *Ph. megasperma* var. *sojae* (lane 2), *Ph. multivesiculata* (lanes 3,5 and 6) and *Ph. porri* (lanes 4, 7 and 8) on native 8–25% polyacrylamide gels (pH=8.8). 1: PD 94/118; 2: PD 94/615; 3: PD 95/8679R; 4: PD 95/683; 5: PD 95/8679R; 6: PD 95/8680; 7: PD 94/166; 8: PD 94/64.

μm (av. 33.0 μm). No significant differences in dimensions of oogonia, oospores and antheridia were observed between isolates.

Isolates of *Ph. multivesiculata* grew more quickly on V8 (7.4 mm/day) than *Ph. porri* (isolate PD 92/214, 2.9 mm/day), which is fairly typical for this species (Waterhouse, 1963; Stamps et al., 1990), but slightly slower than *Ph. megasperma* (isolate PD 94/118, 9.0 mm/day) (Table 2).

Isolates of *Ph. multivesiculata* can be readily distinguished from *Ph. porri* and *Ph. megasperma* on the basis of the conspicuous formation of hyphal swellings in colonies on solid and liquid media. The swellings are rounded, obpyriform, catenulate and clustered (14–36 μm $\oslash$). New branches are formed at acute angles (Figure 3). In *Ph. porri* isolates, hyphal swellings were mainly rounded with more radiating hyphae. Hyphal swelling formation was not so frequent and abundant in *Ph. megasperma* isolates. They were observed at greater intervals along the hyphae with radiating branches arising almost at right angles.

There were no significant morphological differences among isolates of *Ph. multivesiculata*, also no characteristic growth pattern was observed. Colonies on V8, PDA, OA and CA exhibited moderately fluffy aerial mycelium, a little denser in the middle of the colony on PDA; on CMA mycelium was essentially submerged. On V8 the colony of *Ph. megasperma* (PD 94/118) had more aerial mycelium with irregular growth, the colony of *Ph. porri* (PD 92/214) showed dense low aerial mycelium with a powdery appearance and many coiled hyphae, easily observed on the reverse side of the colony.

Isozym analysis was applied to confirm that *Ph. multivesiculata* is indeed a new species. With malate dehydrogenase (MDH, EC 1.1.1.37) two zones of activity could be observed. The first zone (MDH-1) usually formed a smear, except in one *Ph. porri isolate* (PD 92/214) and in all *Ph. multivesiculata* strains. The second locus (MDH-2) could be interpreted in all isolates used in this study. *Ph. multivesiculata* could thus easily be distinguished from *Ph. porri* and *Ph. megasperma*. (Figure 7, Table 3). According to Nygaard et al. (1989) the *Ph. megasperma* complex can be divided into nine different types, based on morphological, physiological and biochemical data, and pathogenicity and growth. With MDH three different banding patterns were observed, one pattern unique to type I, comprising isolates of *Ph. megasperma* var. *sojae* and one pattern unique to type II, whereas type III-IX had all identical patterns. Although small differences in relative mobility due to different gel systems were observed, MDH patterns as found by Nygaard et al. (1989) could be reproduced. The MDH profile of

Table 2. Growth rates of *Phytophthora* spp. on different media at 20 °C

growth rate (mm/day)	V8	CMA	CA	PDA	OA	
PD 91/1973	*Ph. multivesiculata*	8.0	7.6	7.4	5.8	8.3
PD 94/830	*Ph. multivesiculata*	7.4	7.3	7.2	5.4	7.5
PD 95/8680	*Ph. multivesiculata*	7.4	7.6	6.9	5.2	8.2
PD 92/214	*Ph. porri*	2.9	4.2	3.8	3.9	5.9
PD 94/118	*Ph. megasperma*	9.0	9.2	7.8	8.8	9.2

Table 3. Isozyme-alleles scored at the putative MDH loci

	no. of isolates	ET[c]	MDH-1	MDH-2
Ph.multivesiculata	7	1	BB	BB
Ph. megasperma var. *sojae*	3	2[a]	n.d.	DD
Ph. megasperma var. *megasperma*	3	3[b]	n.d.	AD
Ph. porri	2	4	n.d.	CC
Ph. porri	1	5	AA	CC
Ph. porri	1	6	n.d.	CE

[a] identical to group I, Figure 3 according to Nygaard et al. (1989).
[b] identical to group III-IX, fig 3. according to Nygaard et al. (1989).
[c] ET:Electrophoretic type based on isozyme analysis.
n.d.: not determined due to smearing.

Ph. multivesiculata does not fit any profile found in *Ph. megasperma sensu lato*.

In *Ph. porri* two alleles appeared to be present at MDH-2 in the isolates analysed by us, resulting in a three banded pattern, characteristic for a dimeric enzyme. However some *Ph. porri* isolates carry only one MDH allele, resulting in one band (Figure 7). Altogether four isolates of *Ph. porri* were analysed with regard to MDH patterns, resulting in three electrophoretic types (ET's). The MDH profile of *Ph. multivesiculata* differed from the three electrophoretic types found in *Ph. porri* (Table 3).

All seven *Ph. multivesiculata* isolates of different origins in the Netherlands were also analysed for MDHP, IDH (results not shown). With MDHP one zone of activity was observed, containing one band with identical migration distance in all *Ph. multivesiculata* strains. With IDH two zones of activity were generated, each containing one band with identical migration distance in all strains. Intraspecific variation could not be detected with any of these enzymes. The IDH pattern of *Ph. multivesiculata* was different from those found in *Ph. porri* and *Ph. megasperma*.

Discussion

A new *Phytophthora* sp., *Phytophthora multivesiculata*, was isolated from infected *Cymbidium* plants. Artificial inoculation experiments showed that *Ph. multivesiculata* was indeed the causal agent of the disease, causing severe rotting of stems and leaves, eventually resulting in death. Re-isolation of the fungus confirmed the identity of the pathogen, morphologically as well by isozyme analysis.

Having semi-papillate to non-papillate sporangia, and mainly amphigynous antheridia, *Ph. multivesiculata* can be classified as a group IV species according to the revised tabular key devised by Stamps et al. (1990). *Ph. multivesiculata* can easily be distinguished from the other group IV species, because it is the only one which has a maximum temperature for growth of 35 °C and forms catenulate hyphal swellings in both solid and liquid media. A conspicuous feature of *Ph. multivesiculata* is the internal proliferation of sporangia, which is a characteristic feature of groups V and VI. However, of all group V and VI species, only *Ph. humicola*, *Ph. richardiae* and *Ph. insolita* form catenulate hyphal swellings on agar and of these only *Ph. insolita* has a high maximum temperature for growth (38 °C), which is significantly higher than

recorded for *Ph. multivesiculata*. Moreover, oogonia are larger in *Ph. multivesiculata* than in *Ph. insolita*, chlamydospores are observed in *Ph. insolita* but not in *Ph. multivesiculata* and antheridia, present in *Ph. multivesiculata*, are lacking in *Ph. insolita*.

Superficially the features *of Ph. multivesiculata* resembled features of both *Ph. porri* and *Ph. megasperma*. A closer examination, however, revealed small but marked differences with these species. *Ph. porri*, so far only known to be pathogenic to the genera *Allium* and *Brassica*, has a typical slow growth and low optimum and maximum growth temperatures. *Ph. multivesiculata* shows considerably higher growth rates at 20 °C than *Ph. porri*, but lower than those observed for *Ph. megasperma* (Table 2). The maximum temperature for growth of *Ph. multivesiculata* is 35 °C. Catenulate hyphal swellings, typical for *Ph. multivesiculata*, are observed in *Ph. porri* occasionally, but their appearance in this species is mainly rounded with more radiating hyphae. In *Ph. megasperma* hyphal swellings are formed, but not so frequent and abundant as in *Ph. multivesiculata*. Karakaya et al. (1995) rarely observed hyphal swelling in their *Ph. megasperma* isolates and Kröber (1985) noticed that swellings can be produced only under special conditions. Moreover, in *Ph. multivesiculata* antheridia are mainly (95%) amphigynous, whereas in *Ph. megasperma* mostly paragynous antheridia are observed. De Cock et al. (1992) reported the presence of paragynous antheridia in *Ph. porri* isolates from *Allium* and of amphigynous antheridia in isolates from *Brassica*, as well as a fifty-fifty ratio of both types in some other isolates. The dimensions of oospores of *Ph. multivesiculata* are similar to those reported for *Ph. megasperma* by Karakaya et al. (1995).

Isozyme analysis has proven to be a powerful tool in *Phytophthora* taxonomy (Oudemans & Coffey, 1991a/b). Isozyme analysis with MDHP, IDH and MDH (Table 3) of seven *Ph. multivesiculata* isolates revealed a single electrophoretic type. The MDH profiles were not identical to types I and III-IX described for *Ph. megasperma* by Nygaard et al. (1989). Although Nygaard's type II isolate was not available for comparison, it can be deduced from his article that its pattern did not resemble that of *Ph. multivesiculata*. In contrast to the single band present in the MDHP pattern of *Ph. multivesiculata*, our *Ph. megasperma* isolates produced two bands in agreement with results of Nygaard et al. (1989). The enzyme IDH was not investigated by him. The MDH profiles in *Ph. multivesiculata* also differed from those generated by *Ph. porri*.

In order to strengthen the case for creating a new species, the ITS sequence of *Ph. multivesiculata* has been determined and its sequence was found to be unique, but close to that of *P. citricola* (to be published elsewhere, Bonants et al., personal communication). However, *Ph. multivesiculata* can not be confused with *Ph. citricola*, which has mainly paragynous antheridia, a maximum temperature for growth of 31 °C, oogonia and oospores which are considerably smaller, and no hyphal swellings.

Conclusion

We have shown that the *Phytophthora* sp. isolated from *Cymbidium* does not fit the descriptions of *Ph. porri* or *Ph. megasperma*. Isozym analysis and ITS sequence analysis support this idea. We therefore conclude that the *Phytophthora* sp. isolated from *Cymbidium* is indeed a new species for which we propose the name *Phytophthora multivesiculata*.

Phytophthora multivesiculata Ilieva, Man in 't Veld, Veenbaas-Rijks et Pieters. sp. nov.

Mycelium ramosum, hyphae primariae 6.0 μm latae in CMA; vesiculae ex hyphis inflatis oriundae numerosae in agaro et in cultura aquatica, rotundatae, ellipsoideae, catenulatae et aggregatae. Sporangiophora longa, gracilia (2.0–3.0 μm), plerumque tortuosa, vulgo percurrenter per sporangium vacuum vel nonnumquam sympodialiter prope basim proliferentia. Sporangia producta in cultura solida vel liquida, 45.0(30.0–60.0) $\times$ 33.0 (20.0–41.0) μm, ovoidea, obpyriformia, non-papillata vel semi-papillata, poro exitiali 8.0–14.0 μm lato praedita. Oogonia levia, faciliter in planta et in vitro formata, in agaro V8 dicto 28.0–50.0 (in medio 41.0) μm; oosporae praecipue apleroticae 24.0–42.0 (in medio 33.0) μm diam. Antheridia praecipue amphigyna (95%). Cultura irregularis et leviter gossypina. Parasitica in Cymbidio, folia caulesque denigricans. Holotypus cultura exsiccata CBS 6034, viva CBS 545.96, isolatus e foliis *Cymbidii*, Terra Nova, Mijdrecht in Neerlandia, Novembre, 1995, in herbario Centraalbureau voor Schimmelcultures, Baarn, the Netherlands praeservatus.

Phytophthora multivesiculata

Mycelium branched, main hyphae 6.0 μm in CMA; hyphal swellings numerous on agar and in water culture, rounded, ellipsoid, catenulate and clustered.

Sporangiophores long, slender (2.0–3.0 μm), often twisted, proliferating percurrently through the empty sporangium, sometimes sympodially from near the base. Sporangia produced on solid and liquid media, 45.0 (30.0–60.0) μm $\times$ 33.0 (20.0–41.0) μm, ovoid, obpyriform, non-papillate or semi-papillate, exit pore 8.0–14.0 μm (average 10 μm) wide. Oogonia smooth-walled, readily produced, both in host tissue and pure culture; dimensions on V8 agar 28.0–50.0 μm (average 41.0 μm); oospores mostly aplerotic 24.0–42.0 μm (average 33.0 μm) diam. Antheridia mostly amphigynous (95%). Culture irregular and slightly fluffy. Parasitic on *Cymbidium*, causing blackening of leaves and stems.

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