1   **Multiplex PCR assay for the simultaneous detection and differentiation of *Olpidium***

2   ***bornovanus*, *O. brassicae*, and *O. virulentus***

3

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11

12   **ABSTRACT**

13   A multiplex PCR reaction has been developed to detect, differentiate, and confirm the

14   morphological identification of three *Olpidium* species: *O. bornovanus*, *O. brassicae*, and

15   *O. virulentus*. Of 127 samples analyzed, 87 samples were infected by any of these

16   species. Based on the morphology of resting spores, only *O. bornovanus* was detected in

17   21.2% of the samples. It was not possible to establish which of the *Olpidium* species was

18   present in the others samples by morphology. Based on the multiplex PCR, it was

19   possible to determine the *Olpidium* species present in all infected samples, even when

20   resting spores were not observed. This method was also effective for detecting *O.*

21   *bornovanus* in water samples. In addition, the specificity and sensitivity of the multiplex

22   PCR were evaluated. The multiplex PCR method was validated with samples of 9

23   different crops from 10 countries of America, Europe, and Africa.

INTRODUCTION

Olpidium bornovanus (Sahtiyanci) Karling and *Olpidium brassicae* (Woronin) P.A. Dang. sensu lato (sl), two of the chytrid species, are intracellular obligate parasites and root-infecting pathogens, and more importantly, they act as vectors of certain plant viruses (Rochon *et al.* 2004, Sasaya & Koganezawa 2006). The term *O. brassicae* sl is used to denote both *O. brassicae* (a crucifer strain) as a heterothallic fungus and *Olpidium virulentus* (Sahtiyanci) Karling (a non crucifer strain of *O. brassicae*) as a homothallic fungus (Koganezawa *et al.* 2005). *Olpidium* spp. life cycles involve the production of aquatic zoospores as a means of dispersal along with the production of resting spores that enable long-term survival in the absence of the host, which also plays a critical role in the survival of the viruses (Hiruki 1987, Rochon *et al.* 2004).

Olpidium spp. cannot be grown in pure culture, and the differentiation of its species is not possible without tedious and long bioassays (Jiang & Hiruki 1996). Significant differences in host range, host specificity, and virus transmissibility are recognized as the most important characteristics for discriminating *O. bornovanus* from *O. brassicae* sl (Campbell & Sim 1994, Sasaya & Koganezawa, 2006). However, microscopic observation of roots is generally the method used to identity these species by morphology of resting spores (Tomlinson & Thomas 1986). It is therefore essential to have a suitable detection method available for regular and reliable diagnosis of *Olpidium* spp. in plants

46   and also in environmental samples such as the irrigation water or the nutritive solution in

47   hydroponic cultures.

48   The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) have been

49   good targets for the identification and differentiation of fungi using molecular techniques

50   (White *et al*. 1990, Lee & Taylor 1992, Sreenivasaprasad *et al*. 1996, Crous *et al*. 2001,

51   Dunne *et al*. 2002). These regions are less well conserved sequences nested between the

52   highly conserved 18S, 5.8S and 28S rDNA genes. This approach has proved useful for

53   the detection by polymerase chain reaction-restriction fragment length polymorphism

54   (PCR-RFLP) of different *Olpidium* species (Jiang & Hiruki 1996) and others fungi even

55   at the subspecies level (Cubeta *et al*. 1991, Ward & Akrofi 1994). The rDNA-ITS have

56   the limitation that can only be used with pure cultures of fungi because plant DNA also

57   produces bands. In order to detect the fungi in infected plant tissue, specific primers with

58   no homology to plant DNA are required (Kularatne *et al*., 2004).

59   The objectives of this study were (i) to detecting and distinguishing among *O.*

60   *bornovanus*, *O. brassicae*, and *O. virulentus* in root samples by multiplex PCR assay, (ii)

61   to detecting *Olpidium* spp. in water samples, (iii) and to use this technical to confirm the

62   morphological identification of these *Olpidium* species.

63

64   **MATERIALS AND METHODS**

65   **Soil sampling and preparation of samples**

66   One hundred and twenty-seven soil samples were collected from different locations of

67   Brazil, Guatemala, Honduras, Mexico, Panama, Portugal, Spain, Tunisia, Uruguay, and

68   USA over a 10-year period (1999 to 2008). Samples consisted in soils from the root zone

69   of field-grown plants of cucurbit crops [cucumber, *Cucumis sativus* L.; melon, *Cucumis*

70   *melo* L.; watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai; zucchini (*Cucurbita*

71   *pepo* L.)], broccoli (*Brassica oleracea* L. var. *italica* Plenck.), cabbage (*Brassicae*

72   *oleracea* L. var. *capitata* D.C. and var. *acephala* D.C.), escarole (*Cichorium endivia* L.),

73   lettuce (*Lactuca sativa* L.), and tomato (*Solanum lycopersicum* L.) showing virus like-

74   symptoms, were used as sources of *Olpidium* spp. Soil samples of approximately 100 g

75   were put into a tray and covered with a filter paper to avoid airborne contaminations, air-

76   dried for 1 week, and sifted through a 2-mm sieve to remove soil clods prior to

77   processing. The course fraction was used to bait the fungi from soils using plants of crops

78   aforementioned.

79

80   **Isolation with bait plants**

81   *Olpidium* spp. was trapped on bait plants of the host from which it was isolated

82   (homologous host). Seeds of all the crops previously described were germinated in sterile

83   petri dishes containing paper towels and irrigation with Milli-Q water. Three seedlings

84   for each soil sample were transplanted at the cotyledon stage into pots in a 1:7 (wt:wt)

85   mixture of field soil:sterilized sand and placed in a climatic chamber with 60% relative

86   humidity, a 12 h day/night period at 26°C and 18°C, respectively, and irrigation with

87   Milli-Q water. Pots with non inoculated controls were interspersed among the pots of bait

88   plants, remaining free from *Olpidium* spp. in all isolation attempts. Thirty days after the

89   transplant, 1 plant/pot was taken individually with a shovel to not damage the root

90   system. The roots were washed with distilled water to remove the mixture and analyzed

91   morphologically and molecularly to test the suitability of the methods for the

92   determination of *Olpidium* spp. Samples formed by nine root samples, constituted by one

93   centimeter-long slices about 1 mm of diameter, were randomly taken and analyzed by

94   microscopic observation. Approximately 0.1 g of the feeder roots (0.05 g of roots per

95   each extraction procedure evaluated) were analyzed by multiplex PCR. The rest of the

96   bait plants were maintained for subsequent analysis.

97

98   **Morphological identification of *Olpidium* spp.**

99   Root samples were clarified using the method of Jordá *et al*. (2002), placed in water, and

100   visualized directly by a Nikon-YS-100 light microscope (Nikon Corporation, Tokyo,

101   Japan) to observe the presence or absence of resting spores of *Olpidium* spp. *O.*

102   *bornovanus* has smooth-walled resting spores with a honeycomb-like pattern, while *O.*

103   *brassicae* sl has resting spores which are stellate (Lange & Insunza 1977).

104

105   **Molecular detection of *Olpidium* spp.**

106   Multiplex PCR assay was used for molecular detection of *Olpidium* spp. in root and water

107   samples as described later.

108

109   **DNA preparations and extractions: *Olpidium* spp. detection in roots**

110   Two extraction procedures were tested in order to obtain optimum detection conditions.

111   In the first one, zoospores of *Olpidium* spp. were released from approximate 0.05 g of the

112   roots into 1.5 ml microcentrifuge tubes containing cold GS solution (0.05M glycine and

113   1% sucrose) for 45 min at room temperature. The roots were removed from the tubes, the

114   suspension centrifuged at 10000 rpm for 15 min, and approximately nine-tenths of the

115   supernatant fluid was discarded (a modification of Tomlinson & Thomas 1986). The

116   remaining liquid containing the zoospores was stored at -20ºC for further use. In the

117   second procedure, approximately 0.05 g of the roots were placed into small plastic bags

118   and grounded with a pestle. Total DNAs were directly extracted from each extraction

119   procedure using the E.Z.N.A® Plant DNA Miniprep Kit (OMEGA Biotech, Doraville,

120   USA) following the manufacturer's instructions, with some modifications. The extracts

121   were kept at -20ºC until analyzed.

122

Olpidium spp. detection in water

124   Pot containing a mixture of soil of the field-grown melon and sterilized substrate (1:9,

125   wt/wt) was irrigated with Milli-Q water. Eight days after irrigation, 750 ml of the leached

126   were collected, filtered with paper Whatman no. 4, and 250 ml incorporated to the

127   recirculating nutritive solution of melon hydroponic culture at cotyledon stage established

128   under controlled laboratory conditions, with the same environmental conditions

129   aforementioned. The remaining 500 ml were kept at 4-8°C and incorporate gradually in

130   applications to 100 ml each 4 days. Melon hydroponic culture with non-inoculate controls

131   was maintained under same conditions. Ten days after the last application, samples of 1.5

132   ml of recirculated solution were centrifuged at 10000 rpm for 15 min and approximately

133   nine-tenths of the supernatant fluid was discarded. Total DNAs from the resulting liquid

134   were extracted under the same conditions previously indicated. To make sure that

135   *Olpidium* spp. had an opportunity to colonize the root melon plants, these were observed

136   microscopically and total DNAs extracted as previously described.

137

138 **Primers design**

139 The rDNA-ITS sequences for *O. bornovanus*, *O. brassicae, O. virulentus*, which have

140 been deposited in the National Center of Biotechnology Information (NCBI) database and

141 published elsewhere (Sasaya & Koganezawa 2006), were aligned using the CLUSTALX

142 program ver. 1.83 (Jeanmougin *et al.* 1998) and examined for designing species-specific

143 primers using the primer design software OLIGO ver. 4.0 (National Bioscience Inc.,

144 Plymouth, MA). Three primers sets, consisting of one common reverse primer for these

145 species and three species-specific forward primers were obtained (Table 1).

146

147 **Multiplex PCR amplification**

148 Roots and water extracts were analyzed by the multiplex PCR assay to confirm the

149 morphological identification of *Olpidium* spp. Reaction mixtures consisted of 5μl 10x

150 PCR buffer (containing 2 mM $MgCl^{+2}$), 2μl 10 mM dNTP mix, 5μl 10x PVP-40, 1 U/μl

151 of NETZYME® DNA polymerase (NEED S.L., Valencia, Spain), 5μl 10x primers mix

152 (at a final concentration of 0.2 μM each primer), 2.5μl of total DNA extracts, and sterile

153 PCR water to make the volume up to 50μl. DNA amplifications were performed in a

154 Mastercycler personal 5332 thermocycler (Eppendorf, Germany) programmed for 5 min

155 initial denaturation at 94 ℃ followed by 35 cycles of denaturation for 45 s at 94℃,

156 annealing for 1 min at 55℃, extension for 1 min at 72℃, and final extension for 10 min

157 at 72℃ was introduced to finish incomplete PCR fragments, followed by cooling at 10℃

158 until the recovery of samples. The negative control tube, adding total DNA extracts from

159 healthy root and water samples, was incubated under the same conditions in each

160 amplification experiment. PCR amplified products (5μl) were electrophoresed on a 1.2%

161 agarose gel in 1x TAE buffer (40 mM Tris-acetate, and 1 mM EDTA at pH 8.0) at 100 V

162 for approximate 1 h, and stained with 0.2 µg.ml^{-1} ethidium bromide. Fragments sizes

163 were determined by comparison with a Gene Ruler™ 100 bp DNA Ladder Plus (MBI

164 Fermentas, Vilnius, Lithuania).

165

166 **Determination of DNA concentration**

167 The spectrophotometric DNA concentration from root extracts of infected bait plants

168 individually with *O. bornovanus*, *O. brassicae*, and *O. virulentus* was estimated. The

169 DNA concentration of the root extracts of healthy plants, belonging to the same host from

170 which its fungal were isolated, was also estimated under the same conditions. The

171 approximate concentration of fungal template DNA was determinate by a comparison

172 between the DNA concentration of healthy and infected root extracts, because these

173 fungal cannot be cultivated on synthetic media (Gerik *et al*., 1992).

174

175 **Sequencing and analysis of *Olpidium* spp.**

176 To confirm the identity of the *O. bornovanus*, *O. brassicae*, and *O. virulentus*, two

177 amplified PCR products for each *Olpidium* species were purified using a High Pure PCR

178 Product Purification Kit (Roche Diagnostics, Mannheim, Germany), and directly

179 sequenced with the species-specific forward primers using a Big Dye Terminator Version

180 3.1 Cycle Sequencing Kit in a 3100 Genetic Analyzer sequencer (Applied Biosystems,

181 Foster City, CA). The nucleotide sequences were compared by BLAST (Basic Local

182 Alignments Search Tool) (Altschul *et al*., 1997) with *Olpidium* sequences available from

183 the NCBI database. The sequences of *O. bornovanus*, *O. brassicae*, and *O. virulentus* are

184 published in the NCBI GenBank (GenBank accession Nos. EU934039/EU937731,

185 EU981906/EU981907, EU981901/EU981902, respectively).

186

187 **RESULTS**

188 **Morphological identification of *Olpidium* spp.**

189 Morphological identification was firstly used to analyze root bait plants that might

190 possibly be infected with *Olpidium* spp. (Table 2). *Olpidium* was observed in roots of 86

191 plants (67.7%) of the 127 plants analyzed. Based on the morphology of resting spores, *O.*

192 *bornovanus* and *O. brassicae* sl (Fig. 1, A and B) were observed in 27 (21.2%) and 25

193 (19.7%) plants, respectively. Stellate resting spores were referred to as *O. brassicae* sl,

194 because *O. brassicae* and *O. virulentus* could not be distinguished for this morphological

195 characteristic. Mixed infections, *O. bornovanus*/*O. brassicae* sl, were observed in 4 of

196 these plants (3.1%). In their homologous hosts, *Olpidium* spp. not always developed

197 resting spores. Thirty-four samples (26.8%), whose only sporangia formation was

198 observed, are referred to as *Olpidium* spp. (Fig. 2, Table 2).

199

200 **Molecular detection of *Olpidium* spp. in roots samples**

201 Two DNA extraction procedures were tested and multiplex PCR was used for the

202 molecular detection of *Olpidium* spp. Three *Olpidium* species were detected in 87 plants

203 (68.5%) of the total plants analyzed, regardless of the extraction procedure used (Table

204 2). *O. bornovanus*, *O. virulentus*, and *O. brassicae* single infections were detected in 61

205 (48.1%), 20 (15.7%), and 6 (4.7%) plants, respectively. Mixed infections were

206 determined in 6 plants (4.7%), but only the *O. bornovanus*/*O. virulentus* combination was

207   detected (Fig. 3, Table 2). No mixed infection between *O. bornovanus*/*O. brassicae*, or

208   *O. virulentus*/*O. brassicae* was found in the plants analyzed. *O. bornovanus* was detected

209   only in cucurbits plants from all the geographical origins studied. Instead, *O. virulentus*

210   was detected in cucurbits (melon and watermelon), lettuce, and tomato plants in most of

211   the countries studied. *O. brassicae* was only detected in broccoli and cabbage plants from

212   Portugal and Spain (Table 2).

213

214   ***Olpidium* spp. detection in water samples**

215   Root (R) and water (W) samples of melon hydroponic culture, to which was artificially

216   incorporated leached from of a soil of field-grown melon, were analyzed by multiplex

217   PCR assay for *Olpidium* spp. detection. A band of 977 bp corresponding to *O.*

218   *bornovanus* was obtained in both sources (Fig. 4). However, when health roots and sterile

219   water were used no amplified cDNA band was observed. Roots were also

220   morphologically analyzed and only resting spores of *O. bornovanus* were observed.

221

222   **Specificity of the multiplex PCR**

223   To confirm the specificity of the multiplex PCR, single infection root extracts for each

224   *Olpidium* species were mixed equally and subjected to multiplex PCR, as described

225   earlier. The four different patterns expected from these combinations are shown in Figure

226   5. The triple infection pattern with the three expected bands which might possibly occur

227   consists in *O. bornovanus*/*O. virulentus*/*O. brassicae*. The three double infections

228   patterns that might possibly also occur are: *O. bornovanus*/*O. virulentus*, *O.*

229   *bornovanus*/*O. brassicae*, and *O. virulentus*/*O. brassicae* (Fig. 5).

230 **Sensitivity of the multiplex PCR**

231 In order to check whether the presence of the primers cocktail in the multiplex PCR

232 reaction could influence the detection limit of *Olpidium* spp., the sensitivity of this

233 technical with *O. bornovanus*/*O. virulentus*/*O. brassicae* artificial mixed infections was

234 evaluated. The DNA concentration of the same extracts aforementioned was estimated

235 first. To determine the minimum amount of fungal DNA needed to produce a visible band

236 after PCR and gel electrophoresis, approximately 0.126 µg.µl^{-1} was quantified equally for

237 each *Olpidium* species. The infected extracts were equally mixed and serially diluted in

238 five-fold dilutions in water, and the multiplex PCR was performed, as described earlier.

239 Serial dilutions of this *Olpidium* mix were evaluated and *O. bornovanus* and *O. brassicae*

240 were clearly observed until dilution 5^{-9} (approx. 0.064 pg.µl^{-1} of fungal DNA). However,

241 *O. virulentus* was observed until dilution 5^{-6} (approx. 8.064 pg.µl^{-1} of fungal DNA) (Fig.

242 6).

243

244 **Sequence analysis of the *Olpidium* spp.**

245 The sequences from each *Olpidium* species were compared with the sequences published

246 in the NCBI database. In all cases, the obtained DNA sequence was that expected. *O.*

247 *bornovanus*, *O. brassicae*, and *O. virulentus* shows sequence nucleotide homology

248 ranging from 97-99%, 98%-99%, and 98%-99% in the genome zone studied,

249 respectively, with sequences published for these species.

250

251

252

DISCUSSION

Morphologically, the sporangia of *O. bornovanus* differ slightly from *O. brassicae* sl, although this morphological difference is not definitive evidence for discrimination between them (Herrera-Vásquez *et al*. 2007). The morphology of resting spores is accepted as the most important morphological characteristic to differentiate these species (Campbell & Sim 1994). However, the study of these characteristics is completely dependent on the skilled and tedious microscopic examination of root plants for fungal infection (Jiang & Hiruki 1996). The data provided in this work show that resting spores of *Olpidium* spp. were not always observed in the root samples analyzed. Therefore, the morphological identification is often not possible given a lack of resting spores formation.

The increase in the number of the genome sequence and the *Olpidium* spp. role as a vector of certain plant viruses (Rochon *et al.* 2004, Sasaya & Koganezawa 2006), entail the necessity of having a detection method available that allows differentiates the *Olpidium* spp. The multiplex PCR assay has been utilized for the detection of different fungi species, e.g., *Monilinia* and *Monilia* in apple fruits (Côté *et al*., 2004), *Eutypa lata* and *Eutypella vitis* in grapevine (Catal *et al*., 2007), *Polymyxa graminis* f. sp. *temperata* and *P. graminis* f. sp. *tepida* in barley and wheat (Vaïanopoulos, *et al*., 2007). Our laboratory has developed a multiplex PCR assay for the rapid detection and differentiation of three *Olpidium* species, which is a significant advance for the routine diagnosis of *Olpidium* spp. This technique was proved with samples of different crops from several geographic regions. Some samples tested in this work resulted in mixed double infections. To our knowledge, no previous studies report mixed infections of

276   *Olpidium* spp. The differentiation among the *Olpidium* species was possible, even when

277   resting spores were not observed. Therefore morphological identification was impossible.

278   In some cases, the morphological identification apparently has greater sensitivity that

279   detection molecular, perhaps due to the roots were randomly taken for each analysis and

280   *Olpidium* spp. distribution in the root plants is heterogeneous. The use of the multiplex

281   PCR technology in routine diagnosis allows the rapid detection and differentiation of

282   *Olpidium* spp. and their mixed infections, that sometimes elude the detection methods

283   usually performed. The fungal template DNA was successfully amplified from the

284   zoospores and root plants. In the first procedure however, low intensity cDNA

285   amplification was observed, in some cases. Probably, a longer root culture time was

286   needed to collect zoospores. The detection levels of both methods were, in any case,

287   sufficiently good to detect *Olpidium* spp. *O. bornovanus* has been observed in

288   greenhouse-grown melon plants which were irrigated with a water source from Almeria,

289   Spain (Gómez & Velasco 1991a). We reported the first molecular detection of this fungus

290   in water samples. The procedure developed for detecting *Olpidium* spp. in root and water

291   samples may help improve the sanitary status of melon and other cucurbit crops.

292   *Olpidium* spp. are intracellular obligate parasites (Jiang & Hiruki 1996) and DNA

293   quantification resulted approximate. In other fungi, e.g., *Aspergillus carbonarius* and *A.*

294   *japonicus*, it was possible to determine the fungus DNA concentration directly from the

295   pure culture (Perrone *et al*. 2004). The specificity of the multiplex PCR assay was

296   demonstrated with the amplification of a single PCR fragment in single-infected roots

297   compared with the amplification of several PCR fragments in artificially mixed infections

298   using a cocktail of all primers in both cases. The detection limit of the multiplex PCR

13

299   performed with cocktail primers was not affected, except in *O. virulentus*, probably

300   because the DNA concentration in the artificially mixed infection was not the same in all

301   three fungal species analyzed.

302   The multiplex PCR assay is reliable, simple, reduces time, work demands, and can be

303   used to identify *Olpidium* spp. without the need for a morphological analysis and the

304   consequent risks of misidentification. In addition, this method is suitable for research

305   involving a large number of samples because the DNA extraction is rapid and the PCR

306   amplification is simple. Furthermore, this methodology could be used to study the

307   transmission of plant viruses by *Olpidium* spp., the detection of *Olpidium* spp. in water

308   samples or solution nutritive from commercial hydroponic cultures, and the identification

309   of other plant species as natural reservoirs of *Olpidium* spp. Therefore, it is essential to

310   have a suitable detection method available for regular and reliable diagnoses of these

311   fungi. In the long term, this form of research should help develop strategies to control the

312   disease spread caused by the viruses transmitted by *Olpidium* spp.

313

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323

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401

402   **FIGURES**

403   **Fig. 1.** Sporangia of *Olpidium* spp. 40X observation.

404   **Fig. 2.** Resting spores of *Olpidium bornovanus* (A) and *Olpidium brassicae* sl (B). 40X observation.
405

406   **Fig. 3.** Multiplex PCR amplified DNA of three *Olpidium* species from root bait plants. Lanes 1-2, *O. bornovanus*; 3-4,
407   *O. virulentus*; 5-6, *O. brassicae*; 7-8, *O. bornovanus*-dual infection detection with *O. virulentus*; H, Healthy roots; M,
408   Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania).
409

410   **Fig. 4.** Multiplex PCR amplified DNA of *O. bornovanus* from roots (R) or water (W) samples of melon hydroponic
411   culture. Lane H, corresponds to multiplex PCR using non-infected root melon plants as template; M, Gene Ruler™ 100
412   bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania).

413

414   **Fig. 5.** Specificity of the multiplex PCR amplified DNA of *Olpidium* artificial mixed infections from single infection
415   root extracts. Lane 1, *O. bornovanus*/*O. virulentus*/*O. brassicae*-triple infection detection; 2, *O. bornovanus*/*O.
416   virulentus*-dual infection detection; 3, *O. bornovanus*/*O. brassicae*-dual infection detection; *O. virulentus*/*O. brassicae*-
417   dual infection detection; H, Healthy roots; M, Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius,
418   Lithuania).

419

420   **Fig. 6.** Sensitivity of the multiplex PCR amplified DNA of *Olpidium* artificial mixed infections from single infection
421   root extracts. Lane 5^0, Approx. 0.126 ng.μl^{-1} of fungal DNA; 5^{-1} to 5^{-9}, Serial nine-time dilution from 5^0; H, Healthy
422   root; M, Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania).