**UNIVERSIDAD POLITÉCNICA DE VALENCIA**

*ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS*

# Evaluation of weeds as potential hosts of Black foot and Petri disease pathogens on grapevine fields

**TESIS DE MÁSTER**

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Autorizo la presentación de la Tesina de máster "Evaluation of weeds as potential hosts of Black foot and Petri disease pathogens on grapevine fields" y certifico que se adecua plenamente a los requisitos formales, metodológicos y de contenido exigidos en la normativa existente.

(Firma) Director Tesina de máster

Valencia a, 10 de Noviembre de 2010

**Abstract**

Weeds were sampled in grapevine rootstock mother fields, openroot field nurseries and commercial vineyards in Spain between June 2009 and June 2010 and evaluated as potential hosts for black foot and Petri disease pathogens. Isolations were performed from the root system and internal xylem tissues for black foot and Petri disease pathogens, respectively. *Cylindrocarpon macrodidymum* was successfully isolated from the roots of twenty-six weed species belonging fifteen families. Regarding Petri disease pathogens, one isolate of *Phaeomoniella chlamydospora* was obtained from *Convolvulus arvensis*, and three isolates of *Cadophora luteo-olivacea* were obtained from *Bidens subalternans, Plantago coronopus* and *Sonchus oleraceus.* Pathogenicity tests showed that *C. macrodidymum* isolates obtained from weeds were able to induce typical black foot disease symptoms and caused shoot. Isolates of *Ca. luteo-olivacea* and *Pa. chlamydospora* were also shown to be pathogenic on grapevine cuttings. These results indicate that weeds can serve as host for *C. macrodidymum*, *Ca. luteo-olivacea* and *Pa. chlamydospora* and may play roles in the epidemiology of black foot and Petri disease pathogens on grapevine.

**Keywords**

*Cadophora luteo-olivacea*, *Cylindrocarpon macrodidymum*, *Phaeomoniella chlamydospora*, sources of inoculum, *Vitis vinifera*

**Resumen**

Entre Junio de 2009 y Junio de 2010, se muestrearon malas hierbas en campos de plantas madre de vid, en campos de enraizamiento de vivero y en viñedos comerciales, con el objetivo de evaluar las malas hierbas como posibles hospedantes de hongos que causan el pie negro y la enfermedad de Petri en vid. Se realizaron aislamientos de las malas hierbas a partir del sistema radicular y de los tejidos xilemáticos. *Cylindrocarpon macrodidymum* se aisló consistentemente de raíces de cincuenta familias y veintiséis especies de malas hierbas. Respecto a los patógenos causantes de la enfermedad de Petri, sólo se obtuvo un aislado de *Phaeomoniella chlamydospora* procedente de *Convolvuluas arvensis*, y tres aislados de *Cadophora luteo-olivacea* obtenidos de *Bidens subalternans, Plantago coronopus* y *Sonchus oleraceus*, todos ellos aislados a partir del xilema. Los test de patogenicidad a plántulas de vid demostraron que los aislados de *C. macrodidymum* obtenidos de malas hierbas causan los síntomas típicos del pie negro de la vid, provocando una reducción tanto en la brotación como en el desarrollo del sistema radicular. Los aislados de *Ca. luteo-olivacea* y *Pa. chlamydospora* también fueron patógenos a vid, observándose lesiones en el tejido vascular de varetas de vid inoculadas. Estos resultados indican que las malas hierbas pueden servir como hospedantes alternativos de *C. macrodidymum, Ca. luteo-olivacea* y *Pa. chlamydospora,* pudiendo tener un papel importante en la epidemiología del pie negro y de la enfermedad de Petri en vid.

**Palabras clave**

*Cadophora luteo-olivacea*, *Cylindrocarpon macrodidymum*, *Phaeomoniella chlamydospora,* fuentes de inóculo, *Vitis vinifera*

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# I.-INTRODUCTION

Over the last years, a drastic reduction has been noted in the survival rate of grafted grapevines in nurseries and young vineyards worldwide (Halleen *et al*., 2003). Grapevine wood fungi are among the most destructive pathogens either infecting grapevine propagation material or newly planted vines. They have been reported in most grapevine producing regions of the world and are responsible for the decline of young vines, loss of productivity and young vine death (Oliveira *et al*., 2004; Halleen et al., 2006; Mostert et al., 2006).

The main fungal diseases associated with young grapevine decline are black-foot disease, caused by *Cylindrocarpon liriodendri* MacDon. & Butler, *C. macrodidymum* Schroers, Halleen & Crous, *C. pauciseptatum* Schroers & Crous, *Campylocarpon fasciculare* Schroers, Halleen & Crous and *Campyl. pseudofasciculare* Halleen, Schroers & Crous (Halleen *et al*., 2004, 2006; Schroers *et al*., 2008), and Petri disease, caused by *Phaeomoniella chlamydospora* (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams, as well as several species of *Phaeoacremonium* W. Gams, Crous & M. J. Wingf. (Mugnai *et al*., 1999; Mostert *et al*., 2006), mainly *Pm. aleophilum* W. Gams, Crous, M. J. Wingf. & L. Mugnai, the most commonly isolated and studied species of this genus (Mostert *et al*., 2006). Several *Phialophora*-like and *Acremonium* species have also been involved in the decline in young vines, mainly species of the genus *Cadophora* Lagerberg. & Melin (Overton *et al*., 2005; Halleen *et al*., 2007). Of those, *Cadophora luteo-olivace*a (F. H. Beyma) Harrington & McNew has been recently shown to be quite common on grapevines affected by esca and Petri disease in several viticultural areas worldwide (Gramaje *et al*., 2010b).

Field symptoms of black foot and Petri disease affected vines are frequently indistinguible. External symptoms show stunted growth, reduced vigour, retarded or absence of sprouting, shortened internodes, sparse and chlorotic foliage with necrotic margins, wilting and dieback (Sheck *et al*., 1998; Rego *et al*., 2000; Fourie and Halleen, 2004).

Specific symptoms of vines affected by *Cylindrocarpon* spp. and *Campylocarpon* spp. often are sunken necrotic root lesions with a reduction in root biomass and root hairs (Halleen *et al*., 2006; Alaniz *et al*., 2007). Removal of rootstock bark reveals black discoloration and necrosis of wood tissues which develops from the base of the rootstock. Characteristic blackened sectors occurring at the base of the rootstock has given the disease the name of black foot (Halleen *et al.*, 2006). Dissected vines affected by Petri disease show a typical black discoloration of the xylem vessels, which is a result of tyloses, gums, and phenolic compounds formed inside these vessels by the host in response to the fungus growing in and around the xylem vessels (Mugnai *et al*., 1999).

Species of *Cylindrocarpon* are commonly known to be saprobes in soil, or occur on dead plant substrata, or act as weak pathogens of plants infecting wounds of roots and stems of various hosts (Halleen *et al*., 2006; Schroers *et al*., 2008). The production of chlamydospores would allow *Cylindrocarpon* spp. to survive for extended periods in soil (Halleen *et al*., 2004). However, very little information is currently available regarding the survival of these pathogens, and the role of chlamydospores during subsequent infections (Halleen *et al.*, 2006).

Regarding Petri disease pathogens, it has been suggested that *Pa. chlamydospora* is a soilborne pathogen due to its ability to produce chlamydospores in culture (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Sidoti *et al*., 2000). *Pa.*

*chlamydospora* was detected in vineyard soil in New Zealand by nested- Polymerase Chain Reaction (PCR) (Whiteman *et al*., 2002) and in South Africa by species-specific PCR (Damm and Fourie, 2005) or nested-PCR (Retief *et al*., 2006). Rigdway *et al.* (2005) used a nested-PCR/RFLP procedure to determine the persistence of viable and nonviable spores of *Pa. chlamydospora* in soil, and observed that nonviable spores were undetected after 8 weeks whereas viable spores still could be detected at 17 weeks, indicating that spores could persist in soil and the inoculum could build-up over time. Eskalen *et al*. (2001) confirmed the presence of *Pm. aleophlium* in the soil by nested-PCR. This species was also recovered from soil and standing water under grapevines (Rooney *et al*. 2001).

Given these findings, it could be assumed that black foot and Petri disease pathogens could survive in the soil and infest grapevine plants from this media. Colonization of weed hosts could be another possible means of persistence and source of inoculum (Dissanayake *et al*., 1997). Weeds have been shown to be an alternative host for several plant pathogens (Wisler and Norris, 2005). Black *et al.* (1996a and b) found a wide range of weed species as potential hosts for *Diaporthe phaseolorum* var. *caulivora* Kulik and *Rhizoctonia solani* J.G. Kühn AG-1 in soybean fields in Lousiana. Dissanayake *et al*. (1997) isolated *Pythium arrhenomanes* Drechs. from roots of grass species native to the north central plains of North America. *Cyperus rotundus* L. and other weed species associated with peanut crop have also been identified as a host of *Sclerotinia minor* Jagger (Hollowell *et al*., 2003). The genera *Datura, Geranium, Ipomoea* and *Solanum* may be also found as weed hosts for *Phytophthora infestans,* while *Portulaca oleracea* L. as an alternative host for *P. capsici* (French-Monar *et al*., 2006).

The aim of the present work was to study the role that weeds could play as potential hosts of black foot and Petri disease pathogens in the soil. Consequently, grapevine weeds were sampled in rootstock mother fields, openroot field nurseries and commercial vineyards in Spain and evaluated as potential hosts for these pathogens.

# II.-MATERIALS AND METHODS

## II.1.-Field sites

A total of 32 fields (3 grapevine rootstock mother fields, 6 openroot field nurseries and 23 commercial vineyards) were sampled between June 2009 and June 2010 in Albacete, Alicante, Castellón, Murcia and Valencia provinces in Spain (Figure 1). Family weeds prevalent in most field sites in the different seasons were: Amaranthaceae, Ambrosiaceae, Asteraceae, Brassicaceae, Chenopodiaceae, Convolvulaceae, Euphorbiaceae, Geraniaceae, Leguminosae, Liliaceae, Malvaceae, Plantaginaceae, Poaceae, Portulacaceae and Solanaceae.

## II.2.-Sampling and isolation of fungi

In each field, selected weed plants (three plants per species) were carefully dug out from the soil to keep the root system intact and taken back to the laboratory for immediate processing. Roots of each plant were exposed by carefully washing the soil away. Roots were visually inspected for evidence of root necrosis. Then, all the grapevine weeds were cut open and examined for signs of discoloured xylem vessels.

For *Cylindrocarpon* spp. isolation, root sections were cut from necrotic areas, washed under running tap water, surface-disinfested for 1 min in a 1.5% sodium hypochlorite solution and washed twice with sterile distilled water. Small root pieces were plated on potato dextrose agar (PDA) (Biokar-Diagnostics, Zac de Ther, France) supplemented with 0.5 g $l^{-1}$ of streptomycin sulphate (PDAS) (Sigma-Aldrich, St. Louis, MO, USA). For Petri disease pathogens, isolations were made from sections (10 cm


**Figure 1.-** Weed species in grapevine rootstock mother fields, openroot field nurseries and commercial vineyards. A/ Several weed species growing in a commercial vineyard; B/ *Amaranthus blitoides* and *Diplotaxis erucoides* in a young vineyard*;* Weeds growing close to grapevine plants: *C/ Echinocloa crus-galli;* D/ *Sonchus oleraceus;* E/ *Bassia scoparia* and F/ *Amaranthus blitoides*

long) that were cut from the basal stem and disinfected as previously described. Small pieces of internal xylem tissues were plated on malt extract agar (MEA) (Oxoid Ltd., Basingstoke, Hants, England) supplemented with 0.5 g $l^{-1}$ of streptomycin sulphate (MEAS).

Plates were incubated for 10-15 days at 25ºC in the dark and all colonies were transferred to PDA. They were single-spored prior to morphological and molecular identification with the serial dilution method (Dhingra and Sinclair, 1995).

## II.3.-Fungal identification

### II.3.1.-Morphological identification

Species of *Cylindrocarpon* were identified by macroscopic characters such as colony texture, color, and the type of the growing margin on PDA. Colonies grown on PDA were incubated for a further 20 days to determine the presence/absence of chlamydospores. Conidia size was also measured on Spezieller Nährstoffarmer Agar (SNA) with the addition of a 1×1 cm piece of filter paper to the colony surface (Alaniz *et al*., 2007).

*Phaeomoniella chlamydospora* was identified by conidiophore morphology, conidial size and shape, and its cultural characteristics on PDA and MEA (Crous and Gams, 2000). *Ca. luteo-olivacea* was identified by conidiophore morphology, size of phialides and conidia, and colony characters and pigment production on MEA, PDA and OA (Gams, 2000; Harrington and McNew, 2003).

II.3.2.-DNA isolation and sequencing

Fungal mycelium and conidia from pure cultures grown on PDA for 2 to 3 weeks at 25ºC in the dark were scraped and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using the E.Z.N.A. Plant Miniprep Kit (Omega Bio-tek, Doraville, USA) following manufacturer's instructions. DNA was visualized on 0.7% agarose gels stained with ethidium bromide and was stored at –20ºC.

Identification of *Cylindrocarpon* species was confirmed by a multiplex PCR system using a set of three pairs of specific primers (Alaniz *et al*., 2009a). *Pa. chlamydospora* was detected by PCR using primers Pch1-Pch2 (Tegli *et al*., 2000). Identification of *Ca. luteo-olivacea* isolates was confirmed by analysis of the ITS region of DNA amplified using the fungal universal primers ITS1F and ITS4 (Gardes and Bruns, 1993). PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and sequenced in both directions by Macrogen Inc. (Seoul, Republic of Korea).

**II.4.-Pathogenicity tests**

For pathogenicity tests, ten *C. macrodidymum* isolates (MH-1, MH-3, MH-9, MH-11, MH-21, MH-25, MH-29, MH-34, MH-42, MH-44, MH-46 and MH-55) were selected from those isolated in different weed species (Tables 1 and 2). Inoculum was produced on wheat (*Triticum aestivum* L.) seeds (Brayford, 1993). Seeds were soaked for twelve hours in distilled water and then air dried. Seeds were transferred to 300 mL flasks, which were subsequently autoclaved on 3 successive days at 120ºC during 1

hour. Two fungal disks of a two-week old culture of each *C. macrodidymum* isolate grown on PDA at 25ºC were placed aseptically in separate flasks. The flasks were incubated at 25ºC for four weeks, and shaken once a week to avoid clustering of inoculum.

Plastic pots (220 cc) were filled with a mixture of sterilized peat moss and 10 g of inoculum per pot. Seedlings obtained from grapevines cv. Palomino were planted individually in each pot at the two-true-leaf stage. Controls were inoculated with sterile uninoculated seeds. Six replicates (each one in individual pots) for each isolate were used, with an equal number of control plants. After inoculation, plants were placed in a greenhouse at 25-30ºC in a completely randomized design.

Forty-five days after inoculation plants were observed for the development of foliar symptoms, and evaluated using a 0 to 5 rating scale: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100% chlorotic and necrotic leaves, and 5 = dead plant. Then, all plants were gently uprooted and washed free of soil. Root symptoms of individual plants were evaluated on the following scale: 0 = healthy with no lesions, 1 = slight discoloration with 0 to 25% of root mass reduction, 2 = discoloration with 26 to 50% of root mass reduction, 3 = moderate discoloration with 51 to 75% of root mass reduction, 4 = severe discoloration with >75% of root mass reduction and 5 = dead plant. In addition, dry weights of shoot and root were recorded for each plant. Symptomatic roots were aseptically plated on MEAS to reisolate *C. macrodidymum* and complete Koch's postulates. The experiment was repeated.

Shoot and root disease severities were calculated using the McKinney's index (McKinnney, 1923), which expresses the percentage of the maximum severity of disease according to the formula: MI= [$\Sigma$($R$ x $N$)] x 100 / $H$ x $T$, where $R$ = disease rating; $N$= number of plants with this rating; $H$= the highest rating; $T$= total number of

plants counted. The statistical analysis of the experimental results was carried out in a one-way ANOVA with treatment as independent variable, and the following dependant variables: shoot disease severity (%), shoot dry weight (g), root disease severity (%) and root dry weight (g). The Student's Least Significant Difference (LSD) test was used to compare the overall means of each treatment at $P<0.05$. The analyses used the Statistical Analysis System (version 9.0, SAS Institute Inc. Cary, NC, USA).

For Petri disease pathogens, one isolate of *Pa. chlamydospora* (Pch-256) obtained from *Convolvulus arvensis* L. and two isolates of *Ca. luteo-olivacea* (Clo-65 and Clo-66) obtained from *Bidens subalternans* DC. and *Sonchus oleraceus* L. respectively, were used. Pathogenicity tests were conducted in 1-year-old grapevine cuttings of 110 Richter rootstock. Dormant cuttings were cut into uniform lengths containing four to five buds, and then hot-water treated at 53ºC for 30 min to eliminate the possible incidence of fungal trunk pathogens (Gramaje *et al.*, 2009). In order to enhance callusing and rooting, dormant cuttings were buried into sterilized peat moss in plastic boxes, and placed in a callusing room at 25ºC and 100% humidity for 4 weeks. After callusing and rooting, cuttings were wounded between the two upper internodes with a 5 mm cork borer. A 5 mm mycelium agar plug from a 2-weeks-old culture was placed in the wound. Wounds were wrapped with parafilm. Eight cuttings per fungal isolate were used. Additionally, eight cuttings were inoculated with 5 mm noncolonized PDA agar plugs from two different plates for negative controls. Inoculated cuttings were planted immediately in individual pots, placed in a greenhouse at 25ºC and watered every 3 days or as needed. Plants were arranged in a completely randomized design. The experiment was repeated.

Cuttings were collected after 14 weeks and inspected for lesion development. Extent of vascular discolouration was measured upward and downward from the

inoculation point. Small pieces (0.5 to 1 cm) of necrotic tissue from the edge of each lesion were cut and placed on MEAS in an attempt to recover the inoculated fungi and complete Koch's postulates. Fungi were identified as previously described.

Lesion length data were subjected to analyses of variance using SAS and the LSD test was calculated at the 5% significance level to compare the treatment means for the different fungal species.

# III.-RESULTS

## III.1.-Isolation and identification of fungal species from weeds

Xylem tissues were nonsymptomatic in all weed plant samples, but some roots showed slight discolorations or necrotic lesions.

*Cylindrocarpon* spp. were isolated from roots of weeds collected in seventeen out of the thirty two field sites surveyed and in all type of fields (3 grapevine rootstock mother fields, 3 openroot field nurseries and 11 commercial vineyards). All *Cylindrocarpon* isolates were identified by multiplex PCR as *C. macrodidymum*, because they generated a product size of 387 bp characteristic of this species (Figure 2). The families and species of weeds from which *C. macrodidymum* was isolated are shown in Table 1. This pathogen was recovered from fifteen families and twenty-six weed species. In each family, the number of host weed species was variable, Asteraceae being the most prevalent with six species, followed by Amaranthaceae (three species) and Brassicaceae, Chenopodiaceae, Euphorbiaceae and Poaceae (two species each). *C. macrodidymum* was isolated from only one weed species in the remaining families (Ambrosiaceae, Convolvulaceae, Geraniaceae, Leguminosae, Liliaceae, Malvaceae, Plantaginaceae, Portulacaceae and Solanaceae). The frequency of isolation of *C. macrodidymum* was very variable depending on the number of field sites or plants evaluated for each weed species (Table 1).

Regarding Petri disease pathogens, one isolate of *Pa. chlamydospora* was obtained from *Convolvulus arvensis* in a grapevine commercial field, and three isolates of *Ca. luteo-olivacea* were obtained from *Bidens subalternans* (in a grapevine rootstock

mother field), and from *Plantago coronopus* L. and *Sonchus oleraceus* (both in an openroot field nursery).

### III.2.-Pathogenicity tests

In *C. macrodidymum,* data of the two pathogenicity tests were combined because of the lack of significant differences between the two tests and with the studied variables ($P>0.05$; Anova tables not shown). All isolates used in this study were pathogenic to seedlings obtained from grapevines cv. Palomino. Symptoms developed by 10 d after inoculation and consisted in reduced vigour, leaves with internervial chlorosis and necrosis, necrotic root lesions with a reduction in root biomass, and the death of some plants.

The statistical analysis indicated significant ($P<0.0001$) differences in virulence among *C. macrodidymum* isolates in all variables evaluated. All isolates caused a significant increase of shoot and root disease severity and a decrease of shoot and root dry weight when compared to the uninoculated controls. The isolate MH-46 was the most virulent, causing 100 % mortality of grapevine seedlings (Table 2).

For Petri disease pathogens, analyses of variance of the lesion length data on grapevine cuttings indicated a significant treatment effect ($P < 0.0001$; Anova tables not shown). *Pa. chlamydospora* isolate Pch-256 (mean 57.1 mm), *Ca. luteo-olivacea* isolate Clo-65 (66.3 mm), and Clo-66 (65.5 mm) caused lesions on the xylem of grapevine cuttings that were significantly longer than the controls (mean 3.9 mm).

Table 1. Weed families and species from which *Cylindrocarpon macrodidymum* was isolated from roots.

| Family | Species | Frequency of fields[a] | Percentage of plants[b] |
|---|---|---|---|
| Amaranthaceae | *Amaranthus blitoides* | 3/7 | 66.7 |
| | *Amaranthus retroflexus* | 1/4 | 33.3 |
| | *Salsola kali* | 1/6 | 33.3 |
| Ambrosiaceae | *Xanthium stromarium* | 1/1 | 100.0 |
| Asteraceae | *Bidens subalternans* | 1/1 | 33.3 |
| | *Cirsium arvense* | 1/3 | 100.0 |
| | *Helichrysum stoechas* | 1/1 | 100.0 |
| | *Senecio vulgaris* | 2/2 | 66.7 |
| | *Sonchus oleraceus* | 4/10 | 75.0 |
| | *Sonchus terrenimus* | 3/5 | 66.7 |
| Brassicaceae | *Diplotaxis erucoides* | 1/8 | 66.7 |
| | *Hirschfeldia incana* | 1/1 | 100.0 |
| Chenopodiaceae | *Bassia scoparia* | 1/2 | 66.7 |
| | *Chenopodium murale* | 3/9 | 66.7 |
| Convolvulaceae | *Convolvulus arvensis* | 2/2 | 16.7 |
| Euphorbiaceae | *Chrozophora tinctoria* | 1/1 | 66.7 |
| | *Euphorbia segetalis* | 2/2 | 66.7 |
| Geraniaceae | *Erodium malacoides* | 1/2 | 66.7 |
| Leguminosae | *Medicago lupulina* | 1/1 | 100.0 |
| Liliaceae | *Allium oleraceum* | 1/1 | 33.33 |
| Malvaceae | *Lavatera cretica* | 1/1 | 100.0 |
| Plantaginaceae | *Plantago coronopus* | 1/1 | 100.0 |
| Poaceae | *Bromus madritensis* | 1/1 | 33.3 |
| | *Echinocloa crus-galli* | 2/3 | 33.3 |
| Portulacaceae | *Portulaca oleracea* | 1/3 | 33.3 |
| Solanaceae | *Solanum nigrum* | 1/2 | 100.0 |

[a] For each weed species: No. of fields from which *C. macrodidymum* was isolated / No. of total fields in which the weed species was collected.

[b] For each weed species: Percentage of plants from which *C. macrodidymum* was isolated related to the total number of plants collected in positive fields (3 plants per field).

Table 2. Pathogenicity of *Cylindrocarpon macrodidymum* isolates obtained from twelve different weed species to seedlings of grapevine cv. Palomino fourty five days after inoculation.

| | | Shoot | | Root | |
|---|---|---|---|---|---|
| Isolates | Weed species | Disease severity[a] | Dry weight (g) | Disease severity[b] | Dry weight (g) |
| MH-1 | *Salsola kaly* | 68.3[c]a[d] | 0.18 b | 68.8 b | 0.20 b |
| MH-3 | *Echinocloa crus-galli* | 83.3 bcde | 0.05 cdef | 76.7 bc | 0.07 cd |
| MH-9 | *Sonchus oleraceus* | 80.0 bcde | 0.08 bcdef | 68.8 b | 0.16 bc |
| MH-11 | *Euphorbia segetalis* | 88.3 cde | 0.03 ef | 83.3 bc | 0.01 d |
| MH-21 | *Amaranthus blitoides* | 66.7 b | 0.12 bcde | 70.0 b | 0.10 bcd |
| MH-25 | *Chenopodium murale* | 86.7 bcde | 0.06 cdef | 79.6 bc | 0.08 cd |
| MH-29 | *Bassia scoparia* | 73.3 bcd | 0.08 cdef | 81.3 bc | 0.10 bcd |
| MH-34 | *Chrozophora tinctoria* | 75.0 bcd | 0.14 bc | 79.2 bc | 0.17 bc |
| MH-42 | *Convolvulus arvensis* | 91.7 de | 0.05 def | 86.7 bc | 0.06 cd |
| MH-44 | *Hellychrisum stoechas* | 76.7 bcd | 0.08 cdef | 83.3 bc | 0.07 cd |
| MH-46 | *Diplotaxis erucoides* | 100.0 e | 0.02 f | 91.7 bc | 0.01 d |
| MH-55 | *Sonchus terrenimus* | 66.7 b | 0.13 bcd | 70.0 b | 0.14 bc |
| Control | - | 0.00 a | 0.74 a | 16.7 a | 0.68 a |

[a] Shoot symptoms were evaluated on the following scale: 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, 4 = 76 to 100% chlorotic and necrotic leaves, and 5 = dead plant. Shoot disease severity was calculated using the McKinney's index: MI= [$\Sigma$($R$ x $N$)] x 100 / $H$ x $T$, where $R$ = disease rating; $N$= number of plants with this rating; $H$= the highest rating; $T$= total number of plants counted.

[b] Roots symptoms were evaluated on the following scale: 0 = healthy with no lesions, 1 = slight discoloration with 0 to 25% of root mass reduction, 2 = discoloration with 26 to 50% of root mass reduction, 3 = moderate discoloration with 51 to 75% of root mass reduction, 4 = severe discoloration with >75% of root mass reduction and 5 = dead plant. Root disease severity was calculated using the McKinney's index.

[c] Values represent the means of 12 replications for each isolate; six per experiment.

[d] ANOVA. Means in a column followed by the same letter are not significantly different according to Student's least significant difference test at $P$<0.05

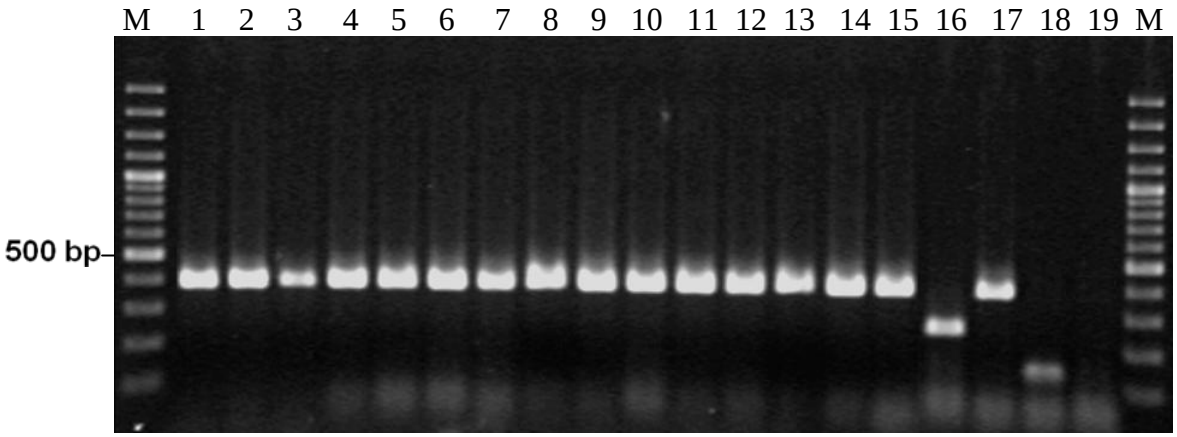


**Figure 2.-** Identification of *Cylindrocarpon macrodidymum* isolates obtained from roots of weeds by polymerase chain reaction (PCR.) PCR amplification products with three primer pairs, Mac1/MaPa2, Lir1/Lir2, and Pau1/MaPa2. Lanes 1 to 15, positive isolates from *Amaranthus blitoides* (Lanes 6 and13), *Amaranthus retroflexus* (Lane 12), *Bassia scoparia* (Lane 2), *Chrozophora tinctoria* (Lanes 3, 4 and 7), *Cirsium arvense* (Lane 10), *Convolvulus arvensis* (Lanes 11 and 14), *Diplotaxis erucoides* (Lane 9), *Hellycrysum stoechas* (Lane 15), *Solanum nigrum* (Lanes 5 and 8), and *Sonchus oleraceus* (Lane 1); lanes 16 to 18, positive control (DNA from pure culture) of *C. liriodendri, C. macrodidymum and C. pauciseptatum*, respectively; lane 19, negative control; lanes M, 100-bp DNA ladder.

## IV.-DISCUSSION

This is the first study that examined weeds as potential hosts for black foot and Petri disease pathogens on grapevine fields. *C. macrodydimum*, *Ca. luteo-olivacea* and *Pa. chlamydospora* were isolated from weeds collected in grapevine rootstock mother fields, openroot field nurseries and commercial vineyards.

We found a high rate of isolation of *C. macrodydimum* from roots of weeds collected in grapevine fields. Although several species of *Cylindrocarpon* have been identified worldwide as a common cause of vine death in nurseries and in young vineyards (Halleen *et al*., 2006; Schroers *et al*., 2008), in our work only *C. macrodidymum* was found. This is in agreement with the results obtained by Alaniz *et al*. (2007), who reported that *C. macrodidymum* is the predominant species in all grapevine growing regions in Spain. To date, *C. macrodidymum* had been shown to be species specific to grapevines, being isolated only from young grapevines in Australia, California, Chile, New Zealand, South Africa, Spain and Uruguay (Halleen *et al*., 2004, 2006; Alaniz *et al*., 2007; Abreo *et al*., 2010), but not from other hosts. There was variation among weed families and species in the percentage of plants colonized by *C. macrodidymum* and its field frequency. This variation could affect their importance as alternative hosts, as described for weed hosts of *Pythium arrhenomanes* in sugarcane fields by Dissanayake et al. (1997).

*Cylindrocarpon* spp. are a soilborne pathogens which produce chlamydospores to survive for extended periods in soil (Halleen *et al*., 2004). However, very little information is currently available regarding the long-term survival of the species of this genus (Brayford, 1993; Halleen *et al.,* 2006). The high frequency of isolation of *C. macrodydimum* obtained in this study demonstrates that this species may have the ability to alternatively survive in weeds. This finding should contribute to a better

understanding of the role of different stages of these fungi in pathogenesis, as well as aid in developing more effective control measures for black foot disease.

The vascular pathogens *Ca. luteo-olivacea* and *Pa. chlamydospora* were also isolated from several weeds within grapevine fields. Recently, *Ca. luteo-olivacea* has been identified in grapevines showing decline symptoms in California (Rooney-Latham, 2005), South Africa (Halleen *et al*., 2007), Uruguay (Abreo *et al*., 2008), New Zealand (Manning and Munday, 2009), Northeastern America (Rolhausen *et al*., 2010), and Spain (Gramaje *et al*., 2010b). Our study provides novel information about the possible role of this species as a soil-borne pathogen of grapevine and could be useful to improve the knowledge of the mode of inoculum survival. Moreover, this work represents the first report of *Pa. chlamydospora* on a host different from grapevine. *Pa. chlamydospora* is regarded as the most important fungal organism associated with Petri disease because of its predominance in affected grapevines (Ridgway *et al*., 2005; Mostert *et al*., 2006); therefore, this finding could indeed be a very important aspect to consider in further epidemiological studies of this species.

In contrast with *C. macrodydimum*, *Ca. luteo-olivacea* and *Pa. chlamydospora* were isolated from asymptomatic xylem tissues. In similar studies, weeds had been also found to be symptomless hosts for other plant pathogens, such as *Rhizoctonia solani* and *Diaporthe phaseolorum* var. *caulivora* in soybean (Black *et al.*, 1996a and b). Our results could indicate that *Ca. luteo-olivacea* and *Pa. chlamydospora* may be surviving in weeds as endophytes. The potential role of these pathogens as grapevine endophytes and the erratic manifestation of leaf symptoms in infected grapevines have been previously reported by different authors. Whiting *et al*. (2001) demonstrated that *Phaeoacremonium* spp. and particularly *Pa. chlamydospora*, were able to adapt to a wide range of water potentials, suggesting that they can possibly to exist as endophytes

or as latent pathogens in grapevine xylem tissue. Infected grapevine rootstock mother plants with no external foliar symptoms were reported by Fourie and Halleen (2004) and Aroca *et al*. (2010). Halleen *et al*. (2005) observed that primary pathogens associated with young vine decline in South Africa, such as *Pa. chlamydospora, Pm. aleophilum* and *Ca. luteo-olivacea*, were already present in the apparently healthy rootstock propagation material as endophytes or latent pathogens. Despite the isolation of *Phaeoacremonium* spp. and *Pa. chlamydospora* from wood discoloration of young grapevines, Zanzotto *et al.* (2007) did not observe classic foliar symptoms of either Petri disease or esca in the vineyards during the 4 years after planting. Gramaje *et al*. (2010a), who inoculated five species of *Phaeoacremonium, Pa. chlamydospora* and *Ca. luteo-olivacea* on rootstock cuttings under field conditions, observed that these species were not able to cause foliar symptoms on inoculated plants during the grapevine growing season.

Several authors indicated that *Pa. chlamydospora* is a soilborne pathogen due to its ability to produce chlamydospores in culture (Bertelli *et al*., 1998; Mugnai *et al*., 1999; Sidoti *et al*., 2000). Chlamydospores of *Pa. chlamydospora* are thought to form conidia that can penetrate uninjured roots of vines in nurseries or vineyards (Bertelli *et al*., 1998; Feliciano and Gubler, 2001). It has been suggested by a number of researchers that the occurrence of this pathogen in nursery soil might act as a potential source of infection for rootstock mother plants and young vines in nursery fields. *Pa. chlamydospora* has been detected using molecular methods in vineyard soils in California and South Africa (Rooney *et al*., 2001; Damm and Fourie, 2005) and nursery soils in New Zealand (Retief *et al*., 2006), although the type and viability of propagules was not determined. In this sense, Ridgway *et al*. (2005) tested the applicability of the nested PCR-RFLP procedure for use in field experiments to determine how long spores

of *Pa. chlamydospora* could persist in soil, and observed that at least some of the spores could still viable in this media for 2 months.

Pathogenicity tests showed that *C. macrodidymum* isolates obtained from weeds were able to induce typical black foot disease symptoms and caused shoot and root mass reduction on grapevine seedlings. Different levels of virulence were also observed in the *C. macrodidymum* isolates inoculated. These results are in agreement with those obtained by Alaniz *et al*. (2009b), who detected a relevant genetic and virulence diversity in *C. macrodidymum* by Inter Simple Sequence Repeat (ISSR) analysis and pathogenicity tests. Isolates of *Ca. luteo-olivacea* and *Pa. chlamydospora* were also shown to be pathogenic on grapevine cuttings. The expression of symptoms caused by *Pa. chlamydospora* and *Ca. luteo-olivacea* on grapevines has been extensively investigated and successfully reproduced with artificial inoculations under field (Mugnai *et al*., 1999; Halleen *et al*., 2007; Gramaje *et al*., 2010a) or greenhouse conditions (Larignon and Dubos, 1997; Halleen *et al.*, 2007; Zanzotto *et al*., 2008; Gramaje *et al*., 2010b).

The results of this work demonstrated that weeds can serve as alternative hosts of black foot and Petri disease pathogens, providing a reservoir from which grapevine infection can occur. Sampling of weeds prevalent in other viticultural regions in different countries might reveal a larger host range for them and contribute to determine if they are likely to play an important role in maintaining or increasing the inoculum density of the pathogens in grapevine fields. In Spain, grapevine weeds are often well-managed during the growing season. During the off-season, when land is fallow, weeds are not managed and their populations increase. High populations of host weed species may increase the amount of infected plant debris in grapevine fields and thus contribute to elevate inoculum levels the following spring. These results point out the importance

of a weed control program, not only for reducing their competition with grapevine, but also in possibly reducing the inoculum levels of soilborne pathogens.

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