Assessment of Fungal Pathogens Affecting the Weed
Conyza bonariensis in Argentina

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Abstract

In the last years *Conyza bonariensis* has become an important weed and control is difficult with the use of current technology in Argentinean pampas region. The increasing prevalence of herbicide-resistant weed species, public concern related to pesticide use and the introduction of government policies for pesticide reduction, is driving the search for alternative methods to chemical control. The aims of the present study were to detect fungal diseases associated with *C. bonariensis*, to identify fungal isolates from the symptomatic leaves and to confirm through Koch's postulates the isolates pathogenicity. Mycological analysis of symptomatic leaves showed the presence of twelve genera of filamentous fungi. Among 116 isolates, *Colletotrichum* spp. was the most prevalent genus followed by *Nigrospora* spp. and *Septoria* spp. In the pathogenicity assays, 22 out of 116 isolates were able to comply with the Koch's postulates. The pathogenic isolates were included into three genera *Alternaria* spp., *Colletotrichum* spp. and *Septoria* spp. This study provides the first report that demonstrates pathogenicity of fungal isolates on *C. bonariensis* in Argentina and represents the first step in a future biocontrol program.

Keywords: *Conyza bonariensis*, foliar, disease, fungal pathogens

1. Introduction

Over the past 25 years Argentina has adopted a productive model based on no-tillage cultivation with scarce or no crops rotation, herbicide overuse with glyphosate predominance and mainly lack of awareness, which has led to weed shift towards difficult to control species and evolution of herbicides resistance (Papa & Tuesca, 2014a). Thus, the number of herbicide-resistant weeds increased markedly in recent years in Argentina, with a rate of 4 biotypes per year with a total of 30 resistant biotypes already confirmed (Acciaresi et al., 2017).

Conyza bonariensis L. Cronquist (Asteraceae) is an annual herb native to South America with abundant presence in Argentina, Uruguay, Paraguay and Brazil (Kissmann & Groth, 1991). This is an annual species that is multiplied by seeds, which germinate mainly in autumn and winter, although a small percentage of seeds are capable of germinating during spring. *Conyza bonariensis* complete their cycle in spring-summer being a cosmopolitan weed present in pastures, annual winter crops, fallows and summer crops, mainly soyabean under reduced and no-tillage system (Leguizamón, 2011). In recent years, this species has appeared in the Argentinean pampas region as an important weed and it is becoming difficult to control using current technology. Furthermore, populations of *C. bonariensis* have been confirmed recently as resistant to glyphosate (Puricelli, Faccinni, & Metzler, 2015). In Argentina several herbicides mixes and sequential applications (double-knock) provide good seedling control, but herbicide performance depends largely on weed size, age, density and growing conditions at spraying. Correct timing of herbicide application is essential for effective *C. bonariensis*

control. It is crucial to apply herbicides when the plant is a small rosette, preferably of 5 cm in diameter or smaller, and definitely prior to stem elongation until 15 cm, as control efficacy declined as plants mature (Metzler, Puricelli, & Papa, 2013; Papa, Tuesca, & Nisensohn, 2010). However, under the current production model more than 60% of the agricultural area in Argentina is under rental with very short term contracts. As a result there is a late start of activities and weeds control is often carried out late to be treated effectively with normal doses of herbicides available. *Conyza bonariensis* is characterized as extremely aggressive weed in soybean and can cause 50% or more yield losses if left uncontrolled (Papa & Tuesca, 2014b). Integrated Weed Management (IWM) is defined as the integration of different control methods to provide the crop with an advantage over the weeds (Lamichhane et al., 2017). It is practiced worldwide with different adoption levels and is intended to restrict weed populations to manageable levels, to reduce the environmental impact of individual practices, to increase the sustainability of cropping systems and to reduce selection pressure for weed resistance to herbicides. However, published articles on chemical control since 1995 are the most numerous compared to any other method of weed control (Harker & O'Donovan, 2013).

Considering the general trend to reduce the entry of synthetic pesticides in the agroecosystem, it is necessary to search biological alternatives for example through the use of bioherbicides considered to be a type of inundative biological control. This biological weed control is relevant to the needs of agriculture and turf management, as it can be implemented through the application of inoculum as liquid sprays or solid granules in a similar way to conventional herbicides (Auld, Hetherington, & Smith, 2003). On the other hand, phytopathogenic fungi can be used as producers of bioactive molecules with herbicidal activity (Castro de Souza et al., 2017). Many steps are involved in the implementation of a biological control program but it always includes surveys aimed at identifying the natural enemies associated with the plant in its centre of origin and often also comprises surveys in the region where introductions will be made (Duarte, Santos, & Barreto, 2016). However, no systematic surveys have been carried out on *C. bonariensis* for suitable agents as it was suggested by Scott et al. (2016). Based on this context, the aims of the present study were: i) to detect fungal disease associated to *C. bonariensis*; ii) to identify fungal isolates from the symptomatic leaves; iii) to confirm through Koch's postulates the isolates pathogenicity.

2. Method

2.1 Sampling Sites and Leaves Symptoms Analysis

Samples were collected during the periods 2015 and 2016 growing season in experimental fields at the Universidad Nacional de Río Cuarto (33°06′31.6″ S; 64°18′01.3″ W) (Río Cuarto, Córdoba). Within this area survey sites were arbitrarily selected according the occurrence of weed populations in the production crops, pastures areas, etc. Whenever diseased plants were found, representative parts bearing symptoms were taken and referenced. In the laboratory, infected plant tissues were observed under a magnifying glass (Motic, model DM-39-N9GO) to observe signs, *i.e.* the presence of fungal structures such as fruiting bodies, conidiophores, sclerotia, etc., establishing associations between signs and disease symptoms. Symptoms on leaves were classified as: central, apical or lateral blight and/or leaf spots. Symptoms were rated using a 0-4 scale according to the approximate leaf blight severity area: 0 = 0%, 1 = 1-10%, 2 = 11-25%, 3 = 26-40%, 4 ≥ 40% (Figure 1). To calculate the percentage of affected leaf an ImageJ 1.51 program was used (Schneider, Rasband, & Eliceiri, 2012).

Figure 1. Ordinal scale according to the approximate leaf blight severity area: 0 = 0%, 1 = 1-10%, 2 = 11-25%, 3 = 26-40%, 4 = 40%

2.2 Fungal Isolation and Identification

The symptomatic leaves were surface disinfected for 1 minute in ethanol 75%, then, for 1 min in 3% sodium hypochlorite (NaOCl) solution, rinsed three times in sterile distilled water and blotted dry on sterile paper towels in laminar flow chamber for 10 min (Agrios, 2005). The fungal isolation was carried out using two methods. On the one hand, symptomatic leaves were placed in moist chambers to allow the pathogen to grow out on the tissue. Then, the isolation in pure cultures were attempted by the direct transfer of spores or other fungal structures using a sterile fine pointed needle onto plates containing potato dextrose agar (PDA) acidified with 25% lactic to pH 5 acid amended with streptomycin (100 mg L^{-1}) (Pioli, Gattuso, Prado, & Borghi, 1997). Incubation was carried out at 25±1 °C and complete darkness for five to seven days. In the second isolation method, small sections of approximately 10 mm square were cut from the margin of the infected lesion so that they contain both disease and healthy-looking tissue (Agrios, 2005) and transferred aseptically to Petri dishes containing PDA acidified with lactic acid 25% amended with streptomycin (100 mg L^{-1}). The plates were incubated in complete darkness for at least 10 days at 25±1 °C. Single-spored cultures from colonies were transferred to PDA and were further identified based on macromorphology and micromorphology according to Barnett and Hunter (1998).

2.3 Pathogenicity Assays

Inoculum production was prepared by growing each isolate on PDA medium and incubated for at least 15 days at 25±1 °C. After this period, conidia were collected by flooding the Petri dish with sterile water and washing them off the media surface. The conidial suspension was filtered through three layers of cheesecloth to reduce the amount of mycelial or other structures. The filtered conidial concentration was determined in a Neubauer chamber (Marienfeld-Superior, Germany) under an optical microscope (Carls Zeiss; Model Primo Star Trinocular) and diluted to obtain 10^7 conidia/mL. Seedlings of *C. bonariensis* were inoculated at rosette stage (approximately 5 cm in diameter) by brushstroke with a suspension of approximately 10^5 conidia/mL supplemented with corn oil to final concentration of 0.01% for better adherence of inoculum to the leaf tissue. Inoculation treatment consisted of a total of six plants by isolate and every experiment was repeated twice in the independent way. For each isolate were utilized two injured plants inoculated with conidia, two plants without injury inoculated with conidia and two plants without injury inoculated with sterile water as negative controls. After inoculation, the plants were placed in a humidity chamber for 72 hs at 25 °C and 90% RH, and then transferred to a greenhouse maintained approximately at 25 °C. After 14 days, plants were removed by hand and transported to the laboratory for more accurate and detailed studies. Leaves were visually assessed for blight severity and the symptomatic leaves were transferred to Petri dishes containing PDA acidified whit lactic acid 25% amended with streptomycin (100 mg L^{-1}) and identified by morphology according to Barnett and Hunter (1998) to complete Koch`s postulates.

3. Results

In most cases where the diseased plants of *C. bonariensis* were detected, the symptoms were observed on living leaves. The most common necrotic symptom on leaves was blight with variable sizes and position showing tissue from dark brown to black and less regularly bluish purple color with yellow-green center or necrotic spots (Figures 2A and 2B). The lesion edges were smooth or irregular, with or without a chlorotic periphery, sometimes thin to more conspicuous. In some cases, signs were observed on the leaf surface with the presence of fruiting bodies (Figures 2C and 2D). A total of 344 symptomatic leaves were analyzed, in which the most common lesions were apical and lateral blight showing frequencies about 49% (n = 168) and 27% (n = 92) respectively. The leaves with symptoms representing central blight and leaf spot showed lower frequency, 22% (n = 75) and 3% (n = 9) respectively. Respect to leaf blight severity, lesions grade 2 (up to 25% of affected leaf area) were the most frequent in the 40% of analyzed leaves, followed by lesions grade 3, 4 and 1 with 23%, 19% and 18%, respectively. The mean disease severity among the total leaves evaluated was around 26.13%.
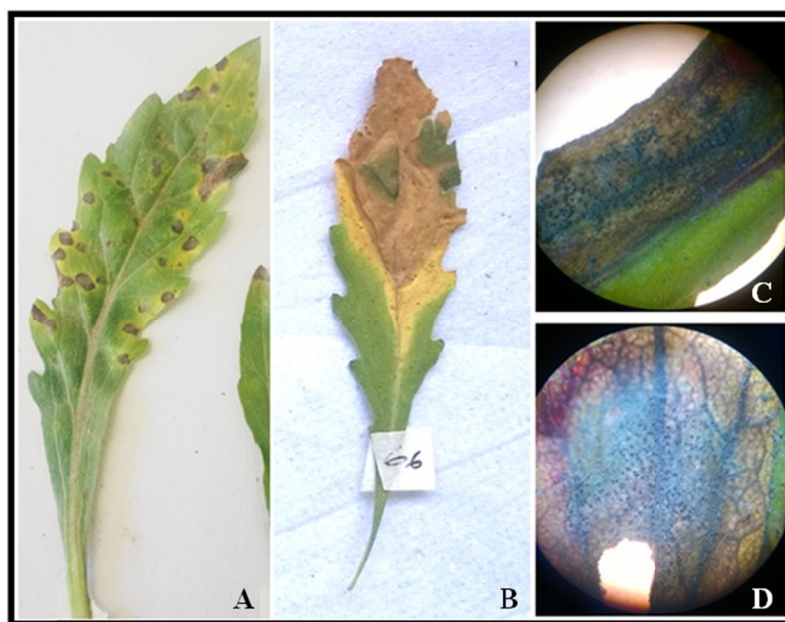

Figure 2. Leaf spots (A) and blight (B) on *Conyza bonariensis*. Presence of fruiting bodies (Pycnidia) of *Septoria* spp. (C, D)

Mycological analysis of symptomatic leaves showed the presence of twelve genera of filamentous fungi. Among the 116 isolates, *Colletotrichum* spp. was the most prevalent genus with 30.2% of isolates followed by *Nigrospora* spp. and *Septoria* spp. account the 15.5% and 13.8% of isolates respectively (Figure 3).
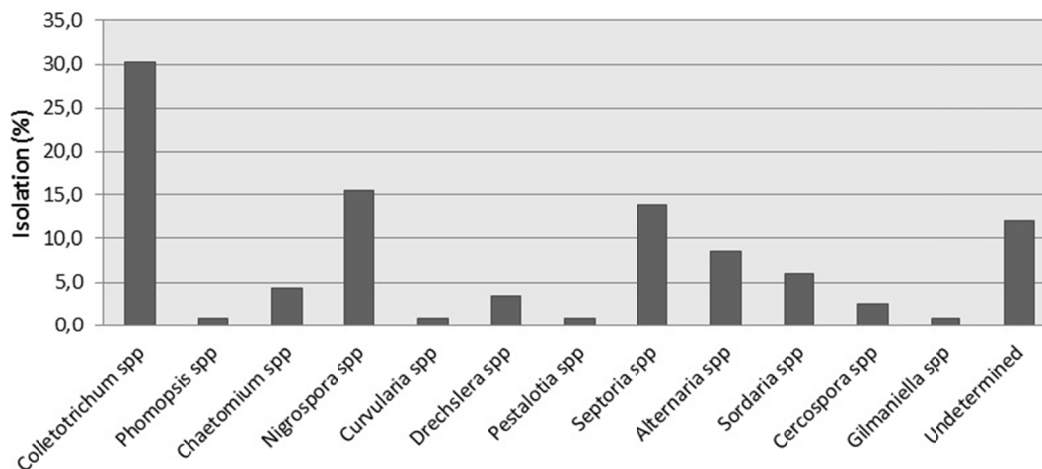
Figure 3. Isolation frequency of fungal genera isolated from symptomatic leaves of *Conyza bonariensis*

Not all isolates belonging to twelve genera recovered from symptomatic leaves were able to develop diseases at leaf level. In the pathogenicity assays, 22 out of 116 isolates (19%) were able to comply with the Koch's postulates. The pathogenic isolates were included into three genera *Alternaria* spp., *Colletotrichum* spp. and *Septoria* spp. Two isolates belonging to *Alternaria* genus were pathogenic for *C. bonariensis* seedling. Lesions were characterized by necrotic lesions with smooth or irregular edges, brown in color with or without a chlorotic periphery (Figures 4A, 4B, and 4C). The visualization time of lesions was 4 to 7 days after inoculation and the lesion size grew until, in some cases, necrotizing all foliar surface. Similar visualization time of lesions was observed in pathogenic *Colletotrichum* isolates. In this case, necrotic lesions were irregular, dark brown more frequently located in the leaf margins (lateral or apical) (Figures 4D, 4E, and 4F). Thirteen out of 35 *Colletotrichum* isolates were able to caused symptoms in the pathogenicity test only in injured leaves and only one isolate induced the seedlings death. Regarding the pathogenic *Septoria* isolates, the visualization time of the lesions was significantly lower than those observed in the pathogenic *Alternaria* and *Colletotrichum* isolates (24 to 48 hs after inoculation). Lesions on leaves were concentric, circular, brown color with edges smooth and defined (Figures 4G, 4H, and 4I). In some cases conidiomata pycnidial were observed adaxially. Although all pathogenic *Septoria* isolates (8 strains) showed varying levels of severity, they were all able to develop symptoms on both damaged and healthy leaves. On other hand, all isolates belonging to the genera such as *Cercospora* spp., *Chaetomium* spp., *Curvularia* spp., *Drechslera* spp., *Gilmaniella* spp., *Nigrospora* spp., *Pestalotia* spp., *Phomopsis* spp., *Sordaria* spp. and the undetermined isolates did not comply with the Koch's postulates and they were not able to develop symptoms even when leaves were injured.
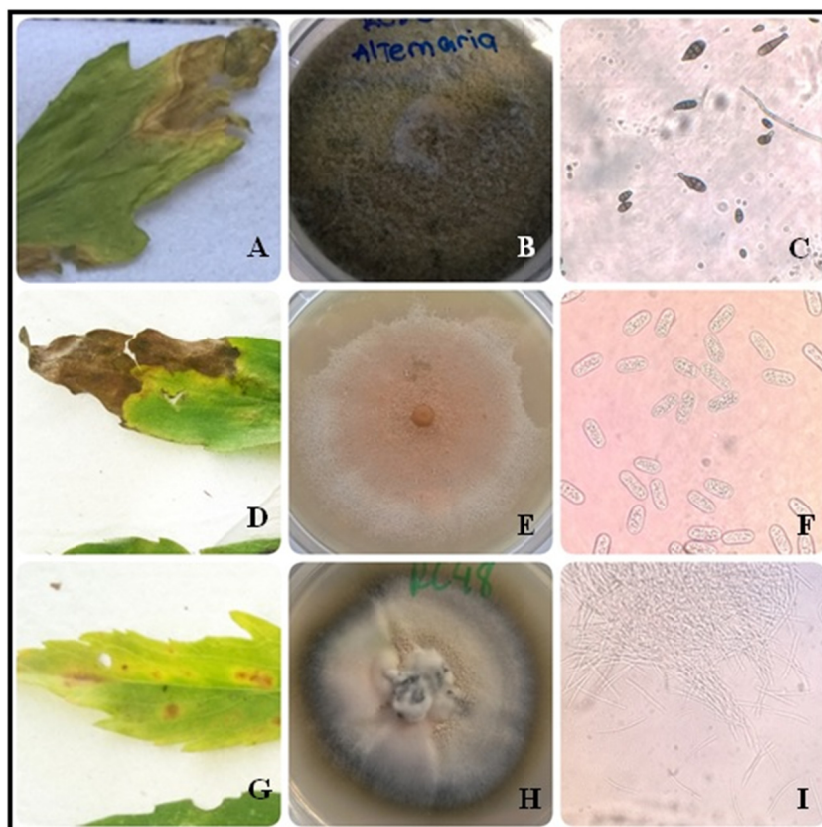
Figure 4. *Alternaria* spp. on *Conyza bonariensis* A) Leaf necrosis B) Colony on PDA C) Conidia.
Colletotrichum spp. on *Conyza bonariensis* D) Necrotic lesions E) Colony on PDA F) Conidia. *Septoria* spp. on
Conyza bonariensis G) Leaf spots H) Colony on PDA I) Conidia

4. Discussion

Conyza bonariensis has become difficult to control weed in Argentinean pampas region favored by absence or lack of weed scouting, lack of crop rotation or soyabean monoculture and the use of herbicides as main management strategy (Papa & Tuesca, 2014a). From a perspective of sustainability it is necessary to develop alternative controls that allow reducing the synthetic herbicide dependence. In this sense, the biological control through the use of a mycoherbicide could be a good option within the framework of an integrated weed management program to increase the weed control efficiency.

A first step in developing a mycoherbicide is the search for fungal pathogens that can cause disease in target weeds. To date, several studies have investigated the mycobiota associated to several *Conyza* species (Duarte et al., 2016; Liu et al., 2012; Pirnia et al., 2012; Caretta et al., 1999; Roy et al., 1994; Lindquist, 1982). However, few researches performed pathogenicity assays to confirm the pathogenic effect of the isolates detected in the weed species (Liu et al., 2012).

In this work, an 81% of the isolates recovered from symptomatic leaves don't comply with Koch's postulates. These isolates could be considered saprophytic fungi that do not cause direct damage to weed such as some *Alternaria* spp., *Phoma* spp., *Nigrospora* spp., *Chaetomium* spp. and *Curvularia* spp. isolates (Caretta et al., 1999) or secondary colonizers of leaves damaged by other fungi or by insects. In Argentina, soybean often is used in rotation with wheat and other cereal crops in a reduced or no-till system. So soybean pathogen such as *Phomopsis* spp. or *Cercospora* spp. isolated in this work from *C. bonariensis*, can use this weed as an alternative host and to provide an inoculum source for crop infections in subsequent year (Ferreira da Silva et al., 2011).

In the present work, 22 isolates belonging to three genera were pathogens for *C. bonariensis* and fulfilling with Koch's postulates; therefore these isolates could be considered potential candidates to be included in a biocontrol program. *Alternaria* is a very ubiquitous genus in the environment considered as a common primary colonizer of leaves in a variety of plants. However, some species affect leaves, stems, flowers and fruits of annual plants,

vegetables, ornamental plants and fruit trees as citric and apple tree (Agrios, 2005). Previous studies demonstrated saprophytic associations between *Alternaria* and *Conyza* species (Caretta et al., 1999; Roy et al., 1994) or related the fungal presence to lesion on leaves (Duarte et al., 2016). In this study, 2 out 10 *Alternaria* isolates were able to develop symptoms in *C. bonariensis* and were subsequently isolated from all lesions. Until now, there is only one worldwide registered bioherbicide called Smoulder® based on *A. destruens* L. Simmons strain 059. This fungus is parasitic on cuscuta's species and can be apply in crops as alfalfa, carrot, tomato and ornamental plants in USA (Bailey, 2014).

The *Colletotrichum* genus was the most frequently isolated from symptomatic leaves of *C. bonariensis*. Similar results were obtained by Roy et al. (1994), and Formento (2013), who demonstrated the presence of *Colletotrichum* or *Glomerella* isolates from foliar lesions of *Conyza* spp. The lesions were similar to those observed in *Alternaria* isolates; however in the pathogenicity tests a 37% of the isolates were able to develop symptoms and could be recovered from affected tissues. Numerous researches showed association between *Colletotrichum* species and weed biocontrol and some of them have been registered such as Collego®/ LockDown®, *C. gloeosporioides f. sp. aeschynomene* ATCC 20358 utilized to control *Aeschynomene virginica* in rice in USA (Bowers, 1986) and Biomal®, *Colletotrichum gloeosporioides f. sp. malvae* ATCC 20767 used to control broadleaf weeds in the family Malvaceae *Malva* in Canadá (Boyetchko, Bailey, Hynes, & Peng, 2007).

The third genus that demonstrated pathogenicity in *C. bonariensis* was represented by isolates belonging to *Septoria* spp. In this case, all isolates recovered from symptomatic leaves reproduced the disease in pathogenicity tests showing similar aggressiveness. Previous works worldwide have detected diseases in *Conyza* related to the presence of *Septoria* species (Duarte et al., 2016; Erper et al., 2010; Priest, 2006; Fatehi et al., 1993). On the other hand, several investigations have reported the use of different *Septoria* species as biocontrol agents such as *S. cirsii* in *Cirsium arvense*, a weed of Asteraceas's family (Leth, 1985), *S. polygonorum* proposed against species of *Polygonum* (Mitchell, 2003) and *S. urticae* proposed against *Urtica urens* L. (Bello, Perelló, & Monaco, 1993).

Taking into account that isolates from three genera pathogenic to *C. bonariensis* have been used as bioherbicides worldwide, such strains could be considered potential candidates as weed biocontrol agents. On the other hand, we know that some *Septoria* species and others belonging to the *Colletotrichum* species complex include pathogens in several crops such as soyabean, maize, wheat, sorghum and grasses (Agrios, 2005). These pathogens can survive in the host absence through the use of alternative hosts as weeds or living as saprophytic on crop residues. For this reason, before including a strain into a biocontrol program of *C. bonariensis* it is necessary to evaluate the environmental risk of the potential bioherbicide using scientific criteria through the evaluation of host specificity, crop tolerance, environmental fate and toxicology. Changes in the public's attitude on the acceptance of synthetic herbicides (and all pesticides) and the introduction of government policies for pesticide reduction, represent an opportunity for the development of new weed control technologies that have reduced risks and are suitable for organic food production (Bailey, 2014). Thus, although only a limited number of isolates were evaluated in the present study, this is the first report that demonstrates pathogenicity of fungal isolates on *C. bonariensis* in Argentina and represents the first step in a future biocontrol program into integrated weed management.

Acknowledgements

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