Fungi pathogenic on wild radish (*Raphanus raphanistrum* L.) in northern Tunisia as potential biocontrol agents

Naceur DJÉBALI[1], John K. SCOTT[2], Mireille JOURDAN[3] and Thouraya SOUISSI[4]

[1]Centre de Biotechnologie à la Technopole de Borj Cedria, BP. 901 Hammam-Lif 2050, Tunisia
[2]CRC for Australian Weed Management and CSIRO Entomology, Private Bag 5, PO Wembley WA 6913, Australia
[3]CSIRO European Laboratory, Campus International de Baillarguet, 34980 Montferrier-sur-Lez, France
[4]Institut National Agronomique de Tunisie, 43 Avenue Charles Nicolle, 1082 Tunis, Tunisia

Summary. The distribution and life cycle of wild radish (*Raphanus raphanistrum* L.) and a survey of the pathogens of this plant are reported for the northern regions of Tunisia. Wild radish is a common weed of cereal crops and legumes. It germinates in early autumn (October), develops a rosette stage in November to December after which stem growth, flowering and pod production occur through to May, with pod maturity completed in June. Fungus isolation from the foliar tissues exhibiting disease symptoms showed that wild radish was infected with the fungi *Albugo candida, Alternaria* spp. including *A. brassicicola,* and *A. raphani, Erysiphe cruciferarum, Stemphylium herbarum, Peronospora parasitica* and *Phoma lingam. Ascochyta* spp.*, Cercospora armoraciae, Cladosporium cladosporioides* and *Colletotrichum higginsianum* are here reported from wild radish for the first time. Inoculation tests of pathogens on wild radish plants showed that the most injurious fungi were *Alternaria raphani* and *Phoma lingam*. The remaining pathogens were weakly to moderately aggressive on this weed. To access the pathogenic effect of fungi spontaneously infecting natural populations of wild radish, the weed was grown in a field experiment with and without the broad-spectrum systemic fungicide Carbendazim. Results showed a statistically significant two-fold decrease in the number and weight of seed pods in the non-treated plants, indicating that the reproductive potential of wild radish was naturally reduced by fungal infection. Foliar pathogenic fungi have a potential in the integrated weed management of wild radish, this role merits further investigations.

Key words: biological control, Brassicaceae, life cycle, phytopathogen, weed.

Introduction

Wild radish (*Raphanus raphanistrum* L.) is a widespread but little studied weed in North Africa (Le Floc'h *et al.*, 1990), particularly in Tunisia, where it is common in cultivated crops. Most studies on the ecology and management of this weed in regions with a Mediterranean climate come from southern Australia, where the plant occurs frequently in cereal and grain legume crops (Cheam, 1986). The persistent seed bank, competitive annual growth habit and high fecundity of wild radish contribute to its weedy nature and ensure that it is likely to be a continuing problem, especially in winter rain-fed crops (Nugent, 1999). Wild radish competes successfully with wheat (*Triticum aestivum*), canola (*Brassica napus*) and lupine (*Lupinus angustifolius* L.). A wild radish density of 16 plants m^{-2} caused a nearly 25% yield loss in a wheat crop in Turkey in 1998 (Boz, 2005). With canola, a wild radish density of 64 plants m^{-2} reduced yield by 77 to 91% (Blackshaw *et al.*, 2002). Competition from 3 to 24

Corresponding author: N. Djébali
Fax: +216 79 412638
E-mail: dnaceur@yahoo.fr

wild radish plants m^{-2} reduced lupine yield by 27 to 66% (Pathan *et al.*, 2005). These authors also stated that contamination of canola grain with wild radish seed reduced canola oil quality by increasing the amount of erucic acid and glucosinolates above marketable levels.

Cultural and chemical control of this weed has received major interest, but has met with only limited success, especially in canola and other cruciferous crops. The dormancy and longevity of wild radish seed enables it to survive as long as six years under continuous cropping systems such as a wheat-lupine rotation (Hashem, 2006). Wild radish has developed multiple-herbicide resistance across at least four modes of action in Australia (Hashem *et al.*, 2001; Walsh *et al.*, 2001; Walsh *et al.*, 2004), making herbicidal control particularly difficult. To date, herbicide-resistant wild radish has not been recorded from Tunisia.

The relative ineffectiveness of chemical control has prompted investigations into integrated weed management (IWM) systems for wild radish. These systems combine the benefits of cultural, mechanical, chemical and biological control techniques. The IWM approach is underpinned by the premise that successful long-term control requires a clear understanding of the biology and ecology of the weed to be controlled. In addition, the use of biological control agents in an IWM system, especially phytopathogenic fungi, offers a promising way to control weeds. Several formulations based on fungi such as DeVine®, Collego® and Casst®, against respectively *Morrenia odorata*, *Aeschynomene virginica* and *Cassia obtusifolia* have been registered as bioherbicides in Canada, South Africa, and the United States (Charudattan and Dinoor, 2000).

Wild radish has been reported as a host of at least 18 pathogenic fungi (Farr *et al.*, 2007), but the use of these fungi for the biological control of wild radish has not been studied previously in Tunisia. A research project to assess the pathogenic fungi of wild radish for the biological control of this weed was therefore started in order to develop a mycoherbicide. The first steps in this project were to determine the distribution and life cycle of wild radish in northern Tunisia, to survey the pathogenic fungi that infect wild radish, and to assess the effect of these fungi on plant growth and pod production under field conditions.

Materials and methods

Distribution of wild radish and field sampling

Wild radish was studied in northern Tunisia, which has a sub-humid to semi-arid Mediterranean-type climate. Wild radish populations, including diseased plants were sampled in roadside and field surveys conducted in October 2001 to May 2002. Where symptoms suggestive of fungal infection were observed, at least three plants per symptom type were collected. Samples of diseased material were placed between filter paper (Whatman 3MM) at room temperature to dry for subsequent microbiological examination.

Fungal isolations

Symptomatic stem and leaf samples were washed with sterile distilled water, placed on filter paper soaked with sterilized distilled water and incubated at 20°C with a 12 h day (100 μE) and 12 h dark for 24 to 72 hours. Pure cultures of each isolate were obtained by transferring single conidia with a sterile needle to sterilized slides covered with a thin layer of 2% water agar (Sigma). Slides were incubated in a moist chamber under the above conditions. After spore germination, pieces of water agar containing conidia were excised using a sterile scalpel and transferred to potato dextrose agar (PDA) plates (Sigma Chemical Co., St. Louis, MO). Seeds of wild radish were surface-sterilized by immersing them in a 3% sodium hypochlorite solution for 5 min, then rinsed 4 times with sterile distilled water, dried on sterile filter paper and placed on PDA medium at 20°C for isolation of the fungi.

Morphological identification of the fungi (Anonymous, 1976; Agrios, 1988; Messiaen *et al.*, 1991) was based on microscopic (Olympus BX41, Japan) inspection of the conidial length, width, shape and color of fungi after mounting in clear lactophenol. At least 25 conidia per isolate were measured. For *Stemphylium herbarum, Cercospora armoraciae*, *Cladosporium cladosporioides* and *Colletotrichum higginsianum*, pure cultures were sent to CABI (http://www.cabi.org/) for identification.

Plant material

Wild radish pods were collected in Hammam Saïala in northern Tunisia (Fig. 1). Seeds were ex-

tracted from the pods and germinated in the dark at 20°C on wet filter paper with 600 ppm of gibberelic acid (Sigma). Germinated seeds were planted in an autoclaved (30 min at 121°C) clay soil-compost mixture (25:75 %) contained in 770 cm^3 pots. Plants were grown in a growth chamber at 20°C with a 12 h day (100 μE) followed by a 12 h dark period and were irrigated twice weekly with 0.25 g L^{-1} nutrient solution (AGLOSPEED: 18 units (U) of nitrogen, 18 U P$_2$O$_5$, 18 U K$_2$O).

Pathogenicity experiment

To prepare fungal inocula, cultures were grown on PDA at 20°C with a 12 h day (100 μE) followed by a 12 h dark period. A conidial suspension was prepared by flooding the plates with sterile distilled water containing 0.05% Tween 80 (Sigma) and manually disrupting the cultures. The resulting suspension was adjusted to 10^6 conidia mL^{-1}. Plants at the 5 to 11 leaf stage were inoculated by depositing the conidial suspension on the leaf surfaces using a sterile brush. The biotrophic pathogens (*Erysiphe cruciferarum*, *Albugo candida* and *Peronospora parasitica*), were inoculated by wiping a freshly collected infected leaf onto the plants. For the first two days after inoculation, the plants were kept in the dark and at humidity near saturation to stimulate the infection. Plants were then transferred to a growth chamber at 20°C, 80% humidity and a 12 h day (100 μE) followed by a 12 h dark period. Fungal pathogenicity tests were made on wild radish plants according to Koch's postulates (Agrios, 1988). Each isolated fungus was inoculated on three wild radish plants. Disease severity of the fungi (IL, infection level) was calculated as a percentage of inoculated leaves that were infected and scored as (-), no disease (0% IL), (+), 1–50% IL, (++), 51–75% IL, (+++), 76–100% IL. Symptoms were recorded 15 days after inoculation.

Field experiment

A field trial was carried out to study the life cycle of wild radish and to determine the effect of naturally occurring fungi on wild radish growth and pod production in natural conditions. The experiment was conducted during 2001–2002 at the National Institute of Agronomy in Tunis (Fig. 1). The soil was slightly alkaline (pH 7.8) calcareous clay with 1.4% of organic matter. Wild radish

seeds were pre-germinated as described above and 15 seedlings (2 to 3 true-leaf stage) were planted per 0.5 m^{-2} in the field, in a randomized complete block design consisting of 4 replicates per treatment. To prevent fungal development, the plants were sprayed with the systemic fungicide Carbendazim (0.8 g L^{-1}) every 21 days corresponding to the withholding period of the product. Control plants received no Carbendazim. To evaluate the effect of spontaneous fungal infection on wild radish growth and pod production, plant density, plant height, dry matter, number of pods per m^2 and the weight of 100 pods were determined. All parameters were recorded after 30 weeks of growth of plants in the field.

Sum of effective temperature and threshold temperature of wild radish

In the field experiment, the "Sum of effective temperature" (SET) of wild radish was calculated according to the daily measured temperature and the threshold temperature of wild radish, following the basic formula (Miller *et al*., 2001): where *Ti* max and *Ti* min are respectively the maximum and the minimum temperatures (°C) of day i, *BZ* is the biological zero (temperature below which no plant growth occurs) of wild radish which is 5°C (Cheam, 1996), and *n* is the number of days needed for each development stage to be traversed.

Statistical analysis

The field experimental data were subjected to analysis of variance and comparison of means using STATISTICA software version 5.1H (StatSoft, France). Mean values of each treatment were compared using the LSD test at 5% probability.

Results

Distribution of wild radish

Wild radish was found throughout northern Tunisia (Fig. 1) with 64% of infested fields located in the north-east. The weed was associated with several crops especially cereals (wheat and barley) and grain legumes (chickpea and bean) which represented 48 and 24% of the sites respectively. The remaining infested sites (28%) were located in orchards (citrus, peach), vegetable crops (potato) and pastures (grass).
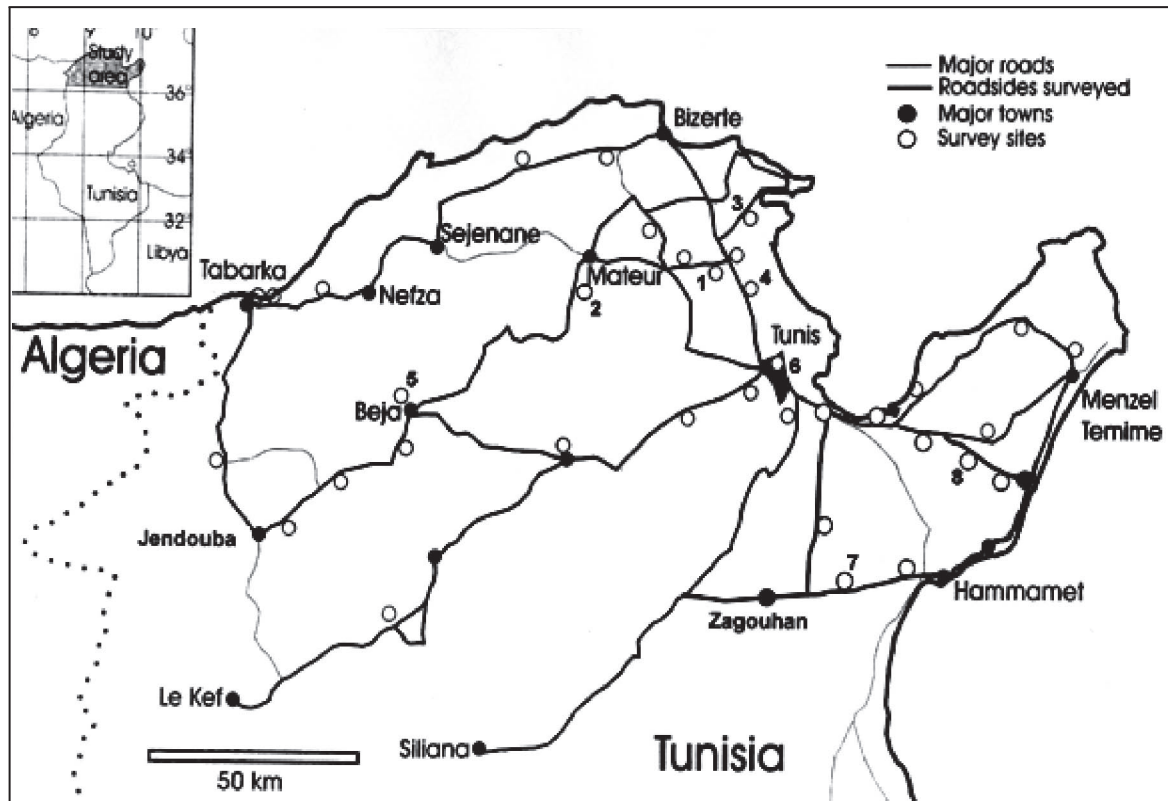
Fig. 1. Location of wild radish (*Raphanus raphanistrum*) sites in northern Tunisia during 2001–2002. Sites (crops) from which samples of diseased wild radish were collected were: (1) Besbessia (bean and orchard of peach), (2) Joumine (pastures), (3) Utique (wheat), (4) Sabbelet Ben Ammar (chickpea), (5) Hammam Saïala (wheat and bean), (6) Tunis (roadside), (7) Zagouwan (wheat), (8) Beni Khalled (barley and orchard of citrus).

Pathogens naturally occurring on wild radish

Three biotrophic, eight necrotrophic and one hemibiotrophic pathogen species of potentially pathogenic fungi were isolated from naturally infected wild radish in field conditions in northern Tunisia (Table 1).

The field surveys showed that downy mildew caused by *P. parasitica*, occurred from the seedling stage to plant maturity. It was the first pathogen to produce disease symptoms on wild radish; it occurred in winter and was not found on neighbouring Brassicaceae plants. White rust infection caused by *A. candida* appeared just after those of downy mildew, but it caused less damage to the plants. White pustules of sporangia of this oomycete were generally located on the leaves and stems and occasionally on the inflorescences and pods. *E. cru-*

ciferarum appeared later than *P. parasitica* and *A. candida*. *E. cruciferarum* caused severe infections on the vegetative and reproductive parts of wild radish plants.

Two *Alternaria* species commonly isolated from wild radish leaves were identified by their conidium size: *Alternaria brassicicola* (55 μm × 15 μm) and *A. raphani* (115 μm × 35 μm). An unidentified *Alternaria* spp. (100 μm × 10 μm) with intermediate-sized conidia was isolated from the seeds. Inoculation tests showed that *A. raphani* isolates were more pathogenic on wild radish than the other two *Alternaria* species. *S. herbarum*, *C. armoraciae*, *C. cladosporioides*, an unidentified *Ascochyta* species and *C. higginsianum* isolated from the vegetative parts of wild radish caused slight damage to wild radish plants when inocu-

lated *in vitro*. These fungal species were isolated from one locality each. *Phoma lingam* isolates found in three localities of north-eastern Tunisia were highly pathogenic, causing severe lesions on leaves and stems, and resulting in the death of wild radish plants in the growth chamber.

Pathogenicity tests of the isolated fungi species (Table 1) showed that the most injurious pathogens on wild radish in growth chamber conditions were *Alternaria raphani* and *Phoma lingam*. These infected more than 75% of all inoculated wild radish leaves and led to plant death. *P. parasitica* and *E. cruciferarum* caused moderately severe infection. The remaining fungi were weakly aggressive on the vegetative part of wild radish, infecting less than 50% of the leaves.

Life cycle of wild radish and its sensitivity to naturally occurring pathogenic fungi

Wild radish emerged just after the autumn

Table 1. Pathogenicity test of fungi isolated from wild radish (*Raphanus raphanistrum*) in eight sites of northern Tunisia during 2001–2002.

Fungus[a]		Organ sampled	Infected leaves (%, ±SD)	Infection level (IL)[b] and origin of isolates[c]
Peronospora parasitica (Pers.) ex Fr.	(bf)	Leaf and stem	53.1 (±3.4)	++ (1, 5, 6, 7)
Albugo candida (Pers. ex. Chev.) Kuntze	(bf)	Leaf and stem	41.5 (±7.1)	+ (1, 3, 4, 5, 6, 7, 8)
Erysiphe cruciferarum Opiz ex Junell	(bf)	Leaf and stem	60.7 (±7.6)	++ (1, 2, 5, 6)
Alternaria sp.		Leaf and stem	0.0	- (1)
Alternaria brassicicola (Schw.) Wiltshire	(nf)	Leaf, stem and seed	39.9 (±11.8)	+ (1, 5, 6)
Stemphylium sp.		Leaf and stem	0.0	- (1)
Alternaria sp.		Leaf and stem	0.0	- (1)
Fusarium sp.		Leaf and stem	0.0	- (1)
Alternaria sp.		Seed	0.0	- (5)
Stemphylium sp.		Seed	0.0	- (5)
Alternaria sp. (nf)		Seed	37.5 (±0)	+ (5)
Alternaria sp.		Seed	0.0	- (5)
Alternaria sp.		Seed	0.0	- (5)
Aspergillus sp.		Seed	0.0	- (5)
Alternaria sp.		Seed	0.0	- (5)
Stemphylium herbarum E.G. Simmons	(nf)	Leaf	10.3 (±4.1)	+ (6)
Stemphylium sp.		Leaf	0.0	- (6)
Fusarium sp.		Leaf	0.0	- (6)
Cercospora armoraciae Sacc.	(nf)	Leaf	25.4 (±0)	+ (6)
Alternaria raphani J.W. Groves & Skolko	(nf)	Leaf	85.7 (±3)	+++ (6)
Cladosporium cladosporioides (Fresen.)	(nf)	Leaf	40.3 (±10.8)	+ (6)
Ascochyta sp.	(nf)	Leaf	28.6 (±0)	+ (6)
Phoma lingam (Tode ex Fr.) Desm.	(nf)	Leaf	81.4 (±9.4)	+++ (6, 7, 8)
Colletotrichum higginsianum Sacc.	(hf)	Leaf and stem	31.3 (±4.4)	+ (2)

[a] bf, biotrophic fungus; nf, necrotrophic fungus; hf, hemibiotrophic fungus.
[b] -, no disease (0% IL); +, 1–50% IL; ++, 51–75% IL; +++, 76–100% IL.
[c] The numbers refer to the origin of isolates as shown in Fig. 1.

Table 2. Growth and pod production of wild radish (*Raphanus raphanistrum*) in field conditions with and without Carbendazim fungicide treatment.

Treatment	Final No. of plants m⁻²	Plant height (cm)	Plant dry matter (g m⁻²)	No. of pod m⁻²	Weight of 100 pods (g)
Carbendazim	29 (±1) a [a]	79.0 (±20.8) a	1525.0 (±139.5) a	1073.5 (±159.9) a	15.0 (±0.8) a
Unsprayed control	27 (±2.4) a	88.3 (±27.9) a	1312.5 (±112.5) a	479.0 (±205.3) b	7.4 (±2.8) b

[a] Means (± standard deviation) with the same letter are not significantly different at $P \leq 5\%$.

rains (beginning of October). The flat rosette stage was observed in November and December after accumulating about 472°C (SET=472°C). The first flowers appeared after SET=1501°C with full bloom and first pod formation occurring after SET=1778°C in spring (March to April). Flowering was completed after SET=2152°C (May), with pods maturing at the beginning of June (summer) during which the vegetative part of the plants desiccated. The entire life cycle of wild radish plants was completed after SET=3195°C, corresponding to a period of about 8 months.

Naturally occurring fungal infections had no observable effect on the growth of wild radish in the field assay. Fungicide treated and untreated plants did not differ in their development phenology and did not differ significantly in the plant density, plant height or dry matter per m² (Table 2). There was, however, a significant difference in the number of pods produced per m² and in the weight of 100 pods between Carbendazim-treated and untreated plants. In the plots without Carbendazim, pod production per m² and pod weight were less than half those of the Carbendazim-treated plots (Table 2).

Discussion

Wild radish was widespread in northern Tunisia in the period from October 2001 to May 2002 (Fig. 1), confirming a previous report by Maire (1965). This distribution can be partly explained by the preference of wild radish for a sandy soil, which is abundant in this part of the country, particularly in the north-east (Caréme, 1990). Wild radish infested mainly cereal and grain legume crops as described by Caréme (1990).

In the field experiment conducted in Tunis, wild radish was first observed at the two-cotyledon stage in early October, just after the first autumn rains. The early emergence of this weed as compared with the annual cereal and legume crops may explain its strong competitive ability in the field. Yield loss due to wild radish competition was greater with late than with early sowing of lupine in Australia (Pathan *et al.*, 2006).

Wild radish flowers between March and May (Maire, 1965). The first flowers of the wild radish plants were observed after a SET of 1501°C in our field assay; this was much higher than the SET of 600°C reported by Reeves *et al.* (1981). In our study, wild radish was planted relatively early in the season, which could explain this difference in phenology. Cheam (1986) also stated that early germinating wild radish plants required a longer time to reach complete flowering and pod production.

In northern Tunisia wild radish was infected with several pathogens including two oomycete and ten fungal species (Table 1). *P. parasitica* causing downy mildew was the first pathogen found to infect wild radish plants in the study. Infection of wild radish by this biotrophic oomycete has been reported by other authors (McMeekin, 1969; Dickinson and Greenhalgh, 1977). Field observation showed that *P. parasitica* occurred on wild radish in winter without being found on neighboring Brassicaceae plants. Morris and Knox-Davies (1980) separated the races of *P. parasitica* found on radish (*R. sativus* L.) and cabbage (*B. oleracea* L.) on the basis of host specificity. Thus it would be useful to test the host range of Tunisian *P. parasitica* isolates for specificity to wild radish.

Albugo candida causing white rust infected wild radish at the flat rosette stage. A similar finding had earlier been reported by Biga (1955). Morris

and Knox-Davies (1980) suggested that there was physiological specialization in *A. candida* isolates; therefore they assumed that wild radish was an unimportant weed host of *A. candida* in cultivated fields of *B. oleracea*, but it might be an important source of inoculum in commercial plantings of *R. sativus*.

Erysiphe cruciferarum causing powdery mildew was found to be a late destructive pathogen on wild radish, attacking both the vegetative and the reproductive parts of the plants. This pathogen infects many Brassicaceae species including species from the genus *Raphanus* and *Brassica* (Winter and Gindrat, 1993). Unlike the field surveys, the pathogenicity test showed that *E. cruciferarum* was moderately aggressive to wild radish plants. This difference probably can be explained by the fact that symptoms were recorded 15 days after inoculation, which was not long enough for the fungus to produce any severe infection on plants like wild radish in the field, where severe symptoms were only observed later in the season.

Three species in the genus *Alternaria* were isolated from wild radish, *A. brassicicola*, *A. raphani* and unidentified species. All these species infected *B. napus* and *R. sativus* in controlled conditions (data not shown). The unidentified *Alternaria* sp. was isolated from the seed, so that it is likely to be transmitted when infected wild radish seed gets mixed with commercial Brassicaceae seed. Rude *et al*. (1999) reported that infection of seeds by three species of *Alternaria* was an important factor in reducing seed germination of turnip (*Brassica rapa* L.). Consequently, *Alternaria*-contaminated wild radish seeds are likely to be an important source of inoculum for early infection of cultivated Brassicaceae species.

Phoma lingam (teleomorph *Leptosphaeria maculans*), the causal agent of blackleg disease of Brassicaceae, also infected wild radish naturally in northern Tunisia. Wild radish isolates of *P. lingam* infected *B. napus* and *R. sativus* (data not shown). This indicates that wild radish is an alternate host for blackleg in Brassicaceae cultivated fields (Barbetti, 1978; Nugent, 1999) limiting the application of *P. lingam* as a potential biocontrol option for wild radish.

To our knowledge, this is the first report of infection of wild radish in the field by *Ascochyta* sp., *C. cladosporioides*, *C. armoraciae* and *C. higgin-sianum*. These fungi are very weak and not very wide spread pathogens on wild radish in northern Tunisia. *C. cladosporioides* infects several plant species such as *Phaseolus vulgaris* (Bhattacharjee and Dkhar, 2005) and the weed *Mitracarpus hirtus* (Pereira and Barreto, 2005). *C. higginsianum* and *C. armoraciae* infect cultivated Brassicaceae species such as *Brassica pekinensis* and *Armoracia rusticana* (Babadoost *et al.*, 2001) respectively. However, in a preliminary specificity test the Tunisian isolates did not induce infection 15 days after inoculation on *R. sativus* and *B. napus* in controlled conditions (data not shown).

Spontaneous fungal infection did not significantly decrease the vegetative growth of wild radish in the field. However, the number and weight of pods was reduced by more than half in Carbendazim-untreated wild radish plants. The reduction in pod production by fungal infection can affect the fecundity and proliferation of this weed under natural conditions. Moreover, the reduction in seed production reduces the seed bank and hence field infestation. Fungal infection reduces pod production but not the vegetative growth of Carbendazim-untreated wild radish plants, indicating that the flower structures of wild radish are the most sensitive to fungal attack, so that any biological agent should be preferentially used at the flowering stage. Treatments at this late stage may also reduce competition of wild radish, which is more intense during the reproductive stages than during the vegetative stage on lupine grain yield (Pathan *et al.*, 2006). *Cercospora armoraciae* and *Colletotrichum higginsianum* isolates showed some interesting results in a preliminary specificity test, but their use as biological control agents is not so attractive because of their low agressivity on this weed. In view of its activity in the field and in growth chamber, *P. parasitica* is a potential biological control agent that warrants further investigation for its host specificity to wild radish and effectiveness at controlling this weed.

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