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Alternaria brassicae causes leaf spots on *Eruca sativa* in Brazil

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Abstract
Leaf spots on *Eruca sativa* (arugula) were observed in Brazil and shown to be caused by a fungus belonging in the genus *Alternaria.* Morphological and molecular features were analysed and identity of the fungus was confirmed as *Alternaria brassicae*. A pure culture was established, and Koch's postulates were fulfilled. Inoculation studies have confirmed that the isolate from arugula attacks other crops in the Brassicaceae.

Keywords Dematiaceous asexual morph · Disease occurrence · Koch's postulates · Leafy vegetable · Pleosporales

Arugula or garden rocket – rúcula in Brazil - (*Eruca sativa*) is originally from the Mediterranean and has been grown as a vegetable since early Roman and Egyptian times (Zeven and de Wet 1982). It is an economically important crop in various Mediterranean countries as well as in Asia (Morales and Janick 2002). In Brazil it was a minor crop until the 1990s when it became increasingly popular and is now one of the most important leafy vegetables in the Brazilian market, particularly in the South and Southeast states (Amorim et al. 2007; Moura et al. 2008; Filgueira 2013). However, there is a lack of basic research on this crop in Brazil in most respects, including diseases. Only three fungus or fungoid pathogens appear in published records as attacking arugula in Brazil (Farr and Rossman 2019; Mendes and Urben 2019), namely *Albugo candida*, *Plasmodiophora brassicae*, and *Rhizoctonia* sp.

In September 2019, leaf spots were observed on *E. sativa* plants growing in a demonstration plot in the Infectarium (plant disease garden) of the Departamento de Fitopatologia, Universidade Federal de Viçosa (UFV) - state of Minas Gerais, Brazil. The disease was more evident on leaves and stems of plants after flowering, initially being small (< 1 mm diam.), circular, dark brown and necrotic and surrounded by a chlorotic halo. Over time the spots enlarged, became elongated and irregular, and coalesced, (Fig. 1 a–c) leading to yellowing and premature death of leaves and even entire plants. A sample of diseased plants was selected and transported to a laboratory for detailed examination. Upon observation under a stereoscopic microscope (Olympus SZX7) reproductive structures of a dematiaceous fungus were found regularly associated with the leaf spots. A representative subsample of diseased leaves and stems was dried in a plant press and deposited in the herbarium of the Universidade Federal de Viçosa (Acc. No. VIC 47378).

Fungal structures were scraped from lesions on infected plants and transferred to a drop of lactoglycerol over which a coverslip was placed. Structures in each slide were then examined under a light microscope (Olympus BX 51) equipped with differential interference contrast and to which a digital image capture system (Olympus Q-Color 3 ™) was attached. The fungus had the following morphology: conidiophores cylindrical, straight or proliferating sympodially at pronounced angles, geniculate, 74–125 × 6–17 μm, 3–5 septate, pale brown; conidiogenous cells 6–26 × 5–7 μm; conidiogenous loci 3 μm diam darkened and thickened; conidia solitary or in short chains (2–3), obclavate, 57–220 × 12–30 μm, with 5–14 transverse septa and 0–9 oblique or longitudinal septae, hilum darkened and thickened, pale brown, smooth (Fig. 1 e–f). The morphology of the fungus on arugula was almost identical to that of *Alternaria brassicae* (Simmons 2007).

Pure cultures were obtained by aseptic transfer of groups of conidia to potato dextrose-agar (PDA) in 9-cm-diameter Petri plates, with a sterile fine pointed needle. After a series of transfers of conidia to the plate, a drop of sterile water was transferred to the center of the plate and the conidia were spread over the surface of the medium with a sterile loop in order to separate individual conidia. Small blocks of the medium on which single conidia were found, upon observation under stereoscopic

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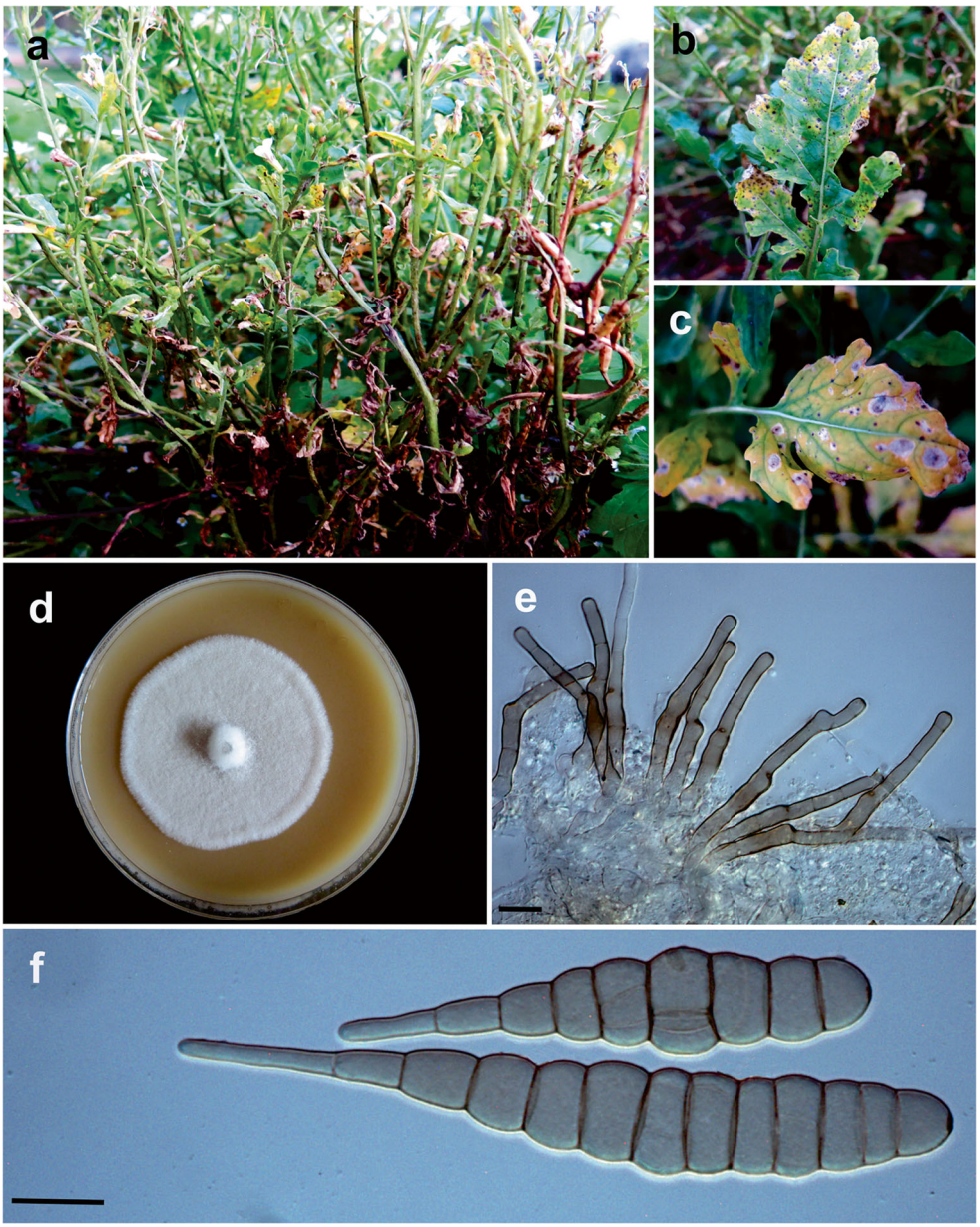

Fig. 1 *Alternaria brassicae* on arugula (*Eruca sativa*). Old stand of arugula plants attacked by *Alternaria* leaf spot (**a**). Numerous tiny necrotic spots on individual leaf (**b**). and coalescing spots with general yellowing of severely infected leaf (**c**). COAD 2990 *A. brassicae* colony on V8 juice-agar medium (**d**). Group of conidiophores of *A. brassicae* formed on arugula leaf (note strong geniculation and darkened and thickened conidial scars) (**e**). Two young conidia (**f**). Bars = 20 μm

microscope, were then removed with a sterile needle and transferred to separate plates, in order to generate single spore pure cultures. A single representative pure culture was selected for use in the study and deposited in the culture collection of the Universidade Federal de Viçosa (COAD 2990). The fungus was grown on malt extract-agar (MEA), potato carrot-agar (PCA), PDA, and on V8 juice-agar, at 25 °C under a 12 h light regime for 7 days for observation and description of culture morphology, as follows: growth rate variable - up to 2.6 cm diam in PCA and up to 5 cm diam. on PDA and on V8 juice-agar (after 7 d at 25 °C); flat or effuse, edge entire, aerial mycelium cottony, white with pale luteous to umber center; reverse mouse ochreous or gray followed by pale luteous ring; sporulation scarce to absent (Fig. 1 d).

For molecular identification, genomic DNA was extracted from COAD 2990 10-day-old mycelium growing on potato dextrose broth (PD) using a Wizard Genomic DNA Purification Kit (Promega) by following the manufacturer's instructions. Molecular analyses were performed using Alt-for (5′-ATGCAGTTCACCACCATCGC-3′) / Alt-rev primers (5′-ACGAGGGTGAYGTAGGCGTC-3′) (Hong et al. 2005). The sequencing was performed by Macrogen (Korea). The resulting sequence was deposited in GenBank (Accession No. MT081235). A BLASTn search of the Alt a 1 sequence revealed a 100% homology of the COAD 2990 sequence with the reference sequence of Hong et al. (2005) for *Alternaria brassicae* (AY563309).

The pathogenicity of *A. brassicae* COAD 2990 was evaluated on healthy 30-day-old arugula plants grown in 1 L pots containing an equal part mixture of pasteurised sand, soil and cow manure. Five plants were sprayed until runoff with a 1.6×10^5 conidia/ml suspension. The suspension was produced by scraping the surface of two-week-old sporulating COAD 2990 colonies formed on PDA plates, after flooding the plates with 30 mL of sterile distilled water. The concentration of the conidial suspension was adjusted after calibration with an haemocytometer. Two control plants were sprayed with sterile distilled water only. All plants were left for 48 h in a dew chamber and later transferred to a greenhouse bench at *ca.* 25 °C and irrigated twice a day. Plants were observed daily for the emergence of symptoms. Two days after inoculation necrotic spots started to appear on all inoculated plants. Seven days after inoculation typical conidiophores and conidia of *A. brassicae* were formed on the lesions. Colonies identical to those of COAD 2990 were obtained after isolation from the necrotic lesions, using the methodology described above. Controls remained healthy. Observation of the morphology of the conidiophores and conidia formed in those cultures confirmed that the identity of the fungus causing the disease was *A. brassicae.*

Parallel to inoculations aimed at fulfilling Koch's postulates, COAD 2990 inoculum was also applied on other related and important vegetable Brassicaceae (*Brassica oleracea*), namely cabbage, cauliflower, and broccoli. Five 30 days-old individuals of each vegetable variety were inoculated and incubated as previously described. As a result, between 48 to 72 h after inoculation, necrotic spots similar to those produced on arugula were observed on all inoculated plants, but controls remained healthy.

Also, another isolate of *A. brassicae* was obtained from the same locality (Infectarium UFV) from leaf spots on cauliflower (Acc No COAD 2993) and inoculated on arugula by following the same methodology described above and it was also proven to be pathogenic to arugula. It was also noticed that disease levels which resulted from this inoculation were higher than those obtained through COAD 2990 inoculations, suggesting that there may be variability in the aggressivity of *A. brassicae* within local populations. This is an issue which deserves further investigation in the future, particularly since our observations were in disagreement with those of Reis and Boiteux (2010) who found no significant differences for aggressiveness levels among different isolates from cultivated Brassicaceae.

Alternaria brassicae has been reported on many species belonging to the Brassicaceae and other families (Farr and Rossman 2019). Nevertheless, this is the first detailed report of *A. brassicae* causing leaf spot on arugula in Brazil. *Alternaria brassicae* has been previously reported on *E. sativa* only from a single, and obscure, record from India (Sarbhoy et al. 1971) which was not accompanied by detailed morphological or molecular identification nor any confirmation of pathogenicity. Two records also appear in a list given in Reis and Boiteaux (2010) of isolates of *A. brassicae* obtained from *E. sativa* in Brazil: one from one locality in the Distrito Federal and another from the state of Santa Catarina. Unfortunately, no detailed description of morphology or molecular information was provided for such isolates and further details on their pathogenicity status are also lacking from that publication. It is likely that the association between *A. brassicae* and *E. sativa* is widespread in Brazil, but remained overlooked because of the small size of lesions, and the fact that readily observable symptoms of infection appear only on older plants. The recognition that arugula is another host for *A. brassicae* is of relevance for disease management in vegetable gardens and to broad scale vegetable production as inoculum of *A. brassicae* from one species of Brassicaceae may promote the disease on another neighboring related crop or on another cruciferous crop in a rotation scheme. Another aspect worthy of attention is the production of healthy seeds of arugula. *Alternaria brassicae* is a well-known seed-borne pathogen and the possibility of infected arugula seeds serving to spread the disease deserves investigation.

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