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Alternaria alternata: A new seed-transmitted disease of coriander in South Africa

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Abstract This is the first report of *Alternaria* leaf spot disease on coriander (*Coriandrum sativum* L.) in South Africa. Using the agar plate method, *Alternaria alternata* was isolated from coriander seed lots together with four other fungal genera, which included *Aspergillus, Fusarium*, *Penicillium* and *Rhizopus.* Standard seed germination tests of coriander seed lots infected with seed-borne mycoflora showed a positive correlation with the number of diseased seedlings ($r = 0.239$, $p < 0.01$). Pathogenicity tests demonstrated that this seed-borne *A. alternata* was pathogenic on coriander and symptoms on leaves first appeared as small, dark brown to black, circular lesions (<5 mm diam.) that enlarged and coalesced to form dark brown blotches as time progressed. Leaf spot disease was most severe (64%) on wounded leaves inoculated with *A. alternata*. Re-isolation of *A. alternata* from diseased coriander plants satisfied the Koch's postulates, thus confirming it as the causal agent of *Alternaria* leaf spot disease. Parsimony analysis based on *rpb*2 (GenBank Accession No. KT895947), *gapdh* (KT895949) and *tef-1α* (KT895945) sequences confirmed identity of the *Alternaria* isolate, which grouped within the *A. alternata* clade. *Alternaria alternata* was shown to be transmitted from infected coriander seed to the developing plants.

Keywords Seed-borne · Seed-transmitted · *Alternaria alternata* · *Coriandrum sativum*

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Introduction

Cultivation of coriander (*Coriandrum sativum* L.) is relatively new in South Africa (SADC Trade 2014). Since coriander is perceived as a minor crop, little priority is given for research on improving local productivity; hence, the 6% net increase in coriander imports over the last decade to meet elevated demands of herbs and spices in South Africa (Phahlane 2013). The growing international trade in plants and plant products is however contributing substantially to the spread of invasive alien plant pests and pathogens (Brasier 2008).

There is a danger of introducing new strains or physiologic races of pathogens that might be more pathogenic than existing genotypes by trans-regional dissemination of infected and/or infested germplasm and other agricultural commodities that may cause outbreaks of new diseases (Neergaard 1969). Some of the most devastating diseases on coriander reported in other parts of the world include bacterial leaf spot by *Pseudomonas syringae* pv. *coriandricola* and footrot disease caused by *Phoma*

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multirostrata (P.N. Mathur, S.K. Menon & Thirum.) Dorenb. & Boerema (Hashmi and Gaffar 1991a; Toben and Rudolph 1996). Establishment and distribution of both diseases were reported to spread over long distances within short time periods by means of infected coriander seeds (Hashmi and Ghaffar 1991b).

Alternaria alternata (Fr.) Keissl. is known to be a common saprotroph on many hosts; however, there are several reports of the pathogen causing disease on different hosts i.e. pomegranate (*Punica granatum* L.) (Berbegal et al. 2014), rubber tree (*Hevea brasiliensis* Muell. Arg) (Cai et al. 2015), spinach (*Spinacia oleracea* L.) (Czajka et al. 2015) and switchgrass (*Panicum virgatum* L.) (Vu et al. 2012). Although *A. alternata* has been reported on onion (*Allium cepa* L.) (Bihon et al. 2015), potatoes (*Solanum tuberosum* L.) (Van der Waals et al. 2011) and apples (*Malus domestica* Borkh.) (Serdani et al. 2002) in South Africa, its association with seeds of coriander and other herbs has never been recorded in this country. Since, a high incidence of *A. alternata* was detected in a preliminary seed health test of coriander seeds produced in South Africa, further studies were conducted to investigate its effect on seed germination, transmission into seedlings and pathogenicity.

Materials and methods

Source of seed

Untreated coriander seed lots CorA, CorB and CorC were supplied by commercial seed companies in South Africa.

Seed health test

The agar plate method was used to detect seed-borne fungi associated with coriander seeds. Two hundred seeds of each seed lot were randomly selected, surface sterilised in 1% sodium hypochlorite solution for five minutes, rinsed in sterile distilled water and left to air-dry. Seeds were plated in Petri dishes containing potato dextrose agar (PDA, Biolabs, Midrand, South Africa) amended with 25 mg L^{-1} streptomycin sulphate (Biolabs, Midrand, South Africa). Petri dishes were incubated at 25 °C for 7 days under alternating cycles of 12 h ultra violet (UV) (365 nm) light and darkness. The experiment was set up in a completely randomized design with four replicates of five Petri dishes (10 seeds per Petri dish) and repeated twice.

Morphological identification of fungi

After 7 days of incubation, fungal colonies on the PDA retrieved from the seeds were identified to genus level using a stereomicroscope. Thereafter, selected fungal cultures were purified using the single spore technique. Fungal cultures identified as *Alternaria* spp. were cultured on potato carrot agar (PCA) (Crous et al. 2009); whereas cultures identified as *Fusarium* were transferred onto carnation leaf agar (Burgess et al. 1994). All other fungal isolates were sub-cultured on PDA.

Fungal cultures were incubated at 25 °C for 7 days under alternating cycles of near UV light and darkness. Morphological characteristics of each isolate were examined at 40× to 100× with a Zeiss (Munich, Germany) light microscope and the identity and incidence of fungal taxa isolated was determined and recorded. Reference manuals of Mathur and Kongsdal (2003), Ellis and Ellis (1997), Leslie and Summerell (2006) were used to identify fungi. Isolated fungi were stored on half strength PDA agar slants at 4 °C.

Standard seed germination test

Seed germination tests (ISTA (International Seed Testing Association) 2017) were performed following a modified between paper method. Four replicates of 50 seeds were placed on top of three layers of moistened germination paper (Agricol, Brackenfell, South Africa) before covering them with a fourth layer of moistened germination paper. The layers of germination paper were rolled, sealed in a polythene bag with an elastic band and incubated at 25 °C. Seedlings were evaluated according to International Seed Testing Association (ISTA 2017) rules, where final counts of normal or abnormal (deformed and diseased) seedlings were recorded after 21 days.

Seed transmission test

Since coriander seed lot CorA had the highest incidence of natural infection by *Alternaria* sp., seed-transmission tests were determined using this cultivar. Coriander seeds were sown singly in twenty-one 5 cm diameter pots filled with pasteurised loamy soil and grown at 25 °C/17 °C day-night temperatures, respectively. The

photoperiod was maintained at 16 h with an average humidity of 80 to 85%. Experimental units were replicated three times with seven pots spaced 90 cm apart per Table (4 m × 2 m) and each replicate arranged randomly. The experiment was repeated twice. Pots were watered each day with sterile distilled water for 8 weeks before evaluation of incidence was determined by observing number of plants that developed disease symptoms in relation to the total number of coriander plants, expressed as a percentage. Severity of leaf spot disease was scored according to the rating scale developed for this study (Table 1). Isolations of the causal organism were made by cutting 5 mm^2 sections from the edges of leaves showing symptoms and plated onto Petri dishes containing PDA and incubated for 3 to 5 days at 25 °C under 12 h alternating cycles of near UV light and darkness. Isolated fungi were purified and morphologically identified as indicated above. A culture of the *Alternaria alternata* isolate was deposited (PPRI 18133) in the National Collection of Fungi, ARC-Plant Protection Research, Roodeplaat, South Africa.

Molecular identification

Identity confirmation of *A. alternata* isolate PPRI 18133 was performed by the polymerase chain reaction (PCR). DNA was isolated using the DNeasy plant mini-extraction kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. Extracted DNA was used as template in PCR and the second largest subunit of RNA polymerase II (*rpb2*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and translation elongation factor-1 alpha (*tef-1α*) gene regions were amplified (Fig. 1) using primer sets described by Woudenberg et al. (2013). The PCR consisted of 1× DreamTaq reaction buffer with $MgCl_2$ (2 mM), dNTPs (50 μM each), primers (0.2 μM each), template DNA (25 ng) and Dream Taq polymerase (0.5 μM) (Inqaba Biotech, South Africa). The PCR conditions for the *tef-1α* region included an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s and elongation at 72 °C for 45 s, with a final elongation step at 72 °C for 7 min. The conditions for *gapdh* differed only with an annealing temperature of 50 °C. The *rpb2* was amplified using a touchdown PCR protocol of 5 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min, followed by 5 cycles with a 58 °C annealing temperature and 30 cycles with a 54 °C annealing temperature. The resulting PCR amplicons were purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and sequenced in both directions using the PCR primers and the ABI PRISMTM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, Warrington, UK). Consensus sequences were compiled from forward and reverse sequencing using BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html). All generated sequences were deposited in GenBank.

Phylogenetic analyses

Reference sequences for *Alternaria* were selected on the basis of BLAST results from GenBank at National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and MycoBank (http://www.mycobank.org/), as well as Woudenberg et al. (2015). Multiple sequence alignments were generated with MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/index.html). Gaps were treated as missing data in the subsequent analyses. Phylogenetic analysis was based on parsimony using PAUP 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods version 4, Swofford 2002) of the individual

Table 1 *Alternaria* leaf spot disease rating scale

Index value	Description of disease severity
0	No infection
1	Leaf lesions (diameter < 5 mm) on surface of leaf ≤2; (1–20%)
2	3 ≤ leaf lesions (diameter < 5 mm) on surface of leaf <5; (21–40%)
3	5 ≤ leaf lesions (diameter > 5 mm) on surface of leaf ≤7; (41–60%)
4	8 ≤ leaf lesions (diameter > 5 mm) with coalescing of lesions to form one necrotic spot; (61–80%)
5	>9 leaf lesions with severe to complete damage of leaf; (81–100%)

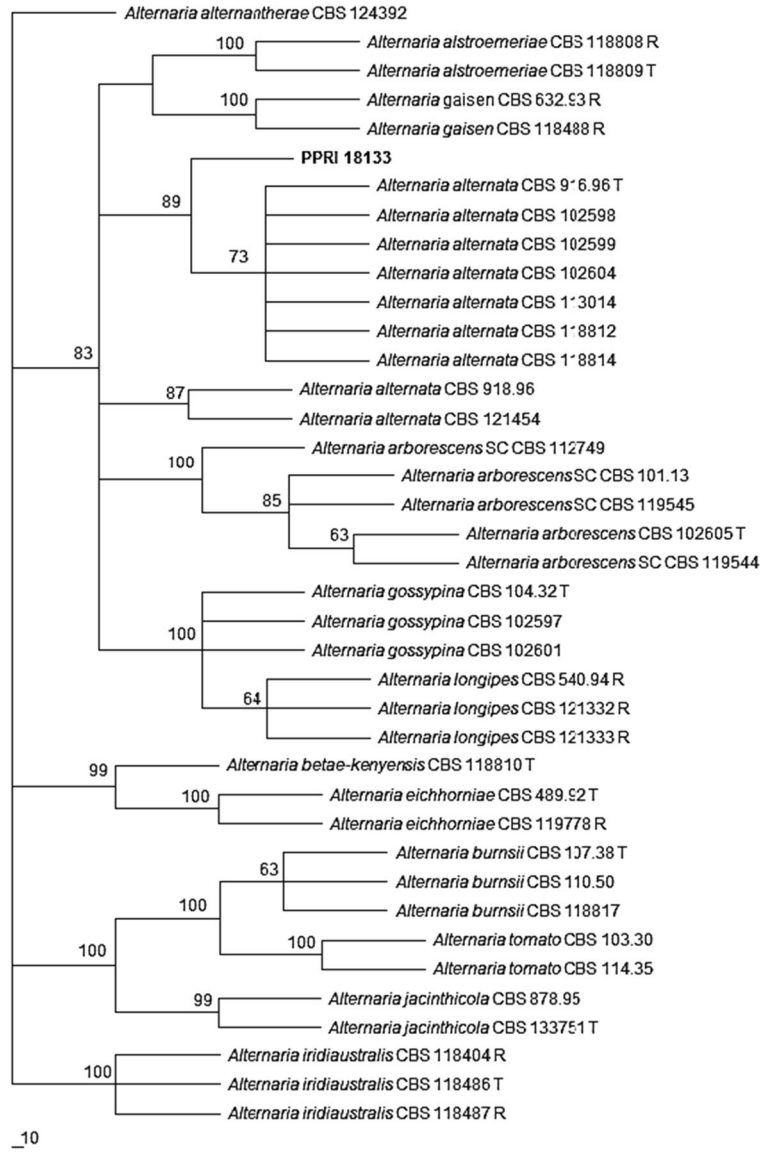

Fig. 1 Most parsimonious tree based on the *gapdh*, *tef-1α* and *rpd2* sequences of 38 *Alternaria* strains within *Alternaria* sect. *Alternaria*

data partitions as well as the combined aligned dataset. Heuristic searches were performed with random addition of sequences (100 replicates), tree bisection-reconnection (TBR) branch swapping, and MULPAR effective and MaxTrees set to auto-increase. The combinability of the data sets was determined by the partition homogenicity test. The consistency (CI) and retention indices (RI) were determined for the data sets. The phylogenetic trees for *Alternaria* were rooted with *Alternaria alternantherae* (CBS 124392). Bootstrap analysis was performed to determine branching point confidence intervals (1000 replicates) for the most parsimonious trees generated for the data sets.

Pathogenicity test

Coriander seed lot CorB was used for pathogenicity tests using a protocol designed by Blodgett and Swart (2002). Seeds were planted and maintained as for the seed transmission test but symptomless 8-week old plants were selected for the pathogenicity test. Fungal inoculum was prepared from a 14-day old culture of *A. alternata* grown on PCA. Spores were harvested, filtered through double cheesecloth to remove mycelium fragments and the concentration was adjusted to 5×10^5 spores mL^{-1}. For wounded inoculations, the 8-week old coriander plants ($n = 30$) were wounded with a

sterile pin with a diameter of 0.5 μm in the centre of the leaves. The wounded plants were inoculated with the *A. alternata* spore suspension with an automatic aerosol sprayer until runoff. Non-wounded plants ($n = 30$) were spray inoculated as above. Pots containing non-inoculated wounded and inoculated non-wounded plants control plants ($n = 15$ respectively) were sprayed with sterile distilled water. A high humidity (>95%) was maintained by covering all plants with polythene bags for 72 h. Disease incidence and evaluation of severity of disease (Table 1) was assessed 14 days after inoculation as described above. Isolations were made from all leaves and isolated fungi were identified as described above.

Data analyses

Data collected from seed health, germination and pathogenicity tests were analysed for variance using SAS Version 9.3 (SAS Institute 2010). The data for the germination test were arcsine transformed. Statistical means for all tests were separated using the Fisher's LSD test, but final germination counts were presented as untransformed data. The correlation between seed germination and seed infection was analysed using the statistical package EViews Version 3.1 (Quantitative Micro Software, 2000).

Results

Seed health test

Six fungal taxa viz. *Alternaria alternata* (Fr.) Keissl, *Aspergillus* section *Nigri*, *Aspergillus* section *Flavi*, *Fusarium oxysporum* Schltdl., *Penicillium* section *Expansa* and *Rhizopus* sp. were detected from coriander seed lots (Table 2). *Alternaria alternata* was isolated with the highest frequency from all seed lots, which ranged from 28.5 to 73%; whereas *Aspergillus* section *Flavi* was detected at the lowest frequency of 0 to 2%. Preliminary pathogenicity tests (results not given) showed that with the exception of *A. alternata*, none of the isolated fungi caused disease and were not studied further.

Seed germination tests

The results of seed germination tests are presented in Table 3. Seed germination ranged from 72.8 to 78.8%; however, there was no statistical difference among the three seed lots. Of the abnormal seedlings, diseased seedlings constituted the larger percentage of abnormalities compared to deformed seedlings. Coriander seed lot CorC and seed lot CorA recorded the highest percentage of diseased seedlings (7.5% and 7.0%, respectively); whereas, coriander seed lot CorB yielded the lowest percentage of diseased seedlings (2.8%) (Table 3) and was subsequently used in pathogenicity tests. However, there were no significant differences recorded for deformed seedlings in all coriander seedlots.

Correlation analysis showed that amount of seed-borne fungi on coriander seeds had a weak correlation with germination ($r = -0.129$, $p < 0.01$). The percentage of diseased seedlings recorded was positively correlated with the incidence of seed-borne fungi detected on coriander seeds ($r = 0.239$, $p < 0.01$). The study indicated

Table 2 Incidence of seed-borne mycoflora associated with South African coriander seed lots

Seed Lot	Incidence (%) of seed-borne fungi[a]					
	A.a*	***A.n****	***A.f****	***F.o****	***P.e****	***R.sp.****
CorA	73 a	2.5 b	0.5 b	0 b	3.5 c	0 b
CorB	28.5 c	1.5 c	2 a	2.5 a	9 a	0 b
CorC	45 b	4 a	0 c	2.5 a	5 b	3 a
LSD	0.136	0.114	0.111	0.061	0.107	0.022
CV%	23.55	68.57	67.76	31.04	29.36	51

[a] Means in the same column followed by the same letter do not differ significantly according to Fisher's LSD test at $p < 0.05$

**A.a* = *Alternaria alternata*; *A.n* = *Aspergillus* section *Nigri*; *A.f* = *Aspergillus* section *Flavi*; *F.o* = *Fusarium oxysporum*; *P.e* = *Penicillium* section *Expansa*; *R.sp.* = *Rhizopus* sp.

Table 3 Standard seed germination tests of coriander seed lots produced in South Africa

Seed Lot	**Normal seedlings (%)***	Abnormal seedlings (%)*	
		Deformed	**Diseased**
CorA	72.8 a	5.5 a	7.0 a
CorB	78.8 a	4.0 a	2.8 b
CorC	77.5 a	4.0 a	7.5 a
LSD	0.1001	0.0803	0.0846
CV%	3.213	15.789	12.857

*Means in the same column followed by the same letter do not differ significantly according to Fisher's LSD test (at $p < 0.05$) using the GLM procedure. Values are means from four replicates of 50 seeds

no correlation between amount of seed-borne fungi and the percentage of deformed coriander seedlings ($r = -0.369$, $p < 0.01$).

Seed-transmission test

Sowing naturally *Alternaria* infected CorA seed displayed transmission of the pathogen, with 18% of 8-week old coriander plants being infected with the leaf spot disease. Symptoms appeared as small (<5 mm diam.), dark brown to black circular lesions on leaves (Fig. 2a). As time progressed, lesions enlarged and coalesced to form dark brown blotches (Fig. 2b). Isolation from leaf lesions and morphological identification showed *A. alternata* to be the pathogen.

Phylogenetic analyses

The aligned sequences of the *gapdh* (579 aligned characters), *tef-1α* (241 aligned characters) and *rpd2* (753 aligned characters) gene regions of the *Alternaria* sp. contained 38, 20 and 44 parsimony-informative characters, respectively. The multi-gene phylogeny based on the three gene regions contained 1573 aligned characters with 102 parsimony-informative characters resulting in a tree length of 134, consistency index of 0.8209 and retention index of 0.9368. Most species formed single clades with high bootstrap support values, except *A. arborescens* species complex and *A. alternata* (Fig. 1) as reported by Woudenberg et al. (2015). The isolate PPRI 18133 grouped within the *A. alternata* clade.

Pathogenicity test

Alternaria alternata was pathogenic on coriander plants of seed lot CorB. Inoculated coriander plants displayed symptoms, as described above, after 9 days. These symptoms were observed on both wounded and non-wounded inoculated plants and were identical to the symptoms on the leaves in the seed transmission test. *Alternaria* leaf spot disease was most severe (64%) on wounded coriander seedlings inoculated with the pathogen compared to non-wounded inoculated control and non-inoculated wounded and unwounded plants (16%, 13% and 10%, respectively). The incidence of the *Alternaria* leaf spot disease was 82% on wounded inoculated seedlings, 35% on non-wounded inoculated plants and 28% and 17% on non-inoculated wounded and unwounded plants, respectively. Identity of *A. alternata* as the cause of leaf spot disease was confirmed as the pathogen was re-isolated thereby fulfilling Koch's postulates.

Discussion

Seed health refers to the presence or absence of disease-causing organisms such as fungi, nematodes, bacteria, and viruses associating with seed lots (Fairey et al. 1999). It is important to determine the seed health status to gain an understanding on the performance of seedlings in a nursery. A seed health report has become the most valuable tool in seed trade, where necessary precautionary steps may be executed to reduce the risk of distributing diseases into non-diseased areas. As such, this study reports an *Alternaria* leaf spot pathogen associated with coriander seed lots produced in South Africa, where its pathogenicity, seed-transmissibility and effects on seed germination were determined.

With reference to the regulations of the International Seed Federation (2017), it was found that all coriander seed lots were above the minimum required germination

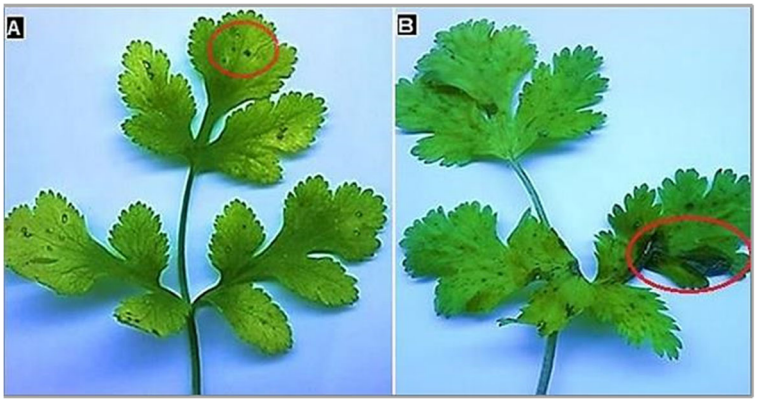

Fig. 2 Symptoms of *Alternaria* leaf spot disease on 8-week old coriander plants. A: Small (<5 mm diam.), dark brown to black circular lesions. B: enlarged lesion forming dark brown blotch

level of 70%; however, the South African Plant Improvement Act set in 1976 does not have standards for certification of coriander seed. Since germination tests showed a significantly higher number of diseased seedlings that were positively correlated with incidence of seed-borne mycoflora ($r = 0.239$, $p < 0.01$), this prompted further investigations into the most commonly isolated seed-borne fungi on germination and plant development.

Seed health tests detected a lower diversity of seed-borne mycoflora compared to other studies done on coriander seeds, where between 13 to 23 genera of fungi were detected (Hashmi and Ghaffar 1991b; Dwivedi et al. 2006). This diversity of seed-borne mycoflora may be attributed to varying environmental conditions in the respective localities where coriander plants were grown or different surface disinfection procedures. The use of the standard agar plate method as the only method may have attributed to the low number of fungi genera detected compared to the findings of Dwivedi et al. (2006), who included the seed washing test and moist blotter techniques together with the standard agar plate method.

Although other studies recorded *A. alternata* on coriander seeds, this is the first time that the fungus is reported to be pathogenic on coriander. *Alternaria alternata* has been reported to cause blights and rots of apples (Serdani et al. 2002) and potatoes (Van der Waals et al. 2011) in South Africa. *Alternaria poonensis* Ragunath is the only fungus in the genus *Alternaria* previously reported to cause blights on coriander (Raghunath 1963; Khare et al. 2017).

The appearance of *Alternaria* leaf spots and its re-isolation from diseased plants demonstrated *A. alternata* to be pathogenic on coriander. Appearance of *Alternaria* leaf spots on coriander in naturally infected, non-inoculated pots in greenhouse trials and seed transmission experiments proved its seed transmission. Similarly, *A. alternata* has been reported as seed-borne and seed-transmitted in maize, sorghum, foxtail millet and many other hosts (Basak and Lee 2002; Fakhrunnisa and Ghaffar 2006; Yago et al. 2011; Meena et al. 2013; Perelló and Larrán 2013).

In conclusion, this is the first time *A. alternata* has been isolated from coriander seeds produced in South Africa. Apart from its seed-borne nature, *A. alternata* caused leaf spots on coriander plants, and was shown to be seed transmitted.

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Animal Studies This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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