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SHORT NOTE

Real-time PCR for rapid in planta detection of *Plectosphaerella cucumerina* on wild rocket (*Diplotaxis tenuifolia*)

Giovanna GILARDI[1], Kathryn WEBB[2], Giuseppe ORTU[1], Maria Lodovica GULLINO[1,3] and Angelo GARIBALDI[1]

[1] Centre for Innovation in the Agro-Environmental Sector, AGROINNOVA, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy
[2] The Food and Environment Research Agency, Sand Hutton, York, North Yorkshire, YO41 1LZ, UK
[3] DISAFA, University of Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy

Summary. *Plectosphaerella cucumerina* that has recently been described as causing a leaf spot on wild rocket (*Diplotaxis tenuifolia*) in Italy, but has not yet been recorded on this host in other countries. This fungus has the potential to cause severe economic losses to the salad-producing industry, especially in Italy. This paper describes the development of a real-time PCR assay based on internal transcribed spacer (ITS) sequence, to aid diagnosis of this pathogen in culture and in plant material. The assay showed no cross reaction with closely related *Plectosphaerella* species or with 18 other commonly found fungi, and was able to detect 10 fg of *Pa. cucumerina* DNA per reaction. The assay was more specific than previously available molecular methods. A comparison of molecular and isolation methods indicated that the real-time PCR method is suitable for detection of the pathogen on plant material.

Key words: diagnosis; leafy vegetables, Taq Man probe.

Introduction

Plectosphaerella cucumerina has recently been described as the causal agent of a leaf spot disease on wild rocket (*Diplotaxis tenuifolia*) (Garibaldi *et al*., 2012). It was first recorded on this host in Italy in 2012, where it was observed causing leaf spots in several commercial glasshouses in northern and southern Italy. The pathogen has been identified as the causal agent of a leaf spot disease on field-grown endive (*Cichorium endivia*) (Garibaldi *et al*., 2013), and cultivated rocket (*Eruca sativa*), also in Italy. *Plectosphaerella cucumerina* is also reported as a pathogen causing severe damage to different crops in different parts of the world (Jimenez and Zitter, 2005; Egel *et al*., 2010; Vallance *et al*., 2011).

Symptoms observed on wild rocket consisted of small (1 mm), irregular shaped, black-brown spots which later coalesced into larger spots (1 cm) surrounded by yellow-grey halos. Spots were mostly located on the foliar limbs, ribs and petioles. Affected leaves were often distorted, appearing hook-like. The disease was severe under 75–90% R.H., at an air temperature of 20–26°C, causing severe production losses. Affected tissue rotted quickly after packaging, during transportation and commercialisation of the processed rocket (Garibaldi *et al*., 2012). *Plectosphaerella cucumerina* has also been associated with root and collar rots of other horticultural crops worldwide (Carlucci *et al*., 2012), and although not generally an important pathogen, it can become severe under appropriate environmental conditions (Uecker, 1993). This fugus has not yet been reported on wild rocket (*D. tenuifolia*) in countries other than Italy (Garibaldi *et al*., 2012).

The use of wild rocket and cultivated rocket as salad leaf or garnish has increased in Italy, and these plants are used widely in Mediterranean cuisine around the world. It is mainly grown in south-

Corresponding author: M.L. Gullino
E-mail: marialodovica.gullino@unito.it

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ern Europe, the Middle East and South-East Asia (Padulosi and Pignone, 1997). There are two types of rocket available on the market in Italy: (i) *Eruca sativa*, known as cultivated rocket; and (ii) several species of *Diplotaxis*, which are wild plants now widely cultivated (Garibaldi *et al*., 2003). According to data gathered in 2009, there are more than 1,100 ha of rocket in protected cultivation in Italy (Bianco, 2009). Due to the importance of this crop in Italy, *Pa. cucumerina* can cause serious economic losses (Garibaldi *et al*., 2012), even considering the only partial disease reduction provided by the control measures available (Gilardi *et al*., 2015).

Circumstantial evidence from surveys in the area affected by the disease suggested that the sudden appearance of this disease may have been due to the transmission of the pathogen by seeds (Gilardi *et al*., 2013). Therefore, long-distance dispersal and the appearance of the pathogen on wild rocket in other countries will most likely result from transportation of infected host material, and through international trade. Rapid and early detection of infected plant material and seeds is important to help reduce the spread of the pathogen. The current morphological detection method involves isolation of the pathogen onto media and incubation for 7–10 d, followed by morphological assessment requiring specialised mycological skills. Molecular methods, such as real-time PCR, were first implemented for routine diagnosis of plant pathogens in the late 1990's (Schaad *et al*., 1999). This methodology has been widely applied to the detection of fungal pathogens, due to its increased sensitivity and specificity compared to conventional PCR, and its rapid turnaround time compared with morphological techniques (Schena *et al*., 2013; Sanzani *et al*., 2014). A real-time PCR assay for the detection of *Pa. cucumerina* has previously been developed (Atkins *et al*., 2003). However, since then, phylogenetic analysis of *Plectosphaerella* combining morphological and molecular data of isolates has shown that there are four new *Plectosphaerella* species, previously thought to be *Pa. cucumerina* (Carlucci *et al*., 2012). Analysis of the sequence data suggested that the assay designed by Atkins *et al*. (2003) would not be specific enough to distinguish between the different *Plectosphaerella* species.

This paper describes the development of a real-time PCR assay based on internal transcribed spacer (ITS) sequence for the specific detection of *Pa. cucumerina*, comparison of the assay with that designed assay (Atkins *et al*., 2003), and the results of a comparison of this new method with the morphological method based on isolation from plant material onto culture media.

Materials and methods

Fungal isolates and DNA extraction

Plectosphaerella cucumerina isolates were taken from the culture collection at Agroinnova, isolated from various hosts. This study also included other *Plectosphaerella* species obtained from Dr. Antonia Carlucci (Department of Sciences of Agriculture, Food and Environment (SAFE), University of Foggia, Italy) and other fungi commonly isolated from rocket (Table 1). Isolates were grown on potato dextrose agar (PDA) at room temperature for 7–10 d, after which 100–200 mg of fresh mycelium of each isolate was harvested and transferred to a 2 mL microcentrifuge tube. The harvested mycelium was subjected to shaking for 3 min in a Qiagen TissueLyser set to 30/s frequencies, with two 3 mm sterile steel beads and 500 µL PL1 lysis buffer from the Nucleospin Plant II kit (Machery Nagel). Following grinding of the tissue, 10 µL of Proteinase K (5-Prime) and 10 µL of RNAase were added to the sample, which was then incubated for 30 min at 65°C. After centrifugation DNA extraction was carried out using the Nucleospin Plant II kit following the manufacturer's protocol for fungi, charging the samples on the extraction column. Extracts for testing assay sensitivity were quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and were diluted to a standard concentration of 5 ng μL^{-1}.

Artificial inoculation and isolation of *Plectosphaerella cucumerina*

One month old *D. tenuifolia* plants were grown in a nursery in 3 L capacity pots (5 plants per pot) filled with steamed peat. Plants were inoculated by spraying a 1×10^6 CFU mL^{-1} conidial suspension of isolate CC1 of *Pa. cucumerina*, prepared from 7-d-old cultures grown on PDA supplemented with 25 mg L^{-1} of streptomycin sulphate. Inoculated plants were maintained in a growth chamber at 25±1°C, at 90% R.H, and monitored daily for the development of symptoms. Plants sprayed with sterile water served as controls. The first symptom of yellow leaves started to be visible 5 d after inoculation. Symptomatic leaf material was asepti-

Table 1. List and characteristics of the organisms tested in this study, and results following testing using real-time PCR primer and probe sets PcRTF1/PcRTR1/PcRTP1 and *Plectosphaerella cucumerina* F2/R2/P1.

Species	Isolate	Host plant/Origin	Origin (City, Region, Country)	Real-time PCR Ct values	
				PcRT assay (Atkins ref)	*P. cucumerina* assay
Plectosphaerella cucumerina	PLC 6	Wild rocket, leaf necrosis	Battipaglia, Campania, Italy	18.77 (1.0)	19.17 (1.7)
Pa. cucumerina	PLC 9	Wild rocket, leaf necrosis	Salerno, Campania, Italy	20.03 (0.1)	22.17 (0.0)
Pa. cucumerina	PLC 12	Wild rocket, leaf necrosis	Salerno, Campania, Italy	17.17 (0.2)	20.41 (0.2)
Pa. cucumerina	PLC 15	Cultivated rocket, leaf necrosis	Agroinnova (Grugliasco, TO), Italy	20.10 (0.1)	29.32 (0.4)
Pa. cucumerina	PLC 24	Wild rocket cv. Grazia, leaf necrosis	Bergamo, Lombardy, Italy	20.30 (0.1)	25.10 (3.2)
Pa. cucumerina	PLC 25	Wild rocket cv. Reset, leaf necrosis	Bergamo, Lombardy, Italy	18.41 (0.0)	21.68 (0.0)
Pa. cucumerina	PLC 27	Wild rocket cv. Selvatica, leaf necrosis	Bergamo, Lombardy, Italy	19.71(0.1)	21.67 (0.1)
Pa. cucumerina	PLC 28	Endive, leaf necrosis	Treviglio, Lombardy, Italy	18.20 (0.2)	20.66 (0.4)
Pa. cucumerina	PLC 29	Escarole cv. Sardana, heart necrosis	Treviglio, Lombardy, Italy	18.37 (0.2)	20.49 (0.4)
Pa. cucumerina[a]	Pt 143	Pepper collar	Rignano Garganico, Foggia, Italy	15.43 (0.0)	19.71 (0.0)
Pa. citrullae[a]	Pt 157	Water melon root	Foggia, Italy	16.46 (0,1)	-
Pa. pauciseptata[a]	Pt 186	Tomato root	Rignano Garganico, Foggia, Italy	15.41 (0.1)	-
Pa. melonis[a]	Pt 228	Melon root	Borgo Cervaro, Foggia, Italy	15.77 (0.1)	-
Pa. plurivora[a]	Pt 365	Asparagus apex turion	Borgo Cervaro, Foggia, Italy	15.21 (0.0)	-
Pa. ramiseptata[a]	Pt 403	Tomato root	Borgo Cervaro, Foggia, Italy	15.02 (0.0)	-
Pythium sp.	-	Wild rocket	Treviglio, Lombardy, Italy	NT	-
Rhizoctonia solani	-	Cultivated rocket	Moncalieri, Torino, Italy	NT	-
Alternaria sp.	-	Cultivated rocket	Moncalieri, Torino, Italy	NT	-
Cladosporium sp.	-	Wild rocket, seed	Anseme Seed Company (Cesena), Italy	NT	-
Botrytis cinerea	-	Cultivated rocket	Moncalieri, Torino, Italy	NT	-
Fusarium oxysporum f. sp. *raphani*	FR-9/03	Wild rocket	Bergamo Lombardy, Italy	NT	

(Continued)

Table 1. (Continued).

Species	Isolate	Host plant/Origin	Origin (City, Region, Country)	Real-time PCR Ct values	
				PcRT assay (Atkins ref)	*P. cucumerina* assay
F. oxysporum f. sp. *raphani*	FR-13/03	Rocket	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *raphani*	FR-30/03	Rocket	Moncalieri, Piedmont, Italy	NT	-
F. oxysporum f. sp. *raphani*	FR-1/06	Wild rocket	Latina, Italy	NT	-
F. oxysporum f. sp. *raphani*	ATCC16601	Radish	USA	NT	-
F. oxysporum f. sp. *matthioli*	FR-4A/02	Wild rocket	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *matthioli*	FR-20/03	Wild rocket	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *spinaciae*	FR-10B/02	Wild rocket	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *spinaciae*	FR-17/03	Wild rocket	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *conglutinans*	ATCC58385	-	USA	NT	-
F. oxysporum f. sp. *conglutinans*	ATCC16600	Wild cabbage	USA	NT	-
F. oxysporum f. sp. *lactucae*	FL10 ATCC MYA 3040	Lettuce	Bergamo, Lombardy, Italy	NT	-
F. oxysporum f. sp. *lactucae*	FL10/9501	Lettuce	Japan	NT	-
F. oxysporum f. sp. *lactucae*	FL ATCC744085	Lettuce	USA	NT	-
F. oxysporum f. sp. *basilici*	6 BASIL BULK	Basil	Savona, Liguria, Italy	NT	-
F. oxysporum f. sp. *basilici*	FOB 009 RB	Basil	Savona, Liguria, Italy	NT	-
F. oxysporum f. sp. *cucumerinum*	ATCC744004	-	American Type Culture Collection	NT	-
F. oxysporum f. sp. *lilii*	-	Lillium	Bagnasco, Piedmont, Italy	NT	-
F. oxysporum f. sp. *lycopersici*	Pallara compost	Tomato	Trofarello, Piedmont, Italy	NT	-
F. oxysporum f. sp. *melonis*	FOM 37	Melon	Alessandria, Piedmont, Italy	NT	-
F. oxysporum f. sp. *dianthi*	Pat (75)	Carnation	Albenga, Liguria, Italy	NT	-
F. oxysporum f. sp. *radicis lycopersici*	960-146	Tomato	Albenga, Liguria, Italy	NT	-
F. oxysporum f. sp. *gladioli*	Ormea – CO 4	Gladiolus	Ormea, Liguria, Italy	NT	-

[a] Isolates from Antonia Carlucci (Department of Sciences of Agriculture, Food and Environment (SAFE), University of Foggia, Italy). Cycle threshold (Ct) values are mean values for triplicate reactions. Standard deviations are indicated in brackets. Dashes indicate negative results (i.e. no Ct value obtained after the completion of 40 cycles); NT indicates the isolates were not tested.

cally excised and the lesions were split; half were used for DNA extraction, and half for plating onto plates of PDA containing 25 mg L^{-1} of streptomycin sulphate. Diseased tissue selected for plating was immersed in a solution containing sodium hypochlorite (1% available chlorine) for 60 s, rinsed in water, dried on sterile filter papers and then placed onto plates of PDA. After 6 d under 12 h per day of fluorescent light at 22°C, a fungus producing whitish-orange mycelium morphologically typical of *Pa. cucumerina* was observed. Five plates each from symptomatic leaves and non-inoculated leaves were prepared.

Extraction of DNA from plant material

The DNA extraction was carried out using the E.N.Z.A. Plant DNA Kit (Omega Bio-Tek) following the manufacturer's protocol for fresh or frozen plant samples. Inoculated plant material (typically 100–200 mg) was cut into small pieces and transferred to a 2 mL microcentrifuge tube and ground for 3 min using a Qiagen TissueLyser set to 30/s frequencies, with two 3 mm sterile steel beads and 600 µL P1 Buffer (E.N.Z.A. Plant DNA Kit, Omega Bio-Tek).

Real-time PCR primer and probe design

The representative ITS sequences of *Plectosphaerella* species, described in Carlucci *et al*. (2012), were retrieved from GenBank and compared using the ClustalW method of the MEGA 5 program (Tamura *et al*., 2011). Primers and a duel-labelled probe for the detection of *Pa. cucumerina* were designed using Primer 3 software (Rozen and Skaletsky, 2000) targeting areas of the ITS sequence with most divergence between the other species in the alignment. A control assay designed in a conserved region of the plant cytochrome oxidase (COX) gene (Hughes *et al*., 2006) was also used when testing plant material, in order to normalize data for differences in DNA extraction efficiency and PCR inhibitors. The *Pa. cucumerina* primer set (PcRTF1-PcRTR1) and probe (PCRTP1) (Atkins *et al*., 2003) were also used as a comparison to the newly developed, more specific method. Primer and probe sequences are shown in Table 2.

Real-time PCR conditions

All real-time PCR reactions were carried out in 48 well plates on a StepOne real-time PCR system (Applied Biosystems), using TaqMan Core Reagents (Applied Biosystems). The reaction mixture for the PcRTF1/PcRTR1 and PcRTP1primer and probe set consisted of 1 × Buffer A and 0.025 U μL^{-1} AmpliTag Gold, plus 0.5 mM of each dNTP, 5.5 mM $MgCl_2$, 900 nM forward primer, 300 nM reverse primer, and 225 nM probe. For each reaction, 1 µL of DNA extract was

Table 2. Sequences of real-time PCR primers and probes used in this study.

Target	Primer/probe name	Sequence
Plectosphaerella cucumerina[a]	PcRtF1	5′-GTGCCCGCCGGTCTC-3′
	PcRtR1	5′-GACAGTTCGCTAAGAACACTCAGAAGT-3′
	PcRtP1	5′-[FAM]-TCAGAATCTCTGTTTTCGAACCCGACGA-[TAMRA]-3′
Plectosphaerella cucumerina	*Pa. cucumerina* F2	5′-CATCAGCCTCGCATTGGGAT-3′
	Pa. cucumerina R2	5′-CTACCTGATCCGAGGTCAAC-3′
	Pa. cucumerina P1	5′-[FAM]-CCCTCGGCGTCCTGCCTCTA-[TAMRA]-3′
Plant[b]	COX F	5′-CGTCGCATTCCAGATTATCCA-3′
	COX RW	5′-CAACTACGGATATATAAGRRCCRRAACTG-3′
	COX probe	5′-[VIC]-AGGGCATTCCATCCAGCGTAAGCA-[TAMRA]-3′

[a] From Atkins *et al*. (2003).
[b] From Hughes *et al*. (2006). Probe modifications: fluorescent reporter dye FAM (6-carboxy-fluorescein) and VIC® (Applied Biosystems), and quencher dye TAMRA (6-carboxy-tetramethylrhodamine).

added, giving a final volume of 25 µL. The cycling parameters and reaction mixture concentrations were those described by Atkins *et al*. (2003). For the COX assay, the cycling parameters and reaction mixture were those described by Hughes *et al*. (2006). The new *Pa. cucumerina* assay was optimised to be species-specific and consisted of 1 × Buffer A and 0.025 U μL^{-1} AmpliTag Gold, plus 0.4 mM of each dNTP, 2.5 mM $MgCl_2$, 300 nM forward primer F2, 300 nM reverse primer R2, and 100 nM probe P1. For each reaction, 1 µL of DNA extract was added, giving a final volume of 25 µL. Cycling conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 65°C. Negative controls containing water instead of DNA were included in all runs. The cycle threshold (Ct) value for each reaction was determined using the StepOne software v2.0. The reactions were carried out in triplicate. The sensitivity of the *Pa. cucumerina* F2/R2/P1 assay was assessed with a ten-fold dilution series of *Pa. cucumerina* DNA (isolate PLC 6) (i) in molecular-grade water and (ii) in a DNA solution obtained from healthy rocket leaves (concentration 50 ng μL^{-1}), with final concentrations of 1 ng μL^{-1} to 1 fg μL^{-1} in a 25 µL reaction (Figure 1).

Comparison of testing inoculated host material by isolation or real-time PCR

A total of 18 inoculated plant samples and one non-inoculated healthy control sample was used to perform a comparison between the newly designed real-time PCR assay, by direct extraction from plant material, and the traditional isolation method followed by morphological assessment.

Results

Assay specificity

The specificity of the newly designed assay was successfully checked against 49 isolates of fungi, including 15 isolates of *Pa. cucumerina*, which gave mean Ct values ranging from 19.17 to 29.32, with non-target species of five closely related *Plectosphaerella* species and other fungi commonly associated with rocket showing no amplification, i.e. Ct >40 (Table 1). The PcRTF1/PcRTR1/PcRTP1 real-time PCR assay (Atkins *et al*., 2003) gave no distinction between the *Plectosphaerella* species, with mean Ct values ranging from 15.02 to 20.30.

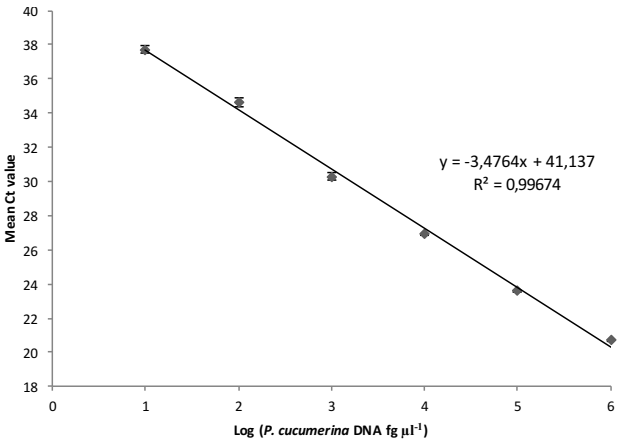

Figure 1. Standard curve and correlation coefficient assessed with a dilution series of *Plectosphaerella cucumerina* DNA for the *Pa. cucumerina* F2/R2/P1 real-time PCR assay. DNA of the pathogen was obtained from healthy rocket leaves (concentration 50 ng μL^{-1}). Mean Ct values are for duplicate reactions; error bars indicate standard deviations.

Assay sensitivity

For both dilution series of *Pa. cucumerina* DNA prepared in molecular-grade water and from healthy rocket leaves, linear relationships between Ct values and log (target concentration) were observed, and the lowest concentration of target DNA detected by the assay was 10 fg. No increase in Ct value or decrease in amplification efficiency were observed with the dilution series in the DNA solution obtained from healthy rocket leaves, indicating that the assay is not affected by the presence of plant DNA or potentially inhibitory substances, which may be present in plant DNA extracts (Figure 1).

Comparison of testing of inoculated host material by isolation and real-time PCR

DNA extraction was successful in all 19 extractions (Table 3), indicated by the detection of COX DNA by real-time PCR, with mean Ct values ranging from 22.05 to 25.58. *Plectosphaerella cucumerina* DNA was detected in all artificially inoculated samples by real-time PCR, with mean Ct values ranging from 24.99 to 32.08. *Plectosphaerella cucumerina* fungal colonies were also successfully isolated from all artificially inoculated samples, using the traditional iso-

Table 3. List of healthy and inoculated rocket samples tested by isolation onto media and real-time PCR, targeting *Plectosphaerella cucumerina* DNA using the *Pa. cucumerina* F2/R2/P1 assay, and plant DNA using the COX F/RW/ probe assay.

Sample	Inoculation	*Pa. cucumerina* isolation	Real-time PCR Ct values[a]	
			COX assay	*P. cucumerina* assay
H1	-	-	22.05 (0.1)	-
I1	+	+	22.58 (0.1)	25.02 (0.1)
I2	+	+	23.80 (1.4)	25.71 (1.0)
I3	+	+	22.45 (0.0)	32.08 (0.5)
I4	+	+	22.78 (0.2)	27.92 (0.0)
I5	+	+	23.12 (0.2)	29.44 (0.1)
I6	+	+	22.51 (0.1)	30.13 (0.1)
I7	+	+	22.33 (0.3)	28.87 (0.1)
I8	+	+	22.19 (0.0)	25.04 (0.0)
I9	+	+	24.97 (0.7)	25.74 (0.6)
I10	+	+	24.20 (2.0)	25.00 (0.2)
I11	+	+	25.58 (3.5)	28.09 (0.0)
I12	+	+	22.32 (0.3)	31.86 (0.0)
I13	+	+	23.93 (1.4)	25.67 (0.1)
I14	+	+	22.56 (0.0)	29.28 (0.0)
I15	+	+	24.40 (2.6)	30.02 (0.0)
I16	+	+	23.02 (0.0)	29.47 (0.1)
I17	+	+	22.66 (0.8)	24.99 (0.3)
I18	+	+	23.74 (0.1)	25.96 (0.1)

[a] Cycle threshold (Ct) values are mean values for triplicate reactions. Standard deviations are indicated in brackets. Dashes indicate a negative result (i.e. no Ct value obtained after the completion of 40 cycles).

lation method. No *Pa. cucumerina* DNA was detected by real-time PCR, and no *Pa. cucumerina* cultures were obtained, using the isolation method from the non-inoculated healthy controls.

Discussion

This paper describes the development of a real-time PCR assay for the effective *in planta* detection of *Pa. cucumerina.* Early and accurate detection of the pathogen are very important, because the pathogen and disease have become of increasing concern to the rocket-producing farmers and industry.

This study has shown that the new *Pa. cucumerina* assay is highly specific, not amplifying five non-target species of *Plectosphaerella or* 18 other commonly found fungal species. The previously designed assay by Atkins *et al*., (2003) showed cross reaction with all *Plectosphaerella* species tested, and therefore can not be used as a tool for species-specific identification of *Pa. cucumerina* (Table 1). The new assay was able to detect a target DNA concentration of 10 fg, which is comparable to levels detected by other real-time PCR assays designed to detect plant pathogens *in planta* (Ioos *et al*., 2009a, 2009b). The sensitivity and efficiency of the assay were not adversely affected by the presence of DNA extracted from healthy wild rocket leaf material. The new *Pa. cucumerina* assay was successfully used to detect the pathogen in artificially inoculated plant tissues. False positives were not obtained from healthy host material. A series of 19 samples (18 inoculated and one non-inoculated) were analysed both by both real-time PCR and isolation onto media, and giving consistent results between the two methods, indicating a 100% relative specificity.

The use of *Pa. cucumerina*-free certified plant propagation material will become an essential prerequisite for worldwide distribution of this crop (Gilardi *et al*., 2013). The turnaround time for molecular testing can be within a few hours of sample receipt in comparison with the classical method of isolation taking 7–10 d, followed by morphological assessment. Detection by isolation of the pathogen in culture is labour intensive and requires specialised mycological skills, whereas the use of molecular methods can mitigate the latter problem, and can be utilised more widely for efficient pathogen detection.

This study has demonstrated the ability of this new assay to detect *Pa. cucumerina* DNA in symptomatic leaf tissues of wild rocket by extraction of plant material followed by real-time PCR.

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