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Validation of a duplex TaqMan real-time PCR for detection of *Colletotrichum coccodes* and *Rhizoctonia solani* AG-3 on potato tubers

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Summary. The potato pathogens *Colletotrichum coccodes*, (causing black dot), and *Rhizoctonia solani* anastomosis group 3 (AG-3) (causing black scurf and stem canker) are economically important, and are common in potato production regions, including Northern Italy. A duplex TaqMan quantitative real-time PCR, based on two previously validated singleplex methods, was tested for identification of these species in potato propagation material. This validation was carried out according to EPPO PM 7/98 standard guidelines. The limit of detection (LOD) for the method, assessed using serial dilutions of targets DNA, was 10 fg. Specificity was assessed by testing ten *C. coccodes* and four *R. solani* AG-3 isolates, and 19 non-target species including other *Colletotrichum* and *Rhizoctonia* spp. and potato pathogens, respecting inclusivity and exclusivity criteria. Selectivity of the test showed no influence of DNA obtained from potato tubers. Repeatability and reproducibility of the duplex qPCR were also validated. The assay was able to detect and distinguish, within a single run, the two fungi allowing early detection in potato tubers.

Keywords. Quantitative PCR, diagnostics, black dot, black scurf, stem canker, *Solanum tuberosum* L.

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INTRODUCTION

Potatoes (*Solanum tuberosum* L.) are one of the most consumed human food crops, with rice and wheat. Asia produces most potatoes, followed by Europe, where five countries are among the ten greatest producers (FAO, 2022; Europatat, 2023). Italy is the fifth largest producer in Europe with approx. 33,000 ha grown, and total production of 10 million tons in 2024 (Çalışkan *et al.*, 2023; ISTAT, 2024).

Potato crops can be affected by several diseases caused by air- or soil-borne pathogens, which can cause severe damage on all parts of potato plants, especially tubers which are economically important plant organ (Fiers *et al.*,

2012). *Colletotrichum coccodes* and *Rhizoctonia solani*, among the most important pathogens of potato, occur commonly in potato-producing regions, and cause, respectively, black dot and black scurf/stem canker.

Colletotrichum coccodes has a wide host range, affecting several horticultural species of *Cucurbitaceae*, *Fabaceae* and *Solanaceae*. Tuber blemish, stem and root lesions are the main symptoms caused by this pathogen (Tsor, 2004; Aqeel *et al*., 2008; Fiers *et al*., 2012). The first record of this fungus in Italy was reported by Buonaurio *et al.* (2002), after detection of black dot symptoms on potato tubers, with incidence ranging from 50 to 100%. *Rhizoctonia solani* causes potato symptoms of tuber blemish and stem lesions (Woodhall *et al*., 2008; Fiers *et al*., 2012), and losses related to *R. solani* in potato crops range from 10 to 30% (Banville *et al*., 1996). Hyphae of *Rhizoctonia* spp. can anastomose with isolates of other anastomosis groups (AGs), and previous studies have shown that *R. solani* AG-3 is the predominant anastomosis group affecting potato crops (Anderson, 1982; Banville *et al*., 1996). Both *C. coccodes* and *R. solani* colonize the surfaces of potato tubers, and can also infect potato plant vascular systems in roots and stems (Stevenson *et al*., 2001; Lehtonen *et al*., 2008; Fiers *et al*., 2012).

Northern Italy is an intensive potato cultivation area, and these pathogens are important causes of potato yield decline. Surveys carried out in specialized potato farms have confirmed *C. coccodes* and *R. solani* as endemic pathogens in this region (Manici and Caputo, 2009; Manici *et al*., 2016). Early detection of these pathogens, in the field and in seed potato material, is important for reducing pathogen spread, and to assist disease management and reduce yield losses.

Detection of *C. coccodes* and *R. solani* was originally based on isolation methods, use of selective media, and morphological characterization, but these methods are not providing quantification of pathogen incidence. These methods are also time-consuming, and are biased by the operator competence (Lees and Hilton, 2003). Polymerase chain reactions (PCRs) are molecular methods widely used for disease diagnoses and identification of several potato pathogens, including *Helminthosporium solani* (Cullen *et al*., 2001), *Alternaria solani* (Niu *et al*., 2022), and *Fusarium coeruleum* (Heltoft *et al*., 2016), and they can be used to assess asymptomatic plant samples.

Several diagnostic methods were developed for specific individual detection of *C. coccodes* and *R. solani*. Specific primer pairs for *C. coccodes* detection were designed for conventional PCR by Cullen *et al.* (2002), and two TaqMan qPCR protocols were developed targeting the ribosomal internal transcribed spacer (ITS) region (Cullen *et al*., 2002) and the glycerol-3-phosphate dehydrogenase (*gapdh*) gene (Ryazantsev *et al*., 2023), to detect *C. coccodes* in potato tubers and soil. The method described by Ryazantsev *et al.* (2023) was developed considering the high variability of this fungus, that can lead to false negatives if a single DNA sequence is used for detection. However, analyses performed in other laboratories using the primers and the probe used by Cullen *et al.* (2002) gave good accuracy for detecting *C. coccodes* (Belov *et al*., 2018). A conventional PCR protocol, specific for *R. solani* AG-3 detection, was validated by Lees *et al.* (2002), while specific quantitative real-time PCR, using SYBR Green and TaqMan chemistries, were developed based on the ribosomal internal transcribed spacer (ITS) by Lees *et al.* (2002), Woodhall *et al.* (2013), and Salamone and Okubara (2020).

Simultaneous presence of multiple pathogens on potatoes is challenging for farmers, and time-consuming for laboratories testing for potato pathogens. Given the challenges in distinguishing and accurately identifying symptoms and the pathogens responsible, development of multiplex qPCR assays could speed up analyses, allowing rapid monitoring and detection of pathogens in the field, and assisting disease management in the field and certification of the propagation material that could be sources of pathogen inoculum. To date, no duplex qPCR protocols have been developed for detection of *C. coccodes* and *R. solani* AG-3 detection.

The aim of the present study was to test and validate a method for detecting and quantifying these two pathogens on potato tubers.

MATERIALS AND METHODS

Fungus isolates

During 2022 and 2023, monitoring activities were carried out on potato crops (cv. 'Monique'), both on seed potatoes and on field-grown plants. For the propagation material, 100 tubers were examined each year. Field monitoring was conducted during the cropping cycle in both years, in two sites located in the province of Alessandria, Piedmont (Northwest Italy), at coordinates 44.9328, 8.9369 and 44.9358, 8.9351, respectively. In addition, observations were extended to the storage phase. Symptomatic tubers and plant portions, collected during the monitoring activities, were surface disinfected with 1% sodium hypochlorite water solution for 1 min, rinsed in sterile deionized water, dried, then cut into small sections (0.2–0.3 cm long) which were placed into Petri dishes containing potato dextrose agar (PDA; Merck) amended with 25 mg L^{-1} of streptomycin sulphate. These plates were then incubated in a cli-

mate chamber at 25±1°C for 72 h, after which resulting colonies were transferred and grown on PDA plates, and were then purified to obtain single conidium or monohyphal cultures.

Selected isolates of *C. coccodes* (eight) and *R. solani* (three) were morphologically and molecularly characterized (as described below), and used in the validation process. One reference isolate (*R. solani* AG-3 MUCL 51930) for comparisons was provided by the *Mycothèque de l'Université Catholique de Louvain* (BCCM/MUCL). Two *C. coccodes* isolates, and 16 isolates of species phylogenetically related to the target species and potato pathogens were provided by the Università di Torino (Agroinnova collection), and isolates of *C. destructivum complex* (CBS 136232) and *C. lentis* (CBS 127604) were provided by the Westerdijk Fungal Biodiversity Institute. Isolates used in this study are reported in Table 1. Monoconidial cultures of the isolates obtained in the present study were stored in tubes of PDA at 4°C.

DNA extraction and molecular identification of Colletotrichum coccodes *and* Rhizoctonia solani *AG-3 isolates*

Eight isolates morphologically identified as *C. coccodes*, and three isolates of *R. solani* were grown on PDA for 7 d at 25±1°C, and 100 mg of mycelium was gently scraped from respective colony surfaces for DNA extraction, using the Omega E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-Tek), following the manufacturer's instruc-

Table 1. Fungal species used in this study for the validation of a TaqMan duplex qPCR assay.

Species	Strain ID	Host	Source	Origin
Colletotrichum coccodes	P178	*Solanum tuberosum*	Tuber	Italy
C. coccodes	P180	*Solanum tuberosum*	Tuber	Italy
C. coccodes	P181	*Solanum tuberosum*	Tuber	Italy
C. coccodes	TS1	*Solanum tuberosum*	Tuber	Italy
C. coccodes	TS2	*Solanum tuberosum*	Tuber	Italy
C. coccodes	TS3	*Solanum tuberosum*	Tuber	Italy
C. coccodes	TS5	*Solanum tuberosum*	Tuber	Italy
C. coccodes	TS11	*Solanum tuberosum*	Tuber	Italy
C. coccodes	T126	*Solanum lycopersicum*	Roots	Italy
C. coccodes	T125.1	*Solanum lycopersicum*	Roots	Italy
C. nigrum	CVG 171	*Salvia greggii*	Leaves	Italy
C. nigrum	CVG 173	*Salvia greggii*	Leaves	Italy
C. karsti	KUM6	*Citrus sinensis*	Fruit	Italy
C. fioriniae	CVG 268	*Origanum vulgare*	Leaves	Italy
C. fructicola	CVG 170	*Salvia greggii*	Leaves	Italy
C. ocimi	CVG 200	*Ocimum basilicum*	Leaves	Italy
C. americae-borealis	CBS 136232	*Medicago sativa*	Stems	United States of America
C. lentis	CBS 127604	*Lens culinaris*	Seeds	Canada
C. lineola	CVG 207	*Campanula trachelium*	Leaf	Italy
C. gloeosporioides	CVG 1940	*Citrus sinensis*	Fruit	Italy
Rhizoctonia solani AG-3	MUCL 51930	*Solanum tuberosum*	Plant tissue	Belgium
R. solani AG-3	P37	*Solanum tuberosum*	Tuber	Italy
R. solani AG-3	P38	*Solanum tuberosum*	Tuber	Italy
R. solani AG-3	P257	*Solanum tuberosum*	Tuber	Italy
R. solani AG-2	St 11.6	*Fragaria ananassa*	Roots	Italy
R. solani	RS 230	*Fragaria ananassa*	Roots	Italy
R. solani	RS 232	*Fragaria ananassa*	Roots	Italy
R. solani	RS 256	*Fragaria ananassa*	Roots	Italy
R. solani	C22	*Allium cepa*	Roots	Italy
Fusarium oxysporum	P149	*Solanum tuberosum*	Tuber	Italy
F. oxysporum	P153	*Solanum tuberosum*	Tuber	Italy
Alternaria alternata	P150	*Solanum tuberosum*	Tuber	Italy
A. alternata	P154	*Solanum tuberosum*	Tuber	Italy

tions. Quality and the concentration of the DNA were checked with a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). Isolates of *Colletotrichum* spp. were identified through amplification of the partial glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*), using GDF1 and GDR1 primers (Guerber *et al.* 2003), and the primers ITS1 and ITS4 (White *et al.* 1990) were used to amplify the internal transcribed spacer regions (ITS) for the *Colletotrichum* spp. and *R. solani* isolates.

For both fungi, PCRs were performed with a Taq DNA polymerase kit (Qiagen), following amplification mixtures and relative cycling conditions described by Damm *et al.* (2012). Amplicons generated were checked by electrophoresis on 1% agarose (VWR), and both strands of the PCR products were sequenced by Eurofins Genomics Service. The generated sequences were analysed and assembled using the program Geneious v. 11.1.5 (Auckland, New Zealand).

Specific primers and duplex qPCR optimization

Specific primers and probes for *C. coccodes* and *R. solani* AG-3 used in this study, both designed on ITS regions, were described, respectively, by Cullen *et al.* (2002) and Lees *et al.* (2002) (Table 2). For each target, qPCR assay optimization and validation were carried out in previous studies (Cullen *et al.* 2002; Lees *et al.* 2002), and were then verified in the present study following the EPPO PM 7/98 guidelines (EPPO, 2021).

Optimum conditions for the duplex amplification of the two target species were evaluated by applying a thermal gradient from 56 to 62°C to assess the optimum annealing temperature, and using different concentrations of primers and probes (from 0.1 to 0.3 μM).

Duplex qPCR assays were carried out with the StepOnePlus qPCR system (Applied Biosystems), using 2× TaqMan Universal Master Mix (Applied Biosystems). Each reaction was carried out in 10 μL total volume, using 2 μL of DNA (10 ng), extracted as described above, and each plate was loaded with standard DNA, a positive control (target pathogen DNA), and a negative control (nuclease-free water), in triplicate. Data collected by the assay instrument were analysed, and presence and quantities of target DNA were assumed by interpolating Ct values from the standard curves generated.

Validation of the duplex qPCR for Colletotrichum coccodes *and* Rhizoctonia solani *AG-3*

The analytical sensitivity, specificity, selectivity, repeatability, and reproducibility of the duplex qPCR assay were assessed, according to the EPPO PM 7/98 standard (EPPO, 2021).

Analytical sensitivity of the reaction was assessed using ten-fold serial dilutions (from 1 ng to 1 fg) obtained from genomic DNA of the targets. The analysis was performed in triplicate, and target DNA was quantified in the samples using the regression line. Analytical sensitivity was assessed both for fungal DNA and inoculated potatoes.

Target and non-target DNA were used to validate analytical specificity of the method. All samples were first analysed with singleplex qPCR, during the verification of the protocols, then the same samples were used in duplex assays to assess any differences in results from the two methods. Ten isolates of *C. coccodes* (eight from potato tubers and plants in this study, and two from *Solanum lycopersicum*), four isolates of *R. solani* AG-3 (three isolated from potato tubers in the present study, and one provided by the *Mycothèque de l'Université Catholique de Louvain* (BCCM/MUCL)), as well as 19 non-target species (Table 2), were used. The non-target isolates included different *Colletotrichum* and *Rhizoctonia* spp. and other potato pathogens.

Selectivity of the assay was evaluated to establish the influence of the host DNA. Healthy potato DNA was added to DNA from *C. coccodes* and *R. solani* AG-3, each at a concentration of 1 ng μL^{-1}. Repeatability was assessed

Table 2. Primer pairs and probes used in this study for the detection of *Colletotrichum coccodes* and *Rhizoctonia solani* AG-3 with the TaqMan quantitative PCR assay.

Primer pairs and probes	Sequence (5'-3')	Target DNA	Reference
CcTqF1	TCTATAACCCTTTGTGAACATACCTAACTG		
CcTqR1	CACTCAGAAGAAACGTCGTTAAAATAGAG	ITS1	Cullen et al. (2002)
CcTqP1	[VIC]-CGCAGGCGGCA CCCCCT-[TAMRA]		
RsTqF1	AAGAGTTTGGTTGTAGCTGGTCTATTT		
RsTqR1	AATTCCCCAACTGTCTCACAAGTT	ITS1	Lees et al. (2002)
RQP1	[FAM]-TTTAGGCATGTGCACACCTCCCTCTTTC-[TAMRA]		

by running three independent duplex qPCRs in the same laboratory on different days, while the reproducibility was evaluated by performing the assay by different operators on different days, in the same laboratory.

Validation of duplex qPCR in artificially inoculated potato samples (cv. 'Monique')

The *C. coccodes* (P178) and *R. solani* AG-3 (MUCL 51930) isolates were grown on PDA plates at 25±1°C for 7 d. Seven potato tubers (cv. 'Monique') were surface disinfected with 5% sodium hypochlorite solution for 5 min, then rinsed twice with sterile deionized water, air dried, and then tested with the assay to confirm absence of pathogens. Five tubers were each inoculated with 5 mm diam. mycelium plugs of both pathogens, taken from 7-d-old PDA cultures, and sealed with parafilm, while two tubers were used as negative controls (one inoculated with the non-target pathogens *C. gloeosporioides* and *R. solani* AG-2, and one inoculated with water). These tubers were then incubated at 25°C for 15 d. The mycelium plugs were removed, and symptomatic portions of the tubers were cut and disinfected to proceed with molecular analyses. The tissues from the inoculated and negative controls were ground with liquid nitrogen, and DNA was extracted with the E.Z.N.A. Plant DNA kit (VWR), following the manufacturer's instructions. Each sample was analysed with duplex qPCR assay in triplicate, and quantities (pg μL^{-1}) of detected *C. coccodes* and *R. solani* AG-3 DNA were determined.

Data analyses

StepOne software automatically generated threshold cycle (Ct) values, baseline range, and real-time PCR standard curves. Data were statistically analysed by variance analysis ANOVA, using Tukey's test ($P \leq 0.05$), with the Statistical Package for Social Science, Version 29.0 (SPSS; IBM, Chicago, Illinois, United States of America).

RESULTS

Isolation and molecular identification of the isolates

During monitoring activities, eight *Colletotrichum* spp. and three *R. solani* isolates obtained from symptomatic potato plants and tubers of cv. 'Monique' were morphologically and molecularly analysed. For molecular identification, all DNA sequences obtained were aligned with reference sequences deposited in the NCBI database, showing similarity percentages from 99% to 100% for all the isolates tested. *Colletotrichum* isolates were all identified as *C. coccodes*, while the three *R. solani* isolates were identified as within the anastomosis group 3. All the isolates were used for the duplex qPCR protocol validation.

Specific primers and duplex qPCR optimization

The specificity of the two sets of primers and probes was verified following EPPO PM 7/98 guidelines (EPPO, 2021).

Optimum conditions for duplex qPCR amplification with 2× TaqMan Universal Master Mix (Applied Biosystems) were: 0.3 μM for CcTqF1/CcTqR1, 0.1 μM CcTqP1, and 0.2 μM for RsTqF1/RsTqR1 and RsP1, and annealing temperature of 60°C for 1 min. Duplex reactions were each carried out in a final volume of 10 μL, using the following thermal protocol: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min.

Duplex qPCR validation

Analytical sensitivity, analytical specificity, selectivity repeatability, and reproducibility of the duplex qPCR protocol were evaluated according to EPPO PM7/98 standard (EPPO, 2021).

Seven serial dilutions, ranging from 1 ng to 1 fg, of the two target DNA were tested to assess the analytical sensitivity of the assay, showing a LOD of 10 fg, for both pathogens. Average values obtained performing the analysis in triplicate were included in the regression line, obtained with the determination coefficient (R^2) values of 0.9977 for *C. coccodes* and of 0.999 for *R. solani* AG-3, and then used to quantify targets DNA in samples tested (Figure 1). The average reaction efficiencies, calculated from the slopes of the regression lines obtained, were of 93% for *C. coccodes* and 90% for *R. solani* AG-3.

In duplex real-time PCR, seven ten-fold serial dilutions of samples containing 1 ng μL^{-1} of both target DNAs, were spiked with healthy potato DNA. Selectivity of the method was validated showing no influence of the host DNA in the duplex qPCR assay, and this showed reliable reaction efficiencies and correlations between the quantities of target DNA and the Ct values detected by the instrument, both when target pathogen DNA was spiked or not with potato DNA (Figure 2).

The duplex assay showed analytical specificity of 100% when tested with a panel of fungal isolates that considered inclusivity and exclusivity criteria, detecting

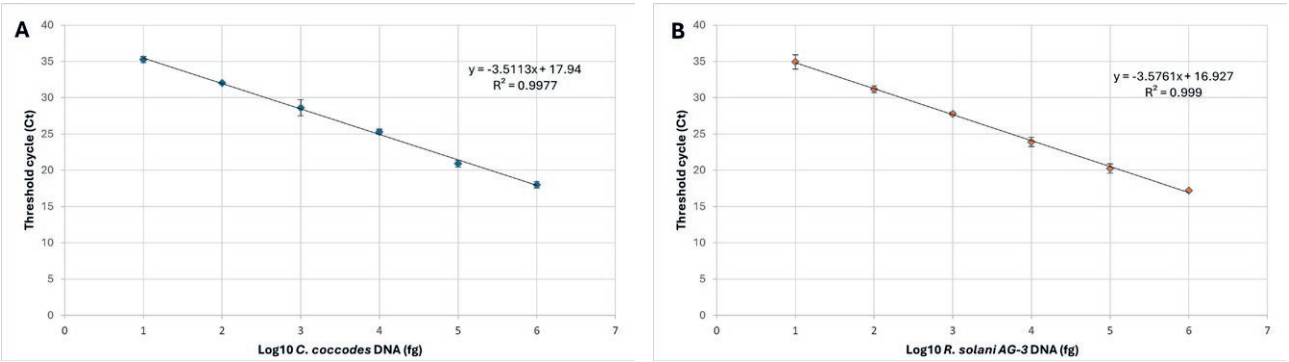

Figure 1. Standard curves obtained in duplex qPCR, running 1:10 serial dilutions (from 1 ng to 10 fg) of DNA of *Colletotrichum coccodes* (A) and *Rhizoctonia solani* AG-3 (B). The coefficients of determination (R2) are indicated, and the standard deviation bars are shown for each mean value in the graphs. Each point of the regression lines was run in triplicate.

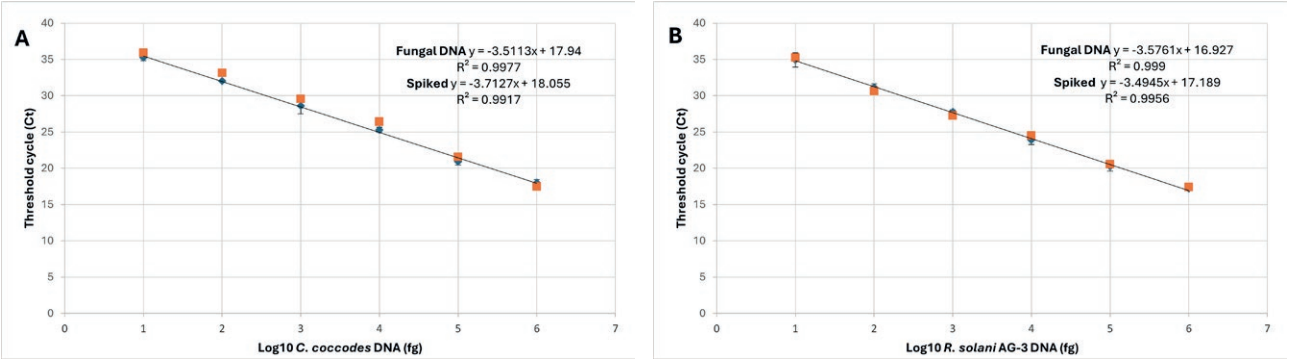

Figure 2. Standard curves obtained in duplex qPCR, running 1:10 serial dilutions (from 1 ng to 10 fg) of DNA of *Colletotrichum coccodes* (A) and *Rhizoctonia solani* AG-3 (B), both spiked with potato tuber DNA. The coefficients of determination (R^2) are indicated, and the standard deviation bars are shown for each mean value in the graphs. Each point of the regression lines was run in triplicate.

amplifications only in DNA samples from target species. The specificity of the assays was compared with the singleplex tests, showing non-significant differences among the Ct values detected (Table 3).

Repeatability and reproducibility of the method were assessed by producing reliable amplifications among biological and technical replications, with no statistically significant differences ($P > 0.05$), when analysed under unchanged or modified test conditions, as described in the EPPO standard PM 7/98.

Validation of duplex qPCR in artificially inoculated potato samples (cv. 'Monique')

Analyses carried out on artificially inoculated potato tubers cv. 'Monique' showed the efficacy of the validated method for detecting and distinguishing true positive and true negative samples. The duplex qPCR assay gave positive amplifications, with Ct values ranging from 21.3 to 33, only for DNA samples extracted from artificially inoculated potato tubers, while no amplifications were observed for negative controls (Table 4).

DISCUSSION

Molecular tools are commonly used for detection of plant pathogens, allowing rapid, accurate and specific identification and quantification of target species affecting crops, even in early stages of plant growth in propagation material and, when more than one species occurs in the same sample, controlling the spread of plant pathogens and the diseases they cause (McCartney *et al.*, 2003; Capote *et al.*, 2012; Hariharan and Prasannath, 2021). In the present study, a sensitive and specific duplex TaqMan qPCR assay was tested and validated for detection and quantification of *C. coccodes* and *R. solani* AG-3 in potato samples.

Currently, two TaqMan qPCR assays based on amplification of the ITS1 region of rDNA, developed by,

Table 3. Fungus isolates used in this study to check specificity of the duplex qPCR assay for detection of *Colletotrichum coccodes* and *Rhizoctonia solani AG-3*, and *Ct* values acquired in singleplex and duplex reactions. Standard deviations are included for each Ct.

Species	Strain ID	Host	Source	Origin	Singleplex *C. coccodes* (Ct)	Singleplex *R. solani* AG-3 (Ct)	Duplex Real-time PCR (Ct)
Colletotrichum coccodes	P178	*Solanum tuberosum*	Tuber	Italy	15.72±0.65	Not detected	15.12±0.11
C. coccodes	P180	*Solanum tuberosum*	Tuber	Italy	15.16±0.08	Not detected	15.27±0.14
C. coccodes	P181	*Solanum tuberosum*	Tuber	Italy	16.15±0.51	Not detected	16.65±0.31
C. coccodes	TS1	*Solanum tuberosum*	Tuber	Italy	18.92±0.27	Not detected	18.51±0.24
C. coccodes	TS2	*Solanum tuberosum*	Tuber	Italy	16.39±0.34	Not detected	16.33±0.46
C. coccodes	TS3	*Solanum tuberosum*	Tuber	Italy	18.73±0.61	Not detected	18.66±0.79
C. coccodes	TS5	*Solanum tuberosum*	Tuber	Italy	15.51±0.24	Not detected	15.82±0.02
C. coccodes	TS11	*Solanum tuberosum*	Tuber	Italy	18.58±0.36	Not detected	18.62±0.23
C. coccodes	T126	*Solanum lycopersicum*	Roots	Italy	17.32±0.22	Not detected	17.26±0.16
C. coccodes	T125.1	*Solanum lycopersicum*	Roots	Italy	16.20±0.34	Not detected	16.32±0.01
C. nigrum	CVG 171	*Salvia greggii*	Leaves	Italy	Not detected	Not detected	Not detected
C. nigrum	CVG 173	*Salvia greggii*	Leaves	Italy	Not detected	Not detected	Not detected
C. kartsi	KUM6	*Citrus sinensis*	Fruit	Italy	Not detected	Not detected	Not detected
C. fioriniae	CVG 268	*Origanum vulgare*	Leaves	Italy	Not detected	Not detected	Not detected
C. fructicola	CVG 170	*Salvia greggii*	Leaves	Italy	Not detected	Not detected	Not detected
C. ocimi	CVG 200	*Ocimum basilicum*	Leaves	Italy	Not detected	Not detected	Not detected
C. americae-borealis	CBS 136232	*Medicago sativa*	Stems	USA	Not detected	Not detected	Not detected
C. lentis	CBS 127604	*Lens culinaris*	Seeds	Canada	Not detected	Not detected	Not detected
C. lineola	CVG 207	*Campanula trachelium*	Leaf	Italy	Not detected	Not detected	Not detected
C. gloeosporioides	CVG 1940	*Citrus sinsensis*	Fruit	Italy	Not detected	Not detected	Not detected
Rhizoctonia solani AG-3	MUCL 51930	*Solanum tuberosum*	Plant tissue	Belgium	Not detected	19.22±0.41	20.40±0.04
R. solani AG-3	P37	*Solanum tuberosum*	Tuber	Italy	Not detected	19.90±0.63	20.53±0.12
R. solani AG-3	P38	*Solanum tuberosum*	Tuber	Italy	Not detected	19.92±0.15	20.36±0.12
R. solani AG-3	P257	*Solanum tuberosum*	Tuber	Italy	Not detected	19.58±0.88	20.22±0.04
R. solani AG-2	St 11.6	*Fragaria ananassa*		Italy	Not detected	Not detected	Not detected
R. solani	RS 230	*Fragaria ananassa*	Roots	Italy	Not detected	Not detected	Not detected
R. solani	RS 232	*Fragaria ananassa*	Roots	Italy	Not detected	Not detected	Not detected
R. solani	RS 256	*Fragaria ananassa*	Rootss	Italy	Not detected	Not detected	Not detected
R. solani	C22	*Allium cepa*	Roots	Italy	Not detected	Not detected	Not detected
Fusarium oxysporum	P149	*Solanum tuberosum*	Tuber	Italy	Not detected	Not detected	Not detected
F. oxysporum	P153	*Solanum tuberosum*	Tuber	Italy	Not detected	Not detected	Not detected
Alternaria alternata	P150	*Solanum tuberosum*	Tuber	Italy	Not detected	Not detected	Not detected
A. alternata	P154	*Solanum tuberosum*	Tuber	Italy	Not detected	Not detected	Not detected

respectively, Cullen *et al.* (2002) and Lees *et al.* (2002), are used for the detection of *C. coccodes* and *R. solani* AG-3, also gave reliable results in testing the isolates obtained in the present study. A specific duplex TaqMan qPCR was tested and validated, based on the above methods, to detect both pathogens using a a single run.

Other singleplex assays have been designed and validated for *C. coccodes* and *R. solani* AG-3, giving similar detection sensitivity. A TaqMan Real-Time PCR was developed for *C. coccodes* detection by the amplification of the *gapdh* genomic locus, to reduce false negatives results, due to the high variability of this fungus (Ryazantsev *et al.*, 2023). However, all the isolates used in the present showed positive amplifications with both, the assay developed by Cullen *et al.* (2002), and the duplex qPCR assay validated in the present study, as observed for isolates tested with Cullen *et al.* (2002) protocol in other studies (Belov *et al.*, 2018). The assay was also able to distinguish *C. coccodes* from *C. nigrum*, a closely related species which has been reported as an important pathogen of solanaceous hosts (Liu *et al.*, 2013). A specific qPCR protocol was also validated for *R. solani* AG-3 by Salamone and Okubara (2020), with the primers designed on a portion of the ITS1 region for the forward primer, and on a conserved portion of the 5.8S rRNA gene for the reverse primer. This allowed identification

Table 4. Mean numbers of cells µL-1 of *Colletotrichum coccodes* and pg DNA µL-1 of *Rhizoctonia solani* AG-3 detected in five artificially inoculated tuber samples. Two tubers inoculated with non-target *Colletotrichum* and *Rhizoctonia* species (*C. gloeosporioides* and *R. solani* AG-2) and with sterile deionized water were used as negative controls.

Sample ID	Ct mean ± SD *C. coccodes*	Concentration of *C. coccodes* (pg DNA µL^{-1})	Ct mean ± SD R. solani AG-3	Concentration of R. solani AG-3 (pg DNA µL^{-1})
PAT_IN_1	23.12±0.24	32.80±0.07	22.10±0.30	35.90±0.08
PAT_IN_2	24.03±0.02	18.00±0.01	23.21±0.06	17.50±0.02
PAT_IN_3	29.00±0.19	0.69±0.05	29.95±0.15	0.23±0.04
PAT_IN_4	21.30±0.38	108.00±0.11	17.90±0.14	536.00±0.04
PAT_IN_5	22.97±0.08	36.10±0.02	29.90±0.10	0.24±0.03
PAT_IN_NCF	Not detected	Not detected	Not detected	Not detected
PAT_IN_NC_water	Not detected	Not detected	Not detected	Not detected

of Pacific Northwest (United States of America) isolates, which were not detectable by Lees *et al.* (2002). The strains revealed as undetectable by the Lees *et al.* (2002) assay were all isolated in Washington State. However, no issues were observed in detecting strains from other origins, indicating the high specificity of the validated assay. In the present study, all analysed isolates were successfully detected and quantified using the validated assay.

Specificity of the duplex qPCR assay was assessed testing ten *C. coccodes* and four *R. solani* AG-3 DNA samples extracted from pure cultures of these fungi, including target isolates from hosts other than potato and coming from other countries, showing positive amplifications for all the tested isolates. No false positive results were observed when phylogenetically related strains or other *Colletotrichum* and *Rhizoctonia* species were analyzed. Low differences were observed in Ct values among the tested isolates.

The LOD of the method was approx. 10 fg for both *C. coccodes* and *R. solani* AG-3 detection, yielding reliable and reproducible results comparable to those obtained in simplex qPCR assays (LOD 10 fg) as reported by Cullen *et al.* (2002) and Lees *et al.* (2002).Furthermore, this method was more sensitive for detecting the target species than the method reported by Ryazantsev *et al.* (2023) with a LOD of 500 fg, while comparable analytical sensitivity results were obtained by Salamone and Okubara, (2020) for *R. solani* AG-3 detection (LOD 10 fg). The results obtained in the present study are similar to those obtained by other TaqMan qPCR methods (Prencipe *et al.*, 2020; Ortega *et al.* 2020) and duplex qPCR assays used for other fungi such as *Botryosphaeriaceae* (*Neofusicoccum parvum* and *Botryosphaeria dothidea*) causing canker diseases in woody crops (Romero-Cuadrado, *et al.*, 2023); *Caliciopsis pinea* and *Fusarium circinatum* in pine samples (Luchi *et al.*, 2018), and for the biocontrol agents *Trichoderma asperellum* and *Trichoderma gamsii* (Gerin *et al.*, 2018).

Colletotrichum coccodes and *R. solani* AG-3 DNA were detected and quantified in the presence of DNA of healthy potato tubers, showing no effects on the sensitivity of the assay, and allowing detection and quantification of both pathogens in artificially inoculated samples, discriminating true negative and true positive samples. The results showed no influence on selectivity of the assay for both pathogens, with concentrations ranging from 1.99×10^3 cells µL^{-1} to 12.8 cells µL^{-1} for *C. coccodes,* and ranging from 0.23 pg DNA µL^{-1} to 536.00 pg DNA µL^{-1} for *R. solani* AG-3.

In conclusion, the assay developed and validated in the present study was able to simultaneously detect *C. coccodes* and *R. solani* AG-3 in infected potato samples. The assay could be used to speed up detection of both pathogens in symptomatic samples during potato cropping cycles, to assess seed tubers allowing quantification of the pathogens, and to promptly intervene in managing the disease in the field, as these pathogens can rapidly infect stems, stolons and roots of potato plants grown from infected propagation material (Lees and Hilton, 2003).

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