Effect of Sugar Beet Variety and Nonhost Plant on *Rhizoctonia solani* AG2-2IIIB Soil Inoculum Potential Measured in Soil DNA Extracts

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ABSTRACT

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A direct soil DNA extraction method from soil samples (250 g) was applied for detection of the soilborne sugar-beet-infecting pathogen *Rhizoctonia solani* anastomosis group (AG) 2-2IIIB using a newly developed real-time polymerase chain reaction assay that showed specificity to AG2-2IIIB when tested against various *R. solani* AG. The assay showed a good relation between cycle threshold and amount of AG2-2IIIB sclerotia detected in three spiked field soils and was also able to detect the pathogen in naturally infested field soil

samples. A field trial was conducted to quantify *R. solani* AG2-2IIIB soil inoculum potential (IP) before and after growing a susceptible and a resistant sugar beet variety as well as after subsequent growth of an expected nonhost winter rye. Plants of the susceptible sugar beet variety displayed a higher disease severity. A more than sixfold increase of the *R. solani* AG2-2IIIB soil IP was observed in contrast to the resistant variety that resulted in a constant IP. Growing winter rye significantly reduced soil IP to the initial level at sowing. Further research is required to better understand the interaction between disease occurrence and soil IP as well as the environmental influence on IP development.

Additional keywords: resistance, *Rhizoctonia* crown and root rot.

The soilborne fungus *Rhizoctonia solani* Kühn is a ubiquitous plant pathogen that affects many crops, causing severe damage and yield losses worldwide (Anees et al. 2010a). *R. solani* is known to represent a species complex of different anastomosis groups (AG) and further subgroups (Ogoshi 1987). The fungus is the causal agent of the *Rhizoctonia* crown and root rot of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*), with AG2-2IIIB isolates identified to be the most often occurring and most aggressive subgroup for sugar beet (Bolton et al. 2010; Engelkes and Windels 1996; Strausbaugh et al. 2011); however, differences in pathogenicity between isolates can be observed (Herr and Roberts 1980; O'Sullivan and Kavanagh 1991). *Rhizoctonia* crown and root rot disease is of increasing economic importance in European growing areas and infests approximately 36,000 ha each year (Garcia et al. 2001). Enormous plant losses and yield decline due to *R. solani* infestation can be observed not only in sugar beet growing areas in Europe but also in several parts of the United States of America; for example, Minnesota and North Dakota (Windels and Nabben 1989), Idaho and Oregon (Strausbaugh et al. 2011), as well as Nebraska, Colorado, and Michigan (Kirk et al. 2008; Ruppel and Hecker 1983).

Like other soilborne pathogens, *R. solani* occurs in patches due to its heterogeneous spatial distribution in the field (Herr 1996). Rotational crops such as maize, sorghum, alfalfa, and different bean species are known to be hosts of *R. solani* AG2-2IIIB and can increase disease severity in subsequently grown sugar beet (Buhre et al. 2009; Engelkes and Windels 1996; Kluth and Varrelmann 2010; Rush and Winter 1994). Integrated control strategies, including cultivation of nonhost plants as rotational crops as well as tillage practices avoiding soil compaction, were shown to be appropriate to decrease disease incidence and severity (Buddemeyer and Märländer 2004; Buhre et al. 2009). In contrast to the United States, no fungicides are registered for *Rhizoctonia* crown and root

rot management in Germany. If host plants are cultivated, less susceptible varieties with quantitative resistance traits are the most suitable measure to avoid high disease severity and yield loss, as shown by Buddemeyer and Märländer (2005) for a susceptible and a tolerant sugar beet variety.

Factors such as chemical and physical soil properties, the soil microbiome, climatic conditions, and their interactions are likely to affect the soil inoculum potential (IP; defined in this study as amount of target DNA per gram of soil) and trigger disease outbreak as well as determine disease severity (Anees et al. 2010a). However, these relations and interactions are not sufficiently understood and, thus, it is currently not possible to implement prediction of disease risk in integrated control strategies. It is supposed that susceptible plant species increase the *R. solani* IP in the soil because successive production of susceptible plants in crop rotations increases disease severity compared with alternate growth of nonhosts (Buhre et al. 2009; Kluth et al. 2010).

For the detection of *R. solani* in soils, methods such as baiting with susceptible host material (Papavizas and Davey 1959), wet sieving and direct microscopy (Boosalis and Scharen 1959), or a soil debris isolation method (Roberts and Herr 1979) have been developed earlier. Davey and Papavizas (1962) compared four methods and found indications for highly variable results between the different methods. Baiting methods are labor intensive and give less reproducible results, as shown for wet-sieving by Van Bruggen and Arneson (1986), and, therefore, are not suitable for large-scale field studies. Indicator plants such as *Vicia faba* L. grown in diseased field soil samples were used as a simple method to estimate the potential *R. solani* AG2-2IIIB disease severity in subsequently grown sugar beet (Dircks et al. 2014). However, pathogen biomass has to be determined for an estimation of absolute pathogen IP values. Consequently, Boine et al. (2014) developed an indirect real-time polymerase chain reaction (PCR) assay to quantify *R. solani* AG2-2IIIB by baiting soil samples with quinoa seed for DNA extraction; which, however, is time consuming, too.

Real-time PCR-based quantification of fungal soil IP from total soil DNA extracts allows for definite identification, high accuracy, and rapid detection compared with wet-sieving and baiting methods

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(Woodhall et al. 2012). In several studies, *R. solani* AG3 (Lees et al. 2002), AG2-1 (Zhou et al. 2014), and AG8 and AG10 (Okubara et al. 2008) were quantified from soil DNA extracts using species-specific primers in real-time PCR assays. However, in these studies, soil DNA was extracted from small soil samples of 1 to 10 g. This amount has to be regarded as too small by far in the background of the heterogeneous spatial distribution of *R. solani* on a field scale (Martin et al. 1983; Otten et al. 2004). Thus, a high number of single core samples is required to obtain one composite sample adequately representing the whole field. Next, thorough mixing of such a composite sample is challenging because the soil usually is moist at sampling and drying might affect IP (Kinsbursky and Weinhold 1988). To alleviate such constraints, Ophel-Keller et al. (2008) and Woodhall et al. (2012) recently developed a soil DNA extraction method from large soil samples of 250 to 500 g combined with real-time PCR detection of soilborne fungal pathogens.

For our study, we modified the direct DNA extraction method from 250 g of soil as described by Woodhall et al. (2012): instead of using commercial DNA kits for DNA purification, we eluted DNA directly from silica particles following the method according to Rott and Jelkmann (2001). For DNA quantification, a real-time PCR assay using new specific primers for *R. solani* AG2-2IIIB was developed. This methodology could contribute to a better estimation of the principal risk for disease occurrence and, in addition, quantify effects of control measures on the soilborne pathogen inoculum density. The assay was evaluated on different soils with regard to extraction efficiency and primer specificity to AG2-2IIIB. Soil samples from fields with and without *Rhizoctonia* crown and root rot history were tested for *R. solani* AG2-2IIIB incidence. More, the assay was applied to detect differences in *R. solani* AG2-2IIIB soil IP after growing different sugar beet varieties (susceptible and resistant) and after subsequently grown winter rye in a randomized field trial. Winter rye, like wheat, has never been reported to represent a host of *R. solani* AG2-2IIIB.

MATERIALS AND METHODS

Fungal cultivation and DNA extraction. Fungal isolates were obtained from the American Type Culture Collection and the Centraalbureau voor Schimmelcultures (CBS). Isolates were cultivated in vitro in potato dextrose broth (PDB; AppliChem) at 20°C in the dark for 7 days on a flat-bed shaker (100 rpm). DNA extraction was performed according to the protocol of Rott and Jelkmann

(2001), with the following modification. Grinding buffer was prepared with 6 M guanidine hydrochloride instead of 4 M guanidine thiocyanate, as originally described by Menzel et al. (2002). Approximately 200 mg of mycelia from the 7-day-old PDB cultures was ground with a pestle in liquid nitrogen prior to homogenization in grinding buffer. DNA integrity was checked by agarose gel electrophoresis (1%) and quantified spectrophotometrically (DS-11 Spectrophotometer; DeNovix).

Production of sclerotia for spiking experiments were carried out by growing *R. solani* AG2-2IIIB (CBS101765) for 2 to 3 weeks on potato dextrose agar in petri dishes. Sclerotia were removed with a scalpel, air dried, and macerated into small fragments.

Real-time PCR assay. To design *R. solani* AG2-2IIIB-specific primers for real-time PCR detection, sequences of the internal transcribed spacers (ITS) regions of different *R. solani* AG listed in Table 1 were compared using MEGA6 software (Tamura et al. 2013). The alignment consisted of a total of 65 sequences from various *R. solani* AG (18 AG2-2IIIB sequences) retrieved from the National Center for Biotechnology Information (NCBI) to cover intraspecific variability. The sequences represent isolates from different geographic origins in Europe and the United States. AG2-2IIIB-specific regions were hand-selected from the ITS1 region to design forward (AG2-2IIIB_F: 5'-GGT TGT AGC TGG CTC CAT TAG-3') and reverse (AG2-2IIIB_R: 5'-G TTY TCC CAT CCA TGT CTC TGC-3') primers for real-time PCR. The forward primer AG2-2IIIB_F comprised nucleotides 16 to 36 and the reverse primer AG2-2IIIB_R nucleotides 100 to 121 of the ITS1 region of *R. solani* AG2-2IIIB isolates, resulting in an amplicon of 104 bp. Finally, primer sequences were checked against the NCBI database to prove their specificity and to identify possible unintended targets. Real-time PCR was performed in 96-well plates using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The iTaq Universal SYBR Green Supermix (1×) (Bio-Rad Laboratories) was used with DNA from pure fungal cultures and soil (test soils [Table 2], soil from the field trial, and individual field locations) in a 20-μl reaction mix containing 1 μl of extracted template DNA (25 ng) and forward and reverse primers at a final concentration of 300 nM, respectively. PCR cycle conditions were 95°C for 10 min and 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s.

A standard curve of pure fungal DNA from *R. solani* AG2-2IIIB (isolate CBS101765, NCBI accession number AB054854.1), which consisted of a 10-fold dilution series with DNA amounts ranging from 520 ng to 0.52 pg, was produced by plotting the cycle threshold

TABLE 1. Isolate code, GenBank accession number, and host origin of different *Rhizoctonia solani* anastomosis groups (AG) used for the evaluation of primer specificity for *R. solani* AG2-2IIIB as well as cycle threshold (Ct) value and amplicon melting temperature (T_m) after real-time polymerase chain reaction^a

AG	Isolate code ^b	GenBank	Original host	Ct value	Decreased sensitivity factor ^c	Amplicon T _m (°C)
AG1-1A	101759 [†]	AB000017.1	<i>Oryza sativa</i>	—	—	—
AG2-1	101763 [†]	DQ279000	<i>Pisum sativum</i>	—	—	—
AG2-2IIIB	101765 [†]	AB054854.1	<i>Scirpus lacustris</i>	17.32	—	78
AG2-2IV	101767 [†]	AB911323.1	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	20.07	6	78
AG2-2LP	RGR-38 [§]	AB054869	<i>Cynodon dactylon</i>	20.08	6	78
AG2-3	101769 [†]	FJ435110	<i>Glycine max</i>	—	—	—
AG2-t	207.97 [†]	TCU57744	<i>Tulipa</i> L.	—	—	—
AG3	363.82 [†]	EF532825	<i>Solanum tuberosum</i>	35.4	—	79.5
AG4-HGI	101775 [†]	JX988994.1	<i>Arachis hypogaea</i>	—	—	—
AG4-HGII	76127 [†]	AB000033	<i>B. vulgaris</i> subsp. <i>vulgaris</i>	—	—	—
AG5	76128 [†]	FJ435115	<i>G. max</i>	—	—	—
AG6	101779 [†]	AF153782	Soil	37.3	—	79.5
AG9	101783 [†]	FJ435118	Soil	—	—	—
AG10	971.96 [†]	FJ435119	<i>Hordeum vulgare</i> L.	—	—	—
AG11	974.96 [†]	FJ435120	<i>Lupinus angustifolius</i>	—	—	—
AG11	973.96 [†]	DQ278967	<i>L. angustifolius</i>	—	—	—
AG12	MYA-982 [‡]	AF153806.1	Leaf litter	—	—	—
AG13	MYA-984 [‡]	AB275641	<i>Gossypium hirsutum</i>	—	—	—

^a A factor of decreased sensitivity was calculated on the basis of the DNA amount using the regression function of a pure 10-fold *R. solani* AG2-2IIIB dilution series; — indicates no specific detection.

^b Sources: [†] = Centraalbureau voor Schimmelcultures, [‡] = American Type Culture Collection, and [§] = Carling et al. (2002).

^c Factor of decreased sensitivity compared with AG2-2IIIB.

(Ct) values against the log₁₀ of the initial DNA concentration. Each sample was analyzed in triplicate and average Ct values were automatically calculated and analyzed by the Bio-Rad CFX Manager software (Bio-Rad Laboratories) using the regression function obtained from the standard curve.

Quantification of target DNA (three technical replications) in soil samples was performed by including the pure fungal DNA standards in each PCR run. The IP (amount of *R. solani* AG2-2IIIB DNA in the soil) was expressed as nanograms of DNA per gram of soil. Further, primer specificity for *R. solani* AG2-2IIIB was tested with real-time PCR and subsequent melting curve analysis, including 25 ng of fungal DNA of different *R. solani* isolates taken from several AG (Table 1).

Soil DNA extraction. Soil samples of 250 g were used for total soil DNA extraction according to the method of Woodhall et al. (2012), with some modifications. Soil (250 g, air dried for 24 h) was mixed with 4 ml of Antifoam B emulsion (S5631; Sigma Aldrich), 500 ml of grinding buffer (0.1 M sodium phosphate buffer [pH 8] and 2% cetyltrimethylammonium bromide), and 15 steel ball bearings (diameter: 20 mm) and thoroughly shaken for 4 min in a Merris Minimix Autopaint Shaker (Merris). A 50-ml subsample was centrifuged at 5,000 × g for 5 min and 20 ml of the resulting supernatant was mixed with 2 ml of 5 M potassium acetate and incubated on ice for 10 min. The incubation step was followed by a 7-min centrifugation at 10,500 × g. Subsequently, the supernatant was transferred to a new 50-ml tube and 15 ml of isopropanol and 800 µl of silicon dioxide suspension was added. The mixture was incubated on a flat-bed shaker at 100 rpm for 10 min followed by 7-min centrifugation at 10,500 × g. The resulting supernatant was discarded and pelleted silica particles remained.

For further purification of extracted soil DNA, we used a modified silica capture method according to Rott and Jelkmann (2001) as subsequently described instead of the commercial silica-column based DNA extraction kit used by Woodhall et al. (2012). The pelleted silica particles were dissolved in 1 ml of washing buffer containing 10 mM TRIS-HCl, 0.5 mM EDTA, 50 mM NaCl, and 50% EtOH (pH 7.5) and transferred into a 2-ml reaction tube. In total, the pellet was thoroughly washed two times in 1 ml of washing buffer by vortexing and subsequent centrifugation at 6,000 × g for 1 min, respectively. The silica pellet was dried under vacuum (SpeedVac; Thermo Fisher Scientific) at 45°C for 5 min after the second washing step. Subsequently, the pellet was eluted in 300 µl of high-performance liquid chromatography-grade H₂O by vortexing and incubated for 10 min at 70°C in a shaking incubator at 100 rpm. Silica particles were separated by centrifugation for 3 min at 13,400 × g and 250 µl of the resulting supernatant was stored at -20°C for further real-time PCR analysis. Finally, DNA yield and integrity were checked by agarose gel electrophoresis (1%, 120 V, 35 min) and DNA concentration was quantified spectrophotometrically (DS-11 Spectrophotometer; DeNovix).

Assay evaluation. *R. solani* DNA detection efficiency and DNA extraction efficiency from soil was evaluated by spiking 250 g of three test soils (Table 2) with 0.5 to 124.5 mg of purified *R. solani* AG2-2IIIB sclerotia. Test soils were taken from fields with no history of any *R. solani*-induced disease in the past. Nine spiked samples and one unspiked control were prepared for each soil in replication for DNA extraction and following real-time PCR tests with three technical replications.

To determine a potential inhibition of extracted soil DNA on real-time PCR amplification efficiency, each *R. solani* AG2-2IIIB DNA dilution of the standard curve was mixed with 25 ng of soil DNA (unspiked and free of *R. solani* AG2-2IIIB).

Field trial design and sugar beet cultivation. A field trial was conducted near Haardorf (Germany, Lower Bavaria; 48°43'07.47" N 12°59'07.98" E) in 2013 to 2015. The soils of this region are naturally infested with *R. solani*. To increase and homogenize the *R. solani* AG2-2IIIB IP, the soil of the trial field (Luvisol: 19.2% clay, 71.5% silt, and 9.2% sand) was artificially inoculated in 2013 with a barley inoculum at 50 kg ha⁻¹ to receive a homogenous soil IP. The barley inoculum was prepared as described by Kluth et al.

(2010). Inoculation was performed immediately before maize (*Zea mays* L. 'AgroLux'; Agromais) was sown as a susceptible rotational crop. The trial consisted of four replicates with five maize blocks each. The maize blocks differed in soil tillage and crop residue removal which, however, is not further considered here. In 2014, two sugar beet varieties were cultivated in plots within each maize block, resulting in 40 sugar beet plots (20 plots per variety) in total. A susceptible and a resistant sugar beet variety, with registration numbers ZR1988 and ZR1555 according to Bundessortenamt (2014), were grown. Each plot consisted of 12 rows of 14 m in length, with a row width of 0.5 m. Pelleted sugar beet seed were sown at a depth of 2 to 2.5 cm (March 2014) at 7 cm of distance followed by manual singling to a final in-row distance of 21 cm at the four- to six-leaf stage. Weeds, pests, and leaf diseases were controlled according to regional standards. After sugar beet harvest in 2014, winter rye (*Secale cereale* L.) was grown on the entire trial and harvested at the end of July 2015.

Rhizoctonia solani disease rating. In October 2014, two rows (±100 sugar beet taproots) in the center of each plot from the field trial were harvested by a trial harvester and beet roots were washed in the tare house. Rhizoctonia solani disease rating was performed on single beet roots. The linear rating scale ranged from 0% (healthy taproot) to 100% (completely mummified or dead taproot) in steps of 10%. Substeps at 2% disease severity (small, black, point-shaped lesions) and 5% disease severity (first clearly visible lesion) were included for a better differentiation between nondiseased taproots and beet roots displaying initial symptoms. A mean value for every plot was calculated from single rating values. Values were displayed as disease severity (percentage of rotten sugar beet taproot surface).

Soil sampling. Soil samples for DNA extraction and determination of *R. solani* AG2-2IIIB incidence of different fields with and without a history of disease in the past were taken during the sugar beet vegetation periods in 2014 and 2015 from sugar beet fields and a long-term crop-rotation field trial located at Lower Saxony (no natural infestation) and fields located at Lower Bavaria (natural infestation area): All Lower Bavarian fields displayed disease symptoms during sampling. In contrast, fields located in Lower Saxony were symptom free, whereas the crop-rotation field trial had shown disease symptoms for the first time and, therefore, was used for soil sampling. Two composite soil samples were analyzed per field. One composite soil sample was obtained from at least 45 soil cores per field at soil depths of 0 to 15 cm, as recommended by Ophel-Keller et al. (2008), to obtain approximately 2 kg of soil. Each 2-kg soil sample was thoroughly hand mixed to receive a homogenous 250-g subsample which was stored at 4°C prior to DNA extraction.

In addition, soil samples for DNA extraction and *R. solani* AG2-2IIIB quantification were taken from the field trial (Haardorf) at sugar beet sowing (March 2014) from the maize blocks, at harvest (October 2014) from the core rows of each variety plot, and after growing winter rye (July 2015) from all former sugar beet variety plots in a W-shaped configuration. One composite soil sample was obtained from at least 25 soil cores per block or plot taken at soil depths of 0 to 15 cm, as recommended by Ophel-Keller et al. (2008) for research plots, resulting in 20 composite soil samples per sugar beet variety for DNA extraction and quantification. Soil samples from the field trial were further processed as described above for soil samples from individual fields.

Data analysis. Statistical analysis was performed with the open-source software R (R Development Core Team 2014). Gaussian

TABLE 2. Soil texture of the soils for evaluating DNA detection efficiency

Soil	Clay	Silt	Sand
	<0.002 mm (%)	0.002 to 0.063 mm (%)	0.063 to 2 mm (%)
A	16.2	73.0	10.7
B	2.1	10.5	87.4
C	2.7	16.2	81.1

distribution was checked by a Shapiro-Wilk-test at $P < 0.05$ using the 'shapiro.test' function of R. Due to non normality, (i) differences of *R. solani* AG2-2IIIB DNA amounts between different soil sampling dates within the sugar beet varieties and (ii) differences of *R. solani* AG2-2IIIB disease severity between the susceptible and resistant variety were calculated using nonparametric Kruskal-Wallis-Test ($P < 0.05$) from the package 'agricolae' (de Mendiburu 2013).

Regressions were calculated using the 'lm' function of R, with significance at $P < 0.05$. Analysis of covariance was performed to evaluate statistical differences between (i) regression lines of the pure fungal DNA with and without soil DNA (Fig. 1) and (ii) regressions of the soil DNA extraction and detection efficiency from three different soil types (Fig. 2), with significance at $P < 0.05$ using the 'lm' function in R. A significant interaction term indicated differences in the slope of the regression lines.

RESULTS

Method evaluation. Real-time PCR tests were performed to evaluate the efficiency and the specificity of the designed *R. solani* AG2-2IIIB-specific primers. Primers showed an efficiency of 102.5% at a 10-fold dilution series of purified *R. solani* AG2-2IIIB DNA, obtained from in vitro grown mycelium, with a detection limit of 0.00052 pg (Ct of approximately 35) (Fig. 1). To evaluate a possible inhibition of DNA amplification efficiency, the pure fungal DNA dilution series was spiked with soil DNA and the real-time PCR was repeated. There was no significant difference between regressions obtained from the fungal DNA dilution series with and without spiking with extracted soil DNA and, therefore, the correlation was best described by a single function (Fig. 1). The results from the cross-reaction tests (*R. solani* AG2-2IIIB specificity) of the new primers with DNA of other *R. solani* AG are displayed in

Fig. 1. Standard curve of a 10-fold *Rhizoctonia solani* AG2-2IIIB DNA dilution series ranging from 520 to 0.00052 ng extracted from a pure culture of in-vitro-grown *Rhizoctonia solani* AG2-2IIIB mycelium performed without (open squares) and with (open circles) an additional 25 ng of soil DNA (spiked to each *R. solani* DNA dilution) for testing interference of soil DNA on amplification during real-time polymerase chain reaction. Points represent single values. Slopes of regression lines did not show significant differences at $P < 0.05$ (analysis of covariance).

Table 1. Primers showed DNA amplification with AG2-2IIIB, AG2-2IV, and AG2-2LP. However, amplification sensitivity for AG2-2IV and AG2-2LP was six times lower compared with AG2-2IIIB. Ct values of AG3 and AG6 were below the detection limit and amplicon melting temperature was 1.5°C higher compared with AG2-2IIIB DNA. All other AG did not show any amplification signal with the AG2-2IIIB primers.

R. solani AG2-2IIIB detection and soil DNA extraction efficiency was evaluated with three different *R. solani*-free soils (Table 2) spiked with *R. solani* AG2-2IIIB sclerotia. For each soil, real-time PCR with DNA extracted from the spiked soils showed a linear relationship between Ct values and the $\log_{10}$ amount of *R. solani* AG2-2IIIB sclerotia (Fig. 2). Ct values increased with decreasing amount of spiked sclerotia and ranged from 0.5 to 124.5 mg of spiked sclerotia per 250 g of soil (Ct of 35 to 20), with mean DNA amounts of 0.04 ± 0.23 ng g^{-1} of soil. The detection limit of 0.5 mg of *R. solani* AG2-2IIIB sclerotia per 250 g of soil was valid for the three soils. No AG2-2IIIB DNA was detected in unspiked control soil samples. There was no significant difference between the slopes of the regressions obtained for the soils, which indicates a high DNA detection efficiency in all cases, with R^2 values ranging between 0.93 and 0.98. The y-intercepts of the regressions were significantly different.

***R. solani* detection in naturally infested field soils.** In all, 11 of 15 soil samples from sugar beet fields with a history of disease were detected positive for *R. solani* AG2-2IIIB (Table 3). *R. solani* AG2-2IIIB was not detected in all four samples from fields with no history of disease. In samples from fields with a history of disease, *R. solani* AG2-2IIIB was detected in 81% of the samples (9 of 11 samples), with a mean amount of DNA of 68.33 ng g^{-1} of soil and a range between 0.06 and 254.10 ng g^{-1} of soil.

Field trial. The disease rating of harvested sugar beet showed a significantly higher disease severity for the susceptible variety

Fig. 2. DNA detection efficiency measured by cycle threshold values of different amounts of *Rhizoctonia solani* AG2-2IIIB sclerotia (milligrams per 250 g of soil) spiked into 250 g of three different test soils: soil A = 16.2% clay, 73.0% silt, and 10.7% sand; soil B = 2.1% clay, 10.5% silt, and 87.4% sand; and soil C = 2.7% clay, 16.2% silt, and 81.1% sand. Amount of sclerotia spiked into the soil ranged from 0.5 to 124.5 mg. Points represent mean values, with $n = 3$. Slopes of regression lines were not significantly different whereas intercepts of lines displayed significant differences at $P < 0.05$ (analysis of covariance).

(16% mean disease severity) compared with the resistant one (8% mean disease severity) (Fig. 3). The mean soil IP at the field trial was DNA at 1.84 ng g⁻¹ of soil for both sugar beet varieties at sugar beet sowing (Fig. 4A and B). Growing the susceptible variety caused an increase of the mean IP after sugar beet harvest by the factor of six to a DNA amount of 12.54 ng g⁻¹ of soil, whereas growing the resistant variety slightly reduced the mean IP to a DNA amount of 1.56 ng g⁻¹ of soil. However, this effect was not significant. Scattering of the soil IP data was increased by growing the susceptible variety and decreased by growing the resistant variety. The growth of different sugar beet varieties resulted in a high IP variation between plots. The lowest value of *R. solani* AG2-2IIIB soil IP after harvest measured in this study was a DNA amount of 0.04 ng g⁻¹ of soil for the resistant variety and 0.10 ng g⁻¹ of soil for the susceptible variety, and highest value was 11.09 ng g⁻¹ of soil for the resistant variety and 63.03 ng g⁻¹ of soil for the susceptible variety (Fig. 4A and B). Furthermore, soil IP variability was increased when the susceptible variety was grown compared with the variability obtained for the soil IP at sugar beet sowing and when the resistant variety was grown. Growing winter rye after sugar beet revealed a significant decrease of the *R. solani* AG2-2IIIB DNA amount in the soil from 12.54 to 1.54 ng g⁻¹ of soil in plots where a susceptible sugar beet variety was previously grown (Fig. 4A). In plots where a resistant sugar beet variety was previously grown, the soil IP was slightly but not significantly reduced (Fig. 4B).

Finally, *R. solani* AG2-2IIIB disease severity and IP at sugar beet sowing as well as IP after sugar beet harvest did not reveal a positive correlation for both varieties when individual plots were analyzed (Fig. 5A and B): a higher IP at sowing did not result in a higher disease severity and a high disease severity did not necessarily result in a higher soil IP at harvest. However, the data of the individual plots of the susceptible variety were more variable at sugar beet harvest compared with data of the resistant variety that clustered mainly at low soil IP and low disease severities.

DISCUSSION

In this study, a new reliable real-time PCR assay based on SYBR Green following direct soil DNA extraction from 250-g soil samples for quantification of the sugar beet pathogen *R. solani* AG2-2IIIB was developed. Accurate and reliable quantification of *R. solani* AG2-2IIIB DNA was demonstrated with three test soils differing in their clay, silt, and sand content. Soil DNA extraction efficiency was affected by soil type, as indicated by differences in the y-intercepts of the regressions. As a consequence, when comparing the IP of different environments, the assay has to be calibrated for different soil types by spiking experiments, as described in this study. Soil DNA did not inhibit real-time PCR amplification efficiency of pure *R. solani* AG2-2IIIB DNA, because there was no significant difference between regressions from the fungal DNA dilution series with and without soil DNA spiking. The method was able to detect *R. solani* AG2-2IIIB DNA in naturally infested field soil samples, with a detection limit of 0.5 mg of sclerotia per 250 g of soil (= DNA amount of 0.04 ng g⁻¹ of soil), and to distinguish whether a field was naturally infested with the pathogen or not. Further, it was applied

to detect differences in the soil IP of *R. solani* AG2-2IIIB before and after growing a susceptible and a resistant sugar beet variety in an *R. solani* field trial and after subsequently growing winter rye.

Specificity of the assay for detection of *R. solani* AG2-2IIIB was demonstrated in cross-reaction experiments with members of various *R. solani* AG. Subgroup IV of AG2-2, also reported to be a pathogen of sugar beet (Büttner et al. 2002; Engelkes and Windels 1996), showed some cross reaction. The unspecific detection of AG2-2LP is undesirable but not a serious issue for the assay because this AG was reported to be nonpathogenic for sugar beet and cause disease only on Asian zoysiagrass and other turfgrasses, for instance (Aoyagi et al. 1998; Hyakumachi et al. 1998). Furthermore, primer sensitivity with AG2-2IV and AG2-2LP was six times lower compared with AG2-2IIIB. In cases where a field is infected with one of these isolates, it would be detected with less sensitivity. AG3 and AG6 were detected; however, amplicons displayed Ct values below the detection limit and a higher melting temperature compared with our target AG2-2IIIB, indicating amplification of unspecific artifacts. For all other AG, no cross reaction was observed in the assay, including AG4, which is known to be pathogenic for sugar beet and causes seedling damping-off (Windels and Nabben 1989), indicating that the assay is not suitable for managing the damping-off disease.

Nevertheless, it is worth mentioning that, in other cropping systems outside of Germany or Europe, where other AG may probably be more prevalent (e.g., AG2-2LP), the assay could still lead to false-positive results or erroneously low-risk indications, limiting the usability of the assay. Moreover, it needs to be mentioned that, although we aligned 65 *R. solani* AG2-2IIIB sequences to design the primers, there is the possibility that some isolates with variable ITS sequence, as described by Strausbaugh et al. (2011), might not be detected with this assay with the same efficiency.

Woodhall et al. (2012, 2013) used additional commercial kit-based DNA purification following DNA elution from the silica particles for automation of the extraction process. This step was replaced by a material cost-saving DNA purification with a modified silica capture method according to Rott and Jelkmann (2001).

TABLE 3. Incidence of *Rhizoctonia solani* AG2-2IIIB in soil samples from sugar beet fields with history and with no history of *Rhizoctonia* crown and root rot disease with mean DNA quantity and range of positive tested samples

Disease history	Samples ^a	Number positive (%) ^b	Mean DNA (ng g ⁻¹ of soil) ^c	Range (ng g ⁻¹ of soil)
Yes	11	9 (81)	68.33	0.06 to 254.10
No	4	0 (0)	–	–

^a Total number of soil samples.

^b Number of samples detected positive.

^c Mean DNA quantity of positive tested samples. Detection limit: sclerotia at 2 × 10⁻⁶ g g⁻¹ of soil = amount of DNA at 0.04 ng g⁻¹ of soil.

Fig. 3. *Rhizoctonia solani* disease severity of a susceptible and a resistant sugar beet variety ($n = 20$) after harvest at the Haardorf field trial in 2014. Black line = median, open circle = mean, and cross = outlier. Different letters indicate significant differences (Kruskal-Wallis, $P < 0.05$).

We directly eluted DNA from the silica particles without further kit-based washing steps. Our results show that direct DNA elution from silica did not lead to coextraction of substances acting as PCR inhibitors (e.g., humic acids) (Ramakers et al. 2003; Schneegurt et al. 2003), resulting in similar detection sensitivity compared with published detection methods of other *R. solani* AG. The detection limit of the assay was at a sclerotia amount of 2×10^{-6} g g⁻¹ of soil spiked in 250 g of soil, with a mean DNA amount of 0.04 ng g⁻¹ of soil. Detection limits previously reported for other *R. solani* AG such as for AG3 sclerotia were 5×10^{-4} g g⁻¹ of soil in 10- to 60-g soil samples (Brierley et al. 2009; Lees et al. 2002) and 8×10^{-7} g g⁻¹

of soil in 250-g soil samples (Woodhall et al. 2013). Nevertheless, there is a potential risk for false-positive results due to the possible detection of nonviable mycelia. This can only be avoided by use of unspecific baiting techniques combined with specific DNA quantification (Boine et al. 2014; Lees et al. 2002; Thornton et al. 1999). Direct sensitivity comparisons between baiting and direct soil-DNA extractions from large volumes, however, are required to identify the most suitable method.

R. solani AG2-2IIIB DNA was detected in naturally infested field soil samples at relatively low levels, with a minimum DNA amount of 0.06 ng g⁻¹ of soil. In all, 9 of 11 field soil samples with a

Fig. 4. *Rhizoctonia solani* AG2-2IIIB inoculum potential (IP). Soil IP was measured before sugar beet sowing ($n = 20$) and after sugar beet harvest ($n = 20$) of **A**, a susceptible and **B**, a resistant sugar beet variety in 2014, as well as after subsequently grown winter rye ($n = 20$) in 2015, at the Haardorf field trial. Black line = median, open circle = mean, and cross = outlier. Different letters indicate significant differences (Kruskal-Wallis, $P < 0.05$).

Fig. 5. Relation between the *Rhizoctonia solani* AG2-2IIIB inoculum potential (IP) in soil **A**, at sugar beet sowing (IP_{sowing}) and **B**, after sugar beet harvest ($IP_{harvest}$) and disease severity of the resistant (SB_{res}) and the susceptible (SB_{sus}) sugar beet variety at the Haardorf field trial in 2014. Points represent values from individual plots.

Rhizoctonia crown and root rot history tested positive in this study, which illustrates the suitability of the assay to recognize Rhizoctonia crown and root rot. DNA concentrations of soil samples that tested positive were comparable with those found by Woodhall et al. (2012) for *R. solani* AG2-1 and AG8 and *Sclerotium cepivorum* or by Brierley et al. (2009) for *Colletotrichum coccodes* and *R. solani* AG3.

Applying the assay for soil IP analysis in a crop-rotation experiment revealed the effect of the sugar beet variety on the IP of *R. solani* AG2-2IIIB in the soil: the susceptible sugar beet variety showed both a significantly higher mean disease severity and a higher mean IP compared with the resistant variety at sugar beet harvest. Nevertheless, the disease severity of 10 to 20% for the susceptible variety was unexpectedly low compared with the results of Kluth et al. (2010) and Büttner et al. (2004), who observed disease severities up to 75% for sugar beet in greenhouse and field experiments following artificial inoculation. However, it is worth mentioning that the susceptible variety increased soil IP even at low disease severities. The reasons for that are unknown. We can only speculate that this may be explained by the higher parasitic activities or by indirect effects (e.g., root exudates) on the pathogen (Boosalis and Scharen 1959; Herr 1976; Naiki and Ui 1977). This underlines the importance of resistant sugar beet varieties in disease control, especially in areas with a potentially higher risk due to a long history of *R. solani*-caused severe and frequent disease occurrence in sugar beet. In these areas, economic sugar beet cultivation without *R. solani*-resistant varieties may be less profitable. Furthermore, it became apparent that the cultivation of winter rye, that has never been reported to be susceptible to *R. solani* AG2-2IIIB, after sugar beet significantly decreased the *R. solani* AG2-2IIIB soil IP left by growing the susceptible sugar beet variety to the level which was determined before sugar beet sowing. These findings are in accordance with Buhre et al. (2009) and Kluth et al. (2010), who detected a positive effect of intercrops and crop rotations with a high proportion of nonhost plants on disease severity, which could have been a result of decreased *Rhizoctonia* soil IP. This is supported by Ruppel (1985), who reported maize residues in soil mixtures to be conducive to *R. solani* AG2 (Rhizoctonia damping-off), whereas barley residues were not conducive, and that persistence of *R. solani* in crop residues may be dependent on the crop species. A positive effect of nonhost crops on pathogen soil IP could possibly be traced to an enhanced antagonistic microbial community in the soil, as described for Rhizoctonia damping-off of sugar beet (Kasuya et al. 2006) or Rhizoctonia root rot of apple (Cohen et al. 2005). To the best of our knowledge, this is the first study that demonstrates the different effect of a resistant and susceptible sugar beet variety on *R. solani* AG2-2IIIB IP in a randomized repeated field trial.

However, there was no correlation between disease severity and soil IP at sugar beet sowing and after sugar beet harvest. The soil IP obtained after growing the susceptible variety showed a high scattering between individual plots and, furthermore, soil IP variability was increased by growing the susceptible variety and decreased by growing the resistant variety compared with the variability of soil IP at sugar beet sowing. Growing winter rye decreased soil IP variability regardless of the previously grown sugar beet variety. This emphasizes the impact of other environmental factors; for example, soil structural properties (Buddemeyer and Märkländer 2004) or the soil microbiome (Anees et al. 2010b), on soil IP. Furthermore, Ruppel et al. (1988) described *R. solani* population densities in a herbicide and crop rotation experiment with sugar beet, pinto bean, maize, and barley and concluded that changes in fungal population densities can be attributed to diverse environmental parameters; however, AG-specific detection of sugar-beet-pathogenic *R. solani* was not performed.

As previously concluded by Herr (1987), disease prediction will still be a challenging task in the future because we have shown that the IP of both sugar beet varieties at sowing did not necessarily represent a measure for disease severity at harvest. Prediction of

disease risks was previously described by Ophel-Keller et al. (2008) for nematodes and soilborne wheat and barley pathogens by allocating DNA levels to disease risk categories, and by Kernaghan et al. (2007), who related DNA concentrations of *Cylindrocarpum destructans* to a disease index of American ginseng.

In summary, it was shown that the assay described in the current study is suitable to detect *R. solani* AG2-2IIIB DNA in naturally infested field soils and, therefore, provides an alternative to other diagnostic methods to detect soilborne pathogens. Furthermore, it can be concluded that increasing the *R. solani* AG2-2IIIB soil IP by growing a susceptible sugar beet variety might increase the risk of an elevated disease level in subsequently grown host crops, whereas growing a resistant sugar beet variety resulted in a constant soil IP on a lower level. However, a reduced soil IP due to the growth of a resistant sugar beet variety or a nonhost crop might decrease disease risk in subsequently grown host crops. Nevertheless, the development of the *R. solani* AG2-2IIIB soil IP within whole-crop rotations consisting of a variety of host and nonhost crops has not yet been examined over a long-term period. The new assay provides a suitable tool to answer these questions. Moreover, the effect of soil structural properties and other environmental factors on soil IP can be investigated. However, due to the lack of a correlation between disease severity and soil IP, further research is required to unravel these interactions on a field scale.

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