Relative contribution of seed tuber- and soil-borne inoculum to potato disease development and changes in the population genetic structure of *Rhizoctonia solani* AG 3-PT under field conditions in South Africa

Norman Muzhinji[ab]*, James W. Woodhall*[c]*, Mariette Truter*[d]*, Jacquie E. van der Waals*[a*]

[a]*Department of Plant and Soil Sciences, University of Pretoria, Private Bag X20, Hatfield, Pretoria 0001, South Africa;* [b]*Tobacco Research Board, P. O. Box 1909, Airport Ring Road, Harare, Zimbabwe;* [c]*University of Idaho, Parma Research and Extension Center, 29603 U of I Ln, Parma, ID 83660, USA;*[d] *Agricultural Research Council-Vegetable and Ornamental Plants, Private Bag X293, Pretoria 0001, South Africa*

*Corresponding author e-mail: Jacquie.vanderwaals@up.ac.za

Understanding the contribution of seed tuber- and soil-borne inocula of Rhizoctonia solani AG 3-PT in causing potato disease epidemics is an important step in implementing effective management strategies for the pathogen. A two-year study was conducted to evaluate the contribution of each source of inoculum using an integrative experimental approach combining field trials and molecular techniques. Two distinct sets of genetically-marked isolates were used as seed tuber-borne and soil-borne inocula in a mark-release-recapture experiment. Disease assessments were done during tuber initiation and at tuber harvest Both inoculuma sources were found to be equally important in causing black scurf disease, whereas soil-borne inocula appeared to be more important for root and stolon infection, and seed-borne inocula contributed more to stem canker. However, seed tuber transmitted genotypes accounted for 60% of the total recovered isolates when genotyped using three PCR-RFLP markers. The changes in population structure of the experimental

R. solani population over the course of the growing season and across two growing seasons were investigated using eight microsatellite markers. The populations at different sampling times were somewhat genetically differentiated as indicated by Nei's gene diversity (0.24 - 0.27) and the fixation index (F_{ST}). The proportion of isolates with genotypes that differed from the inoculants ranged from 13% to 16% in 2013 and 2014, respectively, suggesting the possibility of emergence of new genotypes in the field. Because both soil-borne and tuber-borne inocula are critical, it is important to ensure the use of pathogen-free seed tubers to eliminate seed tuber-borne inoculum and the introduction of new genotypes of *R. solani* for sustainable potato production in South Africa.

Keywords: Inoculum source, marked genotypes, microsatellites, potato, PCR-RFLP, *Rhizoctonia solani* AG 3-PT

Introduction

Rhizoctonia solani Kühn is a major pathogen of potato that negatively impacts the qualitative and quantitative yield, with estimated annual yield losses of approximately 30% (Banville 1989). *Rhizoctonia solani* species complex exists in many different groups and presently, 13 anastomosis groups (AGs) are recognized, with many subgroups (Carling et al. 2002). The predominance and aggressiveness of *R. solani* AG 3 potato-infecting subgroup (AG 3-PT) on potatoes has been confirmed in South Africa (Muzhinji et al. 2015) and elsewhere (Das et al. 2014; Fiers et al. 2011; Woodhall et al. 2007).

Infection of potato by *R. solani* AG 3-PT originates from inoculum present as sclerotia or hyphae on seed tubers or already inhabiting the soil where potato crop is grown (Balali et al. 1997; Sneh et al. 1996). Several strategies are used to control *Rhizoctonia* diseases on potatoes including the use of biocontrol agents, treating seed tubers or field soils

with fungicides and crop rotation (Tsror 2010). However, avoidance of planting in *Rhizoctonia* infested fields and planting pathogen-free certified potato seed are often the most practical disease control measures for the potato grower.

The role of seed tuber- and soil-borne inocula in the development of *Rhizoctonia* diseases on potatoes has been extensively studied but the relative importance of each source of inoculum has not been definitively established (Atkinson et al. 2010; Brierley et al. 2016; Frank and Leach 1980; Tsror (Lahkim) and Peretz-Alon 2005; van Emden et al. 1965). James and Mckenzie (1972) demonstrated that soil-borne inoculum is more important than seed tuber-borne inoculum, while Atkinson et al. (2010) and more recently Brierley et al. (2016) concluded that seed tuber-borne is more strongly associated with potato diseases than soil-borne inoculum. In contrast, other studies have shown that seed tuber- and soil-borne inocula are both important in potato disease development with each source of inoculum playing a different role at different stages of plant growth and development (Atkinson et al. 2010; Brierlery et al. 2016; Frank and Leach 1980; Tsror (Lahkim) and Peretz-Alon 2005). However, these observations and that of the importance of seed tuber-borne inoculum as the primary inoculum in *Rhizoctonia* disease epidemics on potato still lacks conclusive evidence.

The increase in the array of molecular techniques and computing technologies, offers endless possibilities of conducting mark-release-recapture experiments that allow tracking of inoculum sources during disease development and thus determines the inoculum origin (Bardin et al. 2014; Sommerhalder et al. 2010; Zhan et al. 1998; Zhan and McDonald 2013). Mark-release-recapture experiments have been reported for the wheat pathogen *Phaeosphaeria nodorum* (Bennet et al. 2007; Shah et al. 1995) and other pathogens (Zhan and McDonald 2013). The development and validation of selectively neutral, co-dominant and highly polymorphic molecular markers for *R. solani* AG 3-PT (Ceresini et al.

2002) offers practical feasibility of tagging and tracking *R. solani* AG 3-PT genotypes across generations in a mark–release–recapture experiment.

Another possible source of *R. solani* AG 3-PT inoculum is basidiospores that have frequently been observed during canopy closure, as a white-collar on the base of potato stems (Ferrucho et al. 2013; Ogoshi 1987). Their role as an inoculum source in *Rhizoctonia* disease epidemics on potato has not been demonstrated (Jeger et al. 1996). In other crops, such as tobacco and tomato, basidiospores have been reported to serve as primary or secondary inoculum in addition to seed tuber-borne and soil-borne inocula (Bartz et al. 2010; Shew and Melton 1995). Several studies have provided compelling evidence that basidiospores play an important role in shaping the population genetic structure of *R. solani* AG 3-PT on potato (Ceresini et al. 2002; Ferrucho et al. 2013). Changes in population genetic structure allows pathogens to evolve and adapt more rapidly to changing environments, reducing the useful life span of fungicides and resistant cultivars, thus making disease control more difficult (McDonald and Linde 2002).

The production of basidiospores in the field, theoretically, makes *Rhizoctonia* diseases on potato polycyclic, with several generations of *Rhizoctonia* reproduction across growing seasons. A better understanding of how the field population evolves during the course of the growing season or across seasons can be useful for implementing more sustainable disease management strategies (McDonald and Linde 2002).

The aim of this work was two-fold. The first objective was to investigate the role of seed tuber- and soil-borne inocula on *Rhizoctonia* disease development on potatoes using an integrative experimental approach combining both conventional plant pathology procedures and molecular methods. In South Africa, there is little knowledge concerning the effects, importance and relative contribution of different *R. solani* inoculum sources on the epidemiology and severity of *Rhizoctonia* potato diseases. The second objective was to

4

determine if the genetic structure within a field changes over the course of the growing season.

Materials and Methods

Experiment 1: Contribution of seed tuber- and soil-borne inoculum sources to *R. solani* AG 3-PT disease development on potato

Field trials were conducted at the University of Pretoria experimental plots. The field experiments were conducted over two growing seasons; September-December 2013 during year 1 and March-June 2014 during year 2.

Experimental design

The pathogen-free certified seed tubers, cultivar Harmony, were planted in year 1 (2013) and year 2 (2014). Prior to planting the plots, soil was sampled along diagonal transects to check for *R. solani* infestation. The baiting method using autoclaved oat seeds was used to isolate *Rhizoctonia* spp. from field soils (Sneh et al. 1966). The plots had no history of *R. solani* infestation and were disinfested by fumigation with formalin 0.91 kg/9.3 m^3, four weeks prior to planting to minimize the presence of soil-borne pathogens before before the start of the trial. The fumigated soil was rotovated every week until planting. Experimental plots consisted of four rows, 4.5 m long, 1.5 m wide and 70 cm apart; with seed pieces planted 25 cm apart within a row. Four treatments; uninfested soil with uninoculated seed tubers (control treatment), infested soil with uninoculated seed tubers, uninfested soil with inoculated seed tubers, and infested soil with inoculated seed tubers were used. Each plot represented one treatment and the experimental layout was a randomized complete block design (RCBD) with four replicates. Adjacent plots were separated by two

buffer rows of potato plants. The management of the field trial followed the local practice for commercial potato production.

Inoculum preparation

Two groups of *R. solani* AG 3-PT isolates with two distinct multilocus genotypes (MLGs) as assayed with three PCR-RFLPs (Table 1) developed by Ceresini et al. (2002) were used as seed tuber-borne and soil-borne inoculum. Each group of MLG was made up of five isolates. Soil inoculum was composed of isolates Rh24, Rh25, Rh28, Rh106 and Rh114 while seed tuber-borne inoculum was made up of Rh19, Rh29, Rh50, Rh100 and Rh101. The AG identity of the isolates was confirmed by DNA sequencing of the internal transcribed spacer region (ITS) as described in a separate study (Muzhinji et al. 2015). Soil-borne inoculum was prepared according to the method of Atkinson et al. (2010) with slight modifications. In brief, *R. solani* isolates were grown on Potato Dextrose Agar (PDA, Biolabs) plates (90 mm diameter) for 7 days at 25°C. For each isolate, 30 plates covered with mycelia were homogenized by blending in 4 L of sterile water into slurry, mixed with 10 L of vermiculite and incubated for five weeks with shaking after every two days. Soil was inoculated two days prior to planting by spreading the *Rhizoctonia* colonised vermiculite throughout the potato field at an approximate rate of 2 L per 9 m of row. Seed tuber-borne inoculum was prepared according to a modified method of Simons and Gilligan (1997). In brief, *R. solani* isolates were grown on PDA plates for 14 days at 25°C. Seed tubers were coated with a sludge prepared by mixing twenty colonized agar blocks (5 mm x 5 mm) with 5 ml of distilled water and 500 g of autoclaved soil for 20 seed tubers. The coated seed tubers were incubated for 2 days at 25°C to allow fungal colonization.

Table 1. PCR-RFLP markers used in this study (Ceresini et al. 2002).

RFLP locus	Primer Sequence (5'-3')	Annealing T°C	Fragment sizes (bp)	Restriction Enzyme	Alleles	Sizes (bp)
pP09	F-TGTCAGTCGAGTTATCCGCGA	55	530	*Hha* I	1	250
	R- GATCAAGTGTATGCGCATGCG				2	320
pP42	F-GTTTTTGTAGTGCACGGGGT	56	1970	*Hind* III	1	1250
	R- ATTCAACGTCTGTCGGTACGG				2	1020
					3	1970
pP45	F-TTGTTCATCGTCCTCGAGTCTC	56	965	*Hind* III	1	765
	R-ATCGGGTAAATTGCTACGCGA					

Disease assessments

Potato plants were sampled twice during the course of the growing season for disease assessments. Emergence and stem counts were taken 21 days after planting. Emergence counts were conducted to determine the incidence of misses caused by seed piece decay. The numbers of stems per tuber piece were counted on every second plant in the centre two rows of each plot to determine if *R. solani* infection decreased the number of stems per plant. At 60 days, whole plant samples, ten plants per plot were excavated from the two centre rows, and rinsed with tap water to remove soil and examined for stem canker, stolon and root infection and hymenia on the canopy. Isolations were made from cankers on the roots, stems and stolons infections to verify the presence of *R. solani*. An additional ten plants from the centre two rows of each plot were harvested 130 days after planting; 50 tubers per plot were randomly selected, washed and visually rated to estimate the percentage of tuber surface covered with back scurf.

Disease severity rating was done according to Woodhall et al. (2008) using a 0-4 scale where 0 = no damage or lesions present; 1 = one to several lesions less than 5 mm in size; 2 =lesions larger than 5 mm and some girdling present; 3 = larger lesions and girdling; 4 = all root/stems/stolons killed. Black scurf symptoms on progeny tubers were rated using the following scale: 0 = no sclerotia present; 1 = less than 1% of the tuber surface area covered in sclerotia; 2 = 1 to 10% of the tuber surface area covered in sclerotia; 3 = 11 to 20%; 4 = 21 to 50%; 5 = ≥ 51% tuber surface area covered in sclerotia. *Rhizoctonia* diseases on stems and stolons were presented as a disease index (DI). Disease index for stems and black scurf were calculated as described previously (Muzhinji et al. 2015).

***Experiment 2:* Genotypic shift in the *Rhizoctonia solani* AG 3-PT population under field conditions**

Inoculated plots that contained both the seed tuber- and soil-borne inoculum in 2013 were used in 2014 for *R. solani* AG 3-PT population genetic changes experiment without re-inoculation. Pathogen-free certified seed tubers (cultivar: Up-to-date) were planted in plots infested with both soil- and tuber-borne inoculum. As in year 1 (2013), two sets of fungal isolations were made at tuber initiation stage (mid-season) and at harvest stage (end of season).

Fungal isolation and DNA extraction

Rhizoctonia solani isolates were isolated from stem and stolon cankers, hymenia, roots and black scurf on potato tubers. Isolates obtained from the same treatment but different replications were pooled at each time point and were considered a population. Plants were first washed with tap water and placed on 2% water agar amended with streptomycin sulphate (100 μg/L). Colonies identified as *R. solani* under the microscope (Ogoshi 1975) were transferred to PDA and incubated at 25°C for 5 days. DNA extraction was done using the ZR soil microbe DNA kit^{TM} (Zymo Research Corporation, Irvine CA, USA) according to the manufacturer's protocol recommendations. The identification of isolated AGs was done using rDNA ITS sequencing as described previously (Muzhinji et al. 2015).

PCR-RFLP analysis

Rhizoctonia solani isolates recovered from symptomatic tissues were genotyped using PCR-RFLP markers (Ceresini et al. 2002) to determine the most prevalent genotype and infer the source of the inoculum. Amplification of genomic DNA was carried out as described by Ceresini et al. (2002) with minor modifications. PCR conditions for all loci were: initial denaturation at 95°C for 3 min, 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at specific temperature for each RFLP locus (Table 1) and elongation for 45 s at 72°C, and a

final extension cycle of 7 min. In each set, 5 µl of the PCR products were electrophoresed on Metaphor agarose gel (Lonza) and the specific band for each locus determined. A total of 10 µl of dissolved DNA of each isolate was digested with *Hind* III and *Hha* I, according to the manufacturer's instructions. Typical restriction enzyme reactions consisted of 10 µl of PCR product, 7.5 units of enzyme, 2 µl of 10 x reaction buffer, 0.2 µl of Bovine Serum Albumin (BSA) and water to a total volume of 20 µl. Reactions were incubated for 2 h and stopped by incubation at 75°C for 5 min. Restriction fragments were separated on a 2% agarose gel in Tris-Acetate-EDTA (TAE) buffer and stained with ethidium bromide.

Microsatellite analysis

Microsatellite (SSR) analyses of the recovered isolates were done to monitor the shift of the inoculated *R. solani* population over a two-year period. This was done by tracking the changes in genotypes of soil and seed tuber-borne inoculants (Table 2). The population genetic changes experiment, in a broader context, simulated what would occur in a commercial field when inoculum is introduced and the potato growers subsequently prevent re-introduction of the pathogen. The genotype of each isolate was determined using the eight microsatellite markers (Ferrucho et al. 2009) with fluorescently labelled primers using the same amplification protocols as described previously (Muzhinji et al. 2016). The PCR products were first cooled on ice for 2 min and then separated on an ABI 3730xI Genetic Analyser (Applied Biosystems, Forest City, California). Fragment sizes were estimated and alleles were assigned using the program GeneMapper v. 4.1 (Applied Biosystems).

Data analyses

All statistical analyses were carried out using GENSTAT v. 14.0 (VSN International Ltd). Analysis of variance (ANOVA) was used to test the differences between variables and means

Table 2. Multilocus genotypes representing soil-borne and seed tuber-borne inocula used. Alleles (designated by length in base pairs) present at each of the eight microsatellite are shown.

	TC_AG3_0		TC_AG3_1		TC_AG3_6		TC_AG3_7		TC_AG3_10		TC_AG3_16		TC_AG3_18		TC_AG_19	
Soil-borne	151	151	152	165	197	203	244	244	245	248	327	330	346	346	320	356
Tuber-borne	151	151	152	152	197	203	244	244	245	245	323	346	321	324	343	346

separated by Fisher's protected F-test least significant difference. Data in percentages were arcsine-transformed before analysis. Recovered *R. solani* isolates were genotyped using PCR-RFLP to determine if they originated from a seed tuber- or soil-borne inoculum source. Isolated *R. solani* was considered to be seed tuber- or soil-borne if it had an identical PCR-RFLP profile to the seed tuber- or soil-borne genotypes used for inoculations.

Genetic variation and population differentiation

Isolates sampled at the same sampling time points were treated as a single population. Data on the number of alleles per locus, and allelic frequencies per locus and per population were assessed using the program CONVERT v. 1.31 (Glaubitz 2004). Genotypic diversity was computed using the program MULTILOCUS v 1.3 (Agapow and Burt 2001). Allele frequencies were calculated using GeneAIEx version 6.5 (Peakall and Smouse 2006). Each combination of alleles at the eight loci defined a MLG and was implemented in GENOTYPE (Meirmans and van Tienderen 2004). Nei's measures of genetic distance and population differentiation were used to estimate the level of population subdivision between years and were implemented in FSTAT 2.9.3.2 (Goudet 1995). Population differentiation over the sampling periods during the growing season and between years was measured by F_{ST} statistics implemented in program ARLEQUIN v 3.5.2 using 1,100 randomisations (Excoffier and Lischer 2010). To evaluate the associations among loci in each sample, the index of association (I_A) and an unbiased estimate of multilocus linkage disequilibrium (rBarD) were used. The rBarD is much less dependent on the number of loci. They were computed using Multilocus 1.3 software, and 1000 artificially recombined data sets were used to determine the statistical values of the test (Agapow and Burt 2001). A value of zero indicates linkage equilibrium which is expected in random mating populations. A value significantly different from zero indicates linkage between loci, and is high in clonal populations.

Results

Rhizoctonia solani AG 3-PT disease development

The common *R. solani* symptoms on potato, namely; stem canker, stolon and root infection were the major symptoms observed in the mid-season assessments from plots at the University of Pretoria. At the end of the season black scurf was the most conspicuous symptom, although white-collar, malformed or cracked tubers and elephant hide were observed on progeny tubers albeit at low frequencies. *Rhizoctonia solani* symptoms on potato were not observed on control treatments.

Emergence and stem counts. Percentage emergence was significantly higher ($P < 0.05$) in control plots than in treatment plots (Table 3). Emergence counts were not statistically significant between the three treatments at $P < 0.05$. Stem counts were significantly lower ($P < 0.05$) for all the inoculated plots compared to the control.

Stolon and root diseases. Plants grown in plots treated with both seed tuber- and soil-borne inoculum had significantly ($P < 0.05$) higher levels of root infection compared with either of them separately (Table 4). Similarly, treatments containing soil-borne inoculum had significantly higher levels of root and stolon infection than the treatment where the inoculum source was tuber alone.

Stem canker and black scurf. Levels of stem canker and black scurf DI in field plots with a combination of infested soil and infected tubers were significantly higher ($P < 0.05$) than the treatments with soil or seed tuber-borne inoculum alone (Table 4). Stem canker DI was also significantly higher ($P < 0.05$) for plants grown with tuber borne inoculum than when

Table 3. The effects of seed tuber– and/or soil-borne inocula of *Rhizoctonia solani* on the number of stems and percentage emergence (arcsine transformed).

	Number of stems		Percentage emergence	
Inoculum source	**2013**	**2014**	**2013**	**2014**
Control	[z]2.8 a	3.2 a[y]	77.3 a	90.0 a
Soil	2.2 b	2.7 b	59.9 b	55.2 b
Soil and tuber	1.8 c	2.5 c	56.9 b	52.2 b
Tuber	2.0 c	2.5 c	57.4 b	55.2 b

[z] Data was analysed using the Fisher's protected F-test least significant difference (LSD) (*P* = 0.05).

[y] Values in columns followed by the same letter are not significantly different according to Duncan Multiple range test (P = 0.05).

Table 4. The effect of seed tuber- and/or soil-borne inocula of *Rhizoctonia solani* on root, stolon, stem and black scurf disease index (DI).

Inoculum source	Root infection[z]		Stolon canker[y]		Stem canker[x]		Black scurf[w]	
	2013	2014	2013	2014	2013	2014	2013	2014
Control	0.0 c[v]	0.0 c	0.0 d	0.0d	0.0 d*	0.0 d	0.0 c	0.0 d
Soil	14.5 b	10.5 b	8.5 b	15.0a	22.5 c	14.5 c	13.4 b	5.8 c
Soil and Tuber	17.5 a	12.5 a	10.2 a	12.0b	37.0 a	22.5 a	17.6 a	11.3 a
Tuber	13.0 b	6.0 c	5.0 c	8.0 c	29.0 b	17.4 b	12.6 b	7.8 b

[z,y] DI for roots and stolons was calculated by the formula: DI= Σ [0(n0) + 0.2(n1) + 0.4(n2) + 0.6(n3) +0.8(n4) +1(n5)] x 100/ (Nt_{otal}) where n_x = number of roots or stolons in the x rating class and N = total number of roots or stolons.

[xc] DI for stems was calculated by the formula: DI= Σ [0(n0) + 0.2(n1) + 0.4(n2) + 0.6(n3) +0.8(n4) +1(n5)] x 100/ (N_{total}) where n_x= number of stems or stolons in the x rating class and N =total number of stems or stolons.

[w] DI for black scurf was calculated by the formula: DI= Σ [0(n0) + 0.25(n1) + 0.5(n2) + 0.75(n3) +1(n4)] x 100/ (N_{total}), where n_x = number of tubers in the x rating class and N = total number of tubers in each of the category.

[v] Values in columns followed by the same letter are not significantly different according to Duncan's Multiple range test (P = 0.05).

infested soil was the only inoculum source. However, black scurf DI values were comparable in treatments with soil-borne or seed-tuber inoculum alone (Table 4).

Genotypic shift in the *Rhizoctonia solani* AG 3-PT population under field conditions

Rhizoctonia solani AG 3-PT isolates obtained from symptomatic tissues over the two year period at four different sampling time points over two growing seasons were genotyped using PCR-RFLP to quantify and infer the relative contribution of seed tuber-borne and soil-borne inocula to disease development on potatoes. In total, 151 isolates were isolated from symptomatic tissues. Seed tuber-borne genotypes accounted for 60% of the recovered isolates while soil-borne genotypes accounted for 36% of the recovered isolates and the remaining 4% of the recovered isolates did not match either inoculum genotype and were isolated from plants growing in plots where both forms of inocula were present (Table 5). Isolates with genotypes identical to the inoculants were found only in plots where they were inoculated. Further work was therefore carried out to elucidate whether the appearance of non-inoculated genotypes was due to evolutionary forces like sexual recombination and how the established experimental field population changed over two growing seasons.

In plots inoculated with both sources of inoculum, DI values of stolon/stem canker, root infection and black scurf were significantly different from treatments inoculated with soil-borne inoculum and seed tuber-borne inoculum separately. However, disease occurrence could not be linked to a source of inoculum. Therefore, PCR-RFLP genotyping was carried out to correlate *R. solani* AG 3-PT isolates obtained to the genotype of origin. The PCR-RFLP DNA profiles indicated that tuber-borne inoculum was more pronounced in causing

Table 5. Analyses of PCR-RFLP genotypes of isolates of *Rhizoctonia solani* AG 3-PT recovered from infected roots, stolons, stems and progeny tubers.

		Genotypes							
		2013				2014			
Inoculum Sources	Sampling Period[a]	N[b]	Seed tuber-borne[c]	Soil-borne[c]	Non-Released[d]	N[b]	Seed tuber-borne[c]	Soil-borne[c]	Non-Released[d]
Control	Mid-Season	-[e]	-	-	-	-	-	-	-
	End of season	-	-	-	-	-	-	-	-
Soil	Mid-Season	7	-	7	-	8	-	8	-
	End of season	13	-	13	-	11	-	11	-
Soil and Tuber	Mid-Season	23	19	4	-	13	8	4	1
	End of season	24	18	4	2	12	7	3	2
Tuber	Mid-Season	10	10	-	-	8	8	-	-
	End of season	11	11	-	-	11	11	-	-
Total		88	58	28	2	63	34	26	3

[a]Sampling times: Mid-season = Tuber initiation, End of season = Harvest period.

[b]Number of isolates recovered and genotyped.

[c]Number of isolates with genotypes identical to the seed tuber or soil-borne inoculum.

[d]Genotypes different from the inoculated genotypes.

[e] - Indicates no *R. solani* isolated.

black scurf and stem canker while soil-borne inoculum caused more root infection but also contributed significantly to stolon cankers when results for both years were combined (Table 5 and Fig. 1).

Microsatellite analysis

A total of 157 *R. solani* AG 3-PT isolates were obtained from symptomatic tissue sampled over the two growing seasons (Table 6). Novel genotypes were identified at all sampling points with nine non-released genotypes out of 24 recovered at the end of 2014 growing season. Out of the 24 non-released genotypes, nine had new alleles at locus TC_AG3_0 and TC_AG3_19. Tuber-borne inoculum contributed 53% of the recovered genotypes while soil-borne contributed 47% of the released genotypes. A greater proportion of recovered isolates (84%) had genotypes matching the released inoculants, indicating t the importance of the asexual component of *R. solani* AG 3-PT in disease epidemics on potatoes.

Allele frequencies in field samples

Microsatellite analysis indicated that seven of the eight loci were polymorphic at all sampling time points except locus TC_AG3_7 that had the same alleles for all isolates and this was not included in subsequent analyses (Table 7). All alleles that were present in the soil-borne and seed tuber-borne inoculants were observed in the *R. solani* AG 3-PT isolates obtained from experimental plots at each sampling time point (Table 8). Allele frequencies at all loci remained fairly constant, except for the frequency of 321 bp allele at locus TC_AG 3_18 that decreased while the frequency of 165 at locus TC_AG3_1 increased.

Table 6. Analyses of microsatellite genotypes of isolates of *Rhizoctonia solani* AG 3-PT recovered from potato in experimental plots.

Population[a]	N[b]	MLG[c]	Released[d](%)	Seed tuber-borne	Soil-borne	Non – Released[e] (%)
2013Mid	30	6	26(86)	14	12	4 (13)
2013End	32	6	28(87.5)	17	11	4 (12.5)
2014Mid	42	10	35(83)	19	16	7 (16)
2014End	53	11	44 (80)	20	24	9 (16)
Total	157	33	133 (84)	70(53)	63(47)	24 (15)

[a]Populations denoted by year and growth stage of sampling; Mid = Tuber initiation, End = Harvest

[b]Number of isolates with genotypes identical to a released inoculant's genotype.

[c]MLG-clone corrected genotypes.

[d]All released isolates (% of N).

[e]All non-released isolates (% of N)-with different genotypes to the inoculants.

Table 7. Frequencies of alleles observed at eight microsatellite loci in *Rhizoctonia solani* AG 3-PT isolates from four populations isolated from experimental plots at different sampling times.

Locus	Allele sizes (bp)	Expected[a]	2013Mid[b]	2013End	2014Mid	2014End
TC_AG3_0	151	1.00	0.98	0.97	0.96	0.93
	160	0.00	0.02	0.03	0.04	0.07
TC_AG3_1	152	0.72	0.76	0.74	0.72	0.57
	165	0.28	0.24	0.30	0.28	0.43
TC_AG3_6	197	0.50	0.50	0.46	0.54	0.55
	203	0.50	0.50	0.54	0.46	0.45
TC_AG3_7	244	1.00	1.00	1.00	1.00	1.00
TC_AG3_10	245	0.88	0.76	0.69	0.70	0.84
	248	0.12	0.24	0.31	0.30	0.16
TC_AG3_16	323	0.25	0.43	0.26	0.26	0.27
	327	0.25	0.07	0.24	0.24	0.24
	330	0.25	0.07	0.24	0.24	0.23
	346	0.25	0.43	0.26	0.26	0.26
TC_AG3_18	321	0.25	0.43	0.25	0.26	0.29
	324	0.25	0.42	0.26	0.26	0.27
	346	0.50	0.15	0.49	0.48	0.44
TC_AG3_19	320	0.25	0.07	0.25	0.24	0.22
	324	0.00	0.00	0.01	0.01	0.01
	343	0.43	0.26	0.26	0.26	0.30
	346	0.25	0.43	0.25	0.25	0.27
	356	0.25	0.07	0.23	0.24	0.20

[a]Expected allele frequencies based on alleles observed in inoculant isolates.

[b]Sampling points Mid=Mid-season sampled at tuber initiation, End=End of growing season sampled at tuber harvesting stage.

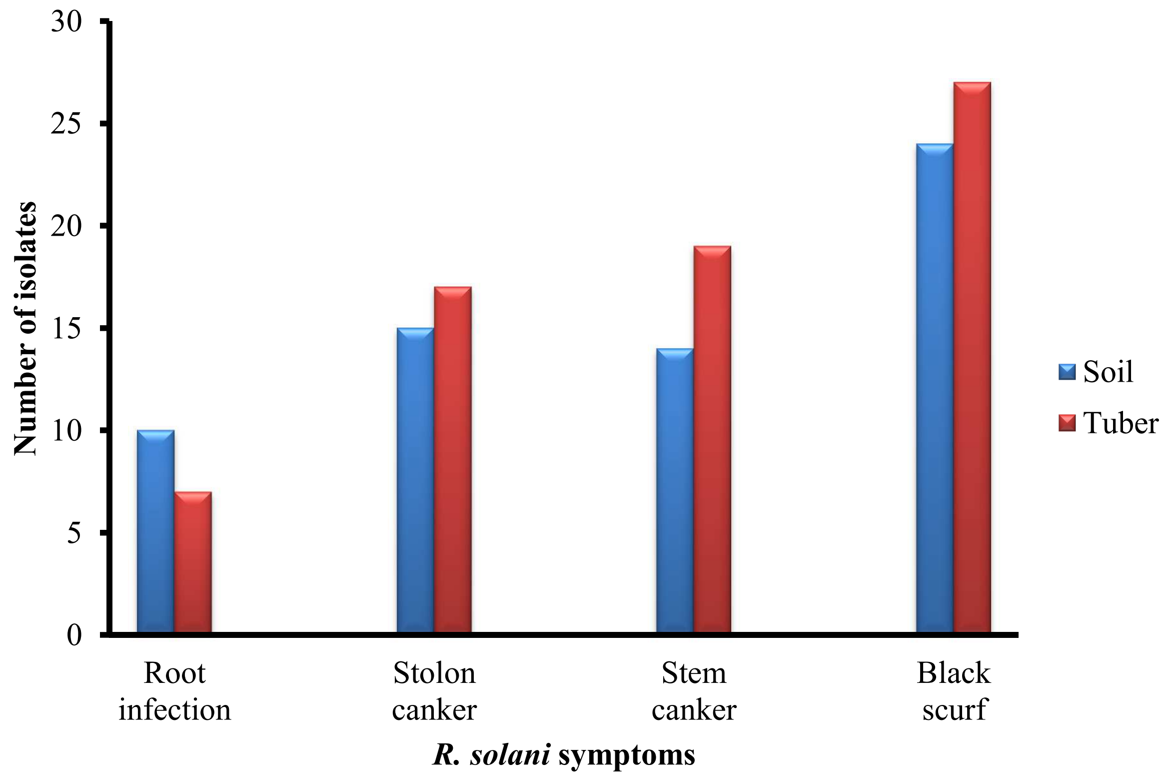

Fig.1. Frequency distribution of seed tuber-borne and soil-borne PCR-RFLP genotypes associated with *Rhizoctonia solani* diseases on potatoes sampled from the experimental plots over two years.

Table 8. Genetic diversity indices for *Rhizoctonia solani* AG 3-PT populations isolated from different sampling times calculated from microsatellite data.

Between Sampling points	N[a]	G[b]	Ne[c]	I_A[d]	rBarD[e]	Allelic richness[f]	E_5[g]	F_{IS}[h]	H_0[i]	H_E[j]
2013Mid	30	0.60	0.24	2.94*	0.72*	2.00	0.54	-0.2	0.6	0.39
2013End	32	0.67	0.26	2.75*	0.58*	1.98	0.56	-0.3	0.59	0.41
2014Mid	42	0.70	0.26	2.69*	0.50*	2.0	0.53	-0.2	0.62	0.41
2014End	53	0.72	0.27	2.72*	0.54*	1.96	0.44	-0.1	0.61	0.40
Between years										
2013	62	0.627	0.26	2.85*	0.67*	2.0	0.54	-0.3	0.6	0.40
2014	95	0.715	0.28	2.72*	0.55*	2.0	0.57	-0.2	0.62	0.41

[a]Number of isolates genotyped.

[b]Genotypic diversity, defined as the probability that two individuals taken at random have different genotypes (Nei 1987).

[c]Nei's unbiased gene diversity based on 1000 permutations computed in FSTAT version 2.9.3.2 (Nei 1987).

[d]I_A: standardized index of association; values that differ significantly from zero indicate departure from linkage equilibrium.

[e]rBarD: standardized index of association.

[f]Calculated according to El Mousadik and Petit (1996).

[g]E_5 Index of evenness.

[h]F_{is} Fixation index.

[i]H_o Observed heterozygosity.

[j]H_e Expected heterozygosity.

*Significant at P < 0.01.

Population differentiation and divergence in field populations

A recovered genotype was considered a possible recombinant if it contained all of the alleles that were common to the released genotypes. Genotypic diversity seemed to increase slightly but not significantly over the successive sampling time points over the two-year study, from 0.6 in 2013 to 0.72 in 2014 (Table 8). The expected heterozygosity (Nei's unbiased gene diversity) remained fairly constant over the sampling time points and growing years (from 0.24 to 0.27). Among the four sampling points, 2013 mid-season had the lowest gene diversity (0.24), whilst the 2014 end of season had the highest (0.27). When the *R. solani* AG 3-PT population were divided by year, Nei's genetic diversity rose slightly from 0.26 in 2013 to 0.28 in 2014 (Table 6). The unbiased expected heterozygosity did not differ significantly in 2013 (H_E = 0.40) as compared to 2014 (H_E = 0.41). The observed heterozygosity remained fairly constant across the years, 0.6 in 2013 and 0.62 in 2014 (Table 8). Negative F_{IS} values were observed for all the populations. Multilocus measures of association (I_A = index of association and rBarD = multilocus linkage disequilibrium) were significant ($P < 0{\cdot}01$) for all populations, providing strong evidence of rejection of the random mating hypothesis (Table 8). The value of I_A ranged from 2.69 (2014 mid-season) to 2.94 (2013 mid-season) and the corresponding values of rBarD ranged from 0.54 (2014 end of season) to 0.72 (2013 mid season) (Table 8). There was, however, a gradual decrease in multilocus measures of association as the seasons progressed.

Population differentiation was estimated using $F_{ST.}$ The F_{ST} values indicated little genetic differentiation among populations across the years. The pairwise F_{ST} values ranged from -0.008 to 0.054 and only 2013 mid-season were significantly differentiated from 2014 end of season ($P < 0.05$). There was no differentiation between populations at other sampling time points with the F_{ST} ranging from -0.008 to 0.054.

Discussion

Rhizoctonia infection affects the quality and quantity of potato tubers (Banville 1989). Integrated management of *R. solani* diseases on potato involves among other strategies, reducing the pathogen population in primary sources of inoculum and preventing the genetic changes of the pathogen in the field. A better understanding of the contribution of different sources of inoculum to *Rhizoctonia* disease development on potato and the concomitant understanding of *Rhizoctonia* pathogen evolution would contribute to long term effective disease management.

Several studies have been conducted to investigate the influence of inoculum source of *R. solani* on potato disease development (Atkinson et al. 2010; Brierley et al. 2016; Carling et al. 1989; Tsror (Lahkim) and Peretz-Alon 2005). However, these studies differ as to which inoculum source is more important for causing Rhizoctonia disease epidemics on potato. In this study, both inoculum sources were important in causing black scurf on progeny tubers. Seed tuber-borne inoculum was more prominent in causing stem canker whereas soil-borne inoculum was associated more with root -and stolon infection (Table 4 and Fig. 1). Soil-borne and seed tuber-borne inoculum of *R. solani* AG 3-PT was demonstrated to have an additive effect in causing potato diseases. Treatments with a combination of soil-borne and seed tuber-borne inoculum had significantly higher stem canker and black scurf disease indices (Table 4). The observations from this study corroborated well with field studies conducted in the Netherlands (van Emden 1965) and Israel (Tsror (Lahkim) and Peretz-Alon 2005), amongst others, that demonstrated the additive effect of seed tuber-borne and soil-borne inoculum in causing diseases on potato.

From the PCR-RFLP genotyping results obtained in this study, both seed tuber- and soil-borne inocula were etiologically important. Seed tuber-borne inoculum resulted in slightly more infections on stems and progeny tubers (Fig. 1). Van Emden (1965) surmised

that seed tuber-borne inoculum is nearest to the developing shoots and is of greater importance in causing stem canker than soil-borne inoculum.

Soil-borne inoculum also appeared to play a role in causing infection on roots, stolons and black scurf on progeny tubers. Tsror (Lahkim) and Peretz-Alon (2005) reported that soil-borne inoculum increased the severity of black scurf while Brierley et al. (2016) proved that soil-borne inoculum is an important source of inoculum in causing black scurf on potatoes. This indicates that if pathogen-free seed tubers are sown in infested soil, the soil becomes the only source of inoculum. Frank and Leach (1980), postulated that as stolons and roots grow through the soil and away from the seed piece inoculum, the inoculum in soil may become the primary source of infection of the stolon. The fact that both seed-tuber- and soil-borne inocula were consistently linked with high stem and black scurf disease indices demonstrates the importance of both inoculum sources -on development of potato diseases in South Africa. This is in contrast to the findings of James and Mackenzie (1972) which demonstrated the importance of soil-borne inoculum alone in potato disease development.

The observation of high genetic diversity among *R. solani* AG 3-PT isolates sampled from the same field in South Africa in a separate study (Muzhinji et al. 2016) might indicate evolution of *R. solani* AG 3-PT within the course of the growing season, perhaps due to gene flow, mutation or recombination. In commercial potato fields it is difficult to ascertain the identity of the founding population or when introductions had occurred, therefore identification of evolutionary forces is done retrospectively. This study on the relative importance of soil- and/or seed tuber-borne inoculum has provided a unique opportunity to understand how genetic diversity is shaped and maintained in fields with minimum gene flow using the mark-release-capture strategy.

Of interest in this study was the identification of non-released genotypes (Table 5 and 6). The identification of non-released isolates leads us to speculate on the role of

recombination between field inoculants. Two lines of evidence to support the assumption were, firstly, that the experiment to evaluate the relative contribution of soil- and/or seed tuber-borne inoculum source was conducted in fumigated soils using disease-free seed tubers minimizing the possibility of any other sources of inoculum. Secondly, white-collar which contains basidiospores involved in sexual recombination was observed on potato stem base during tuber initiation. Furthermore, the contribution of evolutionary forces like mutation to the formation of new genotypes is not expected within the time scale of this experiment. The key to controlling *Rhizoctonia* diseases on potato lies in the use of *Rhizoctonia*-free seed tubers because of their importance in disseminating the disease and adding to the pool of soil-borne inoculum.

Analysis of allelic associations and genotypic diversity is routinely used to infer reproductive strategies of plant pathogens and in most cases an increase in genetic diversity is the hall-mark of sexual recombination (Keller et al. 1997 and 2010). Over the course of the experiment, Nei's unbiased genetic diversity remained fairly constant as expected for clonal and asexually reproducing populations.

Tests for multilocus associations showed that all four populations were in gametic disequilibrium as evidenced by high I_A values (2.69 - 2.90). This suggests high clonality and predominance of asexual reproduction. Furthermore, rBarD was similarly high (0·50 - 0·72) providing further evidence of higher clonality (Table 8). The detection of many isolates (133) with genotypes identical to the soil-borne and tuber-borne inoculants is strong evidence that asexual reproduction contributed significantly to the disease epidemics in the population genetic changes experiment.

Our results demonstrated a significant level of clonality for all the populations with little evidence of sexual recombination. This is supported by high proportion of repeated genotypes, with the inoculant genotypes dominating the population sampled. The discovery

of non-released genotypes and gradual increase in genotypic diversity might suggest that, when the conditions are conducive for the formation of basidiospores, recombination occurs in the field. This might increase the potential formation of novel genotypes through combinations of new alleles. These new novel genotypes might successfully overseason and serve as the founding population for the next *Rhizoctonia* disease cycle. This could lead to significant changes in the genetic structure of field populations from year to year and might have practical implications in *R. solani* AG 3-PT management, given the risk of genetic change demonstrated in this study. The use of pathogen-free tubers or the successful

treatment of seed tubers with fungicides could minimize the introduction of different genotypes of *R. solani* into the same field. Control measures aimed at limiting the sexual phase of *R. solani* AG 3-PT would reduce the emergence of new genotypes in the field. Therefore, a seed health management approach combining clean seed and fungicide seed treatments might result in significant disease control in the absence of substantial gene flow inoculum of *R. solani*.

Acknowledgements

This work was supported by funding from Potatoes South Africa. We thank The Potato Pathology Group at the University of Pretoria for helping with field and greenhouse work. N. Muzhinji received a studentship from the National Research Foundation and University of Pretoria and also thanks the Tobacco Research Board in Zimbabwe. The National Research Foundation (NRF) is acknowledged for supporting this study and purchasing the DNA sequencing instrument (grant UID: 78566) used at the University of Pretoria.

Literature cited

Agapow, P. M., and Burt, A. 2001. Indices of multilocus linkage disequilibrium. Mol. Eco. Notes. 1:101-102.

Atkinson, D., Thornton, M. K., and Miller, J. S. 2010. Development of *Rhizoctonia solani* on stems, stolons and tubers of potatoes. Effects of inoculums source. Am. J. Potato Res. 87:374-381.

Balali, G. R., Neate, S. M., Kasalkher, A. M., Stodart, B. J., Melanson, D. J., and Scott, E. S. 2007. Intraspecific variation of *R. solani* AG 3 isolates recovered from potato fields in Central Iran and South Australia. Mycopathologia. 163:105-115.

Banville, G. J. 1989. Yield losses and damage to potato plants caused by *Rhizoctonia solani* Kuhn. Am. Potato J. 66:821-834.

Bardin, M., Decognet, V., and Nicot, P. C. 2014. Remarkable predominance of a small number of genotypes in greenhouse populations of *Botrytis cinerea*. Phytopathology. 104:859-864.

Bartz, F. E., Cubeta, M. A., Toda, T., Naito, S., and Ivors, K. L. 2010. An in planta method for assessing the role of basidiospores in *Rhizoctonia* foliar disease of tomato. Plant Dis. 94:515-520.

Bennett, R. S., Milgroom, M. G., Sainudiin, R., Cunfer, B. M., and Bergstrom, G. C. 2007. Relative contribution of seed-transmitted inoculum to foliar populations of *Phaeosphaeria nodorum*. Phytopathology. 97:584-591.

Brierley, J. L., Hilton A. J., Wale, S. J., Woodhall, J. W., and Lees A. K. 2016. The relative importance of seed- and soil-borne inoculum of *Rhizoctonia solani* AG-3 in causing black scurf on potato. Potato Res. 59:181-193.

Carling, D. E., Leiner, R. H., and Westphale, P. C. 1989. Symptoms, signs and yield reduction associated with *Rhizoctonia* disease of potato induced by tuber borne inoculum of *Rhizoctonia solani* AG 3. Am. Potato J. 66:693-701.

Carling, D. E., Kuninaga, S., and Brainard, K. A. 2002. Hyphal anastomosis reactions, rDNA internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. Phytopathology. 92:43-50.

Ceresini, P. C., Shew, H. D., Vilgalys, R., Gale, L. R., and Cubeta, M. A. 2002. Genetic diversity of *Rhizoctonia solani* AG-3 from potato and tobacco in North Carolina Mycologia. 94:437-449.

Das, S., Shah, F. A., Butler, R. C., Falloon, R. E., Stewart, A., Raikar, S., and Pitman, A. R. 2014. Genetic variability and pathogenicity of *Rhizoctonia solani* associated with black scurf of potato in New Zealand. Plant Pathol. 63:651-666.

El Mousadik, A. and Petit, R. J. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. Theor. Appl. Gen. 92:832-839.

Excoffier, L., and Lischer, H. E. L. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Res. 10:564-567.

Ferrucho, R. L., Zala, M., Zhang, Z., Cubeta, M. A., Garcia-Dominguez, C., and Ceresini, P. C. 2009. Highly polymorphic in silico-derived microsatellite loci in the potato-infecting fungal pathogen *Rhizoctonia solani* anastomosis group 3 from the Colombian Andes. Mol. Ecol. Res. 9:1013-1016.

Ferrucho, R. L., Ceresini, P. C., Ramirez-Escobar, U. M., McDonald, B. A., Cubeta, M. A., and García-Domínguez, C. 2013. The population genetic structure of *Rhizoctonia solani* AG-3PT from potato in the Colombian Andes. Phytopathology. 103:862-869.

Fiers, M., Chatot, C.,Edel-Hermann, V., Hingrat, L. Y., Konate, A. Y., Gautheron, N., Guillery, E., Alabouvette, C., and Steinberg, C. 2011. Genetic diversity of *Rhizoctonia solani* associated with potato tubers in France. Mycologia. 103:1230-1244.

Frank, J. A., and Leach. S. S. 1980. Comparison of tuber-borne and soil-borne inoculum in the *Rhizoctonia* disease of potato. Phytopathology. 70:51-53.

Glaubitz, J. C. 2004. Convert: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Mol. Ecol. Notes. 4:309-310.

Goudet, J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. J. Hered. 86:485-486.

James, W. C., and Mackenzie A. R. 1972. The effect of tuber-borne sclerotia of *Rhizoctonia solani* Kuhn on the potato crop. Am. Potato J. 49:296-301.

Jeger, M. J., Hide, G. A., van den Boogert, P. H. J. F., Termorshuizen, A. J., and van Barrlen, P. 1996. Pathology and control of soil-borne pathogens of potato. Potato Res. 39:437-469.

Keller, S. M., McDermott, J. M., Pettway, R. E., Wolfe, M. S., and McDonald, B. A. 2007. Gene flow and sexual reproduction in the wheat glume blotch pathogen *Phaeosphaeria nodorum*, (anamorph *Stagonospora nodorum*). Phytopathology. 87:353-358.

Keller, M. D., Waxman, K. D., Brgsrom, G.C., and Schmale, D.G. III. 2010. Local distance of wheat spike infection by released clones of *Gibberella zeae* disseminated from infested corn residue. Plant Dis. 94:1151-1155.

McDonald, B. A., and Linde, C. 2002. The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica. 124:163-180.

Meirmans, P. G., and Van Tienderen, P. H. 2004. GenoType and GenoDive: Two programs for the analysis of genetic diversity of asexual organisms. Mol. Ecol. Notes. 4:792-794.

Muzhinji, N., Truter, M., Woodhall, J. W., and van der Waals, J. E. 2015. Anastomosis groups and pathogenicity of *Rhizoctonia solani* and binucleate *Rhizoctonia* from potatoes in South Africa. Plant Dis. 99:1790-1802.

Muzhinji, N., Truter, M., Woodhall, J. W., and van der Waals, J. E. 2016. Population genetic structure of *Rhizoctonia solani* AG 3-PT associated with potato diseases in South Africa. Fungal Biol. 12: 701 -710.

Nei, M. 1987. Molecular Evolutionary Genetics. New York, USA

Ogoshi, A. 1975. Grouping of *Rhizoctonia solani* Kühn and their perfect stages. Rev.Plant Prot. Res. 8:93-103.

Peakall, R., and Smouse, P. E. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes. 6:288-295.

Shah, D. A., Bergstrom, G. C., and Ueng, P. P. 1995. Initiation of *Septoria nodorum* blotch epidemics in winter wheat by seed-borne *Stagonospora nodorum*. Phytopathology. 85:452-457.

Shew, H. D., and Melton T. A. 1995. Target spot of tobacco. Plant Dis. 79:6-11.

Simons, S. A., and Gilligan, C. A. 1997. Factors affecting the temporary progress of stem canker (*R. solani*) on potatoes (*Solanum tuberosum*). Plant Pathol. 46:642-650.

Sneh, B., Katan, J., Henis, Y., and Wahl, I. 1966. Methods for evaluating inoculum density of Rhizoctonia in naturally infected soil. Phytopathology 56:74-78.

Sneh, B., Jabaji-Hare, S., Neate, S., and Dijst, G. 1996. Rhizoctonia *Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*. Kluwer Academic Publishers Dordrecht, The Netherlands.

Sommerhalder, R. J., McDonald, B. A., Mascher, F., and Zhan, J. 2010. Sexual recombinants make a significant contribution to epidemics caused by the wheat pathogen *Phaeosphaeria nodorum*. Phytopathology. 100:855-862.

Tsror (Lahkim), L., and Peretz-Alon, I. 2005. The influence of the inoculum source of *Rhizoctonia solani* on development of black scurf on potato. J. Phytopathol. 153:240-244.

Tsror, L. 2010. Biology, Epidemiology and Management of *Rhizoctonia solani* on potato. J. Phytopathol. 158:649-658.

van Emden, J. H. 1965. *Rhizoctonia solani*; results of experiments. Eur. Pot J. 8:188-189.

Woodhall, J. W., Lees, A. K., Edwards, S. G., and Jenkinson, P. 2007. Characterization of *Rhizoctonia solani* from potato in Great Britain. Plant Pathol. 56:286-295.

Woodhall, J. W., Lees, A. K., Edwards, S. G., and Jenkinson, P. 2008. Infection of potato by *Rhizoctonia solani*: Effect of anastomosis group. Plant Pathol. 57: 897-905.

Zhan, J., Mundt, C. C., and McDonald, B. A. 1998. Measuring immigration and sexual reproduction in field populations of *Mycosphaerella graminicola*. Phytopathology. 88:1330-1337.

Zhan, J., and McDonald, B. A. 2013. Field-based experimental evolution of three cereal pathogens using a mark–release–recapture strategy. Plant Pathol. 62:106-114.