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PREPARATION OF INOCULUM OF RHIZOCTONIA SOLANI KÜHN FOR AN ARTIFICIALLY INOCULATED FIELD TRIAL

**Herstellung eines *Rhizoctonia solani* Kühn-Inokulums für einen künstlich
inokulierten Feldversuch / Élaboration d'un inoculum *Rhizoctonia solani* Kühn
pour un essai d'inoculation artificielle au champ**

ABSTRACT

Rhizoctonia crown root and rot, caused by *Rhizoctonia solani* Kühn, is a serious disease resulting in substantial economic losses in sugar beet production worldwide. A consistent, uniform disease pressure of the correct intensity is necessary to effectively screen sugar beet for resistance to Rhizoctonia crown and root rot in an artificially inoculated field trial. This study examined the substrate used to grow the *R. solani* inoculum, the method of substrate inoculation, and the pathogenicity of the different particle sizes within the inoculum. It was found that particles greater than 0.425 mm were the most consistently colonized and provided constant flow through the Gandy™ applicator, which is used to inoculate the field plots. The smaller particle sizes did not contain adequate amounts of the pathogen, contained substantial amounts of contaminating bacteria or fungi, and contributed to a varied rate of flow during distribution. We found hull-less barley the best performing substrate to inoculate with *Rhizoctonia solani*, and a liquid suspension of the pathogen in potato dextrose broth provided uniform colonization of the autoclaved barley during the incubation period. Mushroom spawn bags provided the ideal environment to reduce contamination and insure rapid colonization of the barley grain. The techniques described have increased the efficiency of inoculum production, decreased losses due to contamination, and guarantee a homogeneous inoculum in size and disease potential, which results in a uniform, consistent field infection.

INTRODUCTION

Rhizoctonia root rot (caused by the fungus *Rhizoctonia solani* Kühn) continues to be a problem in most sugar beet growing areas in the United States, and has become an increasing concern worldwide. Shorter crop rotations and the expansion of production into infested areas have compounded the issue. The result is a decrease in net returns to the industry from processing losses caused by fewer acres harvested and the reduction of recoverable sucrose. Genetic resistance, coupled with judicious cultural measures and chemical protection (where possible) is a more economical and practical method of reducing losses caused by this fungus than a solely chemical management program. Additionally, in instances of severe rhizoctonia infestation, chemical protection alone may not provide adequate control.

In his 1959 M.S. thesis, V. G. PIERSON states, "Attempts to breed rhizoctonia resistant varieties have been impeded by the lack of satisfactory techniques for creating artificial exposure to attack. Such techniques must provide for a high degree of uniformity in the intensity of disease exposure and also for adjustment of intensity of exposure to levels suitable for root selection and progeny testing purposes" (PIERSON, 1959). PIERSON and GASKILL went on to develop the methodology for creating field epiphytotics of Rhizoctonia crown and root rot (PIERSON & GASKILL, 1961). This method has changed over time as the size of the evaluation nurseries have increased. The manual labor required to apply 0.62 ml (1/8 teaspoon) of inoculum in the crown of each plant (GASKILL, 1968) was no longer feasible. Therefore the inoculation method was adapted to a mechanized delivery system and the rating scale was fixed at 0 (healthy with no sign of disease) to 7 (dead and rotted) (HECKER and RUPPEL, 1977; PANELLA, 1998; RUPPEL *et al.*, 1979). It is imperative that the methods to produce an artificially inoculated field evaluation are continually improved. This study examined the substrate used to grow the *Rhizoctonia solani* (*R. solani*) inoculum, the method of substrate inoculation, and the infectivity of the different fraction sizes within the inoculum.

MATERIALS AND METHODS

All laboratory and greenhouse pathogenicity experiments used barley infested with *R. solani*, created for field inoculum by the Fort Collins Sugar Beet Research Unit. The following experiments were conducted in the fall and winter of 2010. Inoculum also was used for each year's rhizoctonia field nursery evaluations of both research germplasm and commercial cultivars (PANELLA *et al.*, 2011; PANELLA *et al.*, 2010).

Inoculum Composition

Preparation of Inoculum

Field inoculum for 2009 and 2010 was produced in large (16"x 20") stainless steel pans with lids holding 4 l of barley (PANELLA, 1998). Prior to autoclaving, 2400 ml distilled water was added to each pan, which was autoclaved (2 cycles at 45 min each on the liquid setting, and the barley grain stirred after the first cycle). The barley was cooled and inoculated 2.5 cm below the surface of the grain with plugs from plates of potato dextrose agar (PDA) infested with the *R. solani* (AG2-2) isolate, R-9. There were 9 plugs (7 mm) in each pan in 2009 and 13 plugs (18 mm) in 2010. This isolate has been used for over 50 years to test sugar beet for resistance in both field and greenhouse experiments (PIERSON & GASKILL, 1961; HECKER & RUPPEL, 1977; RUPPEL *et al.*, 1979; PANELLA, 1998). The incubation period required was 4 weeks in 2009 and 2 weeks in 2010. The infested barley was air dried (1 week) and ground using a Wiley Mill (#3 diameter screen; 3.175 mm mesh).

Fractionation of Inoculum

Two replicate 4 l samples from each year's inoculum were collected from large storage bins (stored at 16°C) and mixed to assure homogenous consistency. Three 100 ml samples from each replicate (year) were separated into fractions. The separation was accomplished using U.S. Standard Sieve Series (Dual MFG CO, Chicago, IL). Two sterilized sieves (No. 18 and No. 40) and a collection pan were used to separate the inoculum into three fractions: small (<0.425 mm), medium (0.425-0.99 mm), and large (>1.0 mm) (fig. 1). Chaff was then separated by hand from each of the medium and large fractions. Each fraction was weighed and transferred into sterile 50 ml plastic conical tubes and stored at 4°C.

Laboratory Analysis of Fraction Sizes

Examination of particle infectivity

Growth on PDA with and without antibiotics (4.5 µM streptomycin and 1.5 µM penicillin) was used to evaluate at 3 day the viability of *R. solani* from each fraction. The samples were grown in an incubator at 25°C (16 h day, 8 h night). Three samples from each five fractions (fig. 1) of both year's inoculum were assessed for how many particles (10 particles for each sample) contained viable *R. solani* or contaminants, both fungal and bacterial. All handling of media preparation and inoculation was done in a biosafety cabinet to reduce any extraneous sources of contamination.

The large and medium size particles were manipulated easily using standard sterile technique with small forceps. The growth of the small size fraction was tested in several ways with unsatisfactory results. Using a dilution series, no viable *R. solani* was observed (even at 1/10 dilution), however, numerous contaminants were observed over the surface of the plate. A 1 g/200 ml dilution, using both sterile water and sterile 5% water agar, did not produce any viable *R. solani*. Even when plated on KO & HORA's Media semi-selective for *Rhizoctonia* (KO & HORA, 1971), only a few colonies grew. Finally, using forceps, approximately 0.04 g of the small fraction was placed 10 times on the surface of the plate – to represent 10 single sources, comparable to the individual particles of the other fraction sizes.

Greenhouse Pathogenicity Testing

Greenhouse potting mix (Fafard 2-SV) was used following steam pasteurization (30 min @ 180°C) in surface sterilized plastic pots (15 cm diameter). Two genotypes of sugar beet were used in both greenhouse pathogenicity tests – FC709-2 (19921024) (PANELLA, 1999) as a resistant control and FC901/C817 (19941025) the susceptible control used in our field nursery (PANELLA *et al.*, 2014). Seeds were planted to a depth of 1.25 to 2.5 cm within each pot and thinned to three plants per pot, with two pots per treatment. Each plant was inoculated with the designated treatment following a randomized complete block design. At the 8-10 leaf growth stage, approximately 0.62 ml of the designated inoculum was placed 1.25 cm from the hypocotyl and 2.5 cm deep. Each root was evaluated using the 0 to 7 (healthy to dead) disease index (DI) 28 days after inoculation (HECKER & RUPPEL, 1977; RUPPEL *et al.*, 1979).

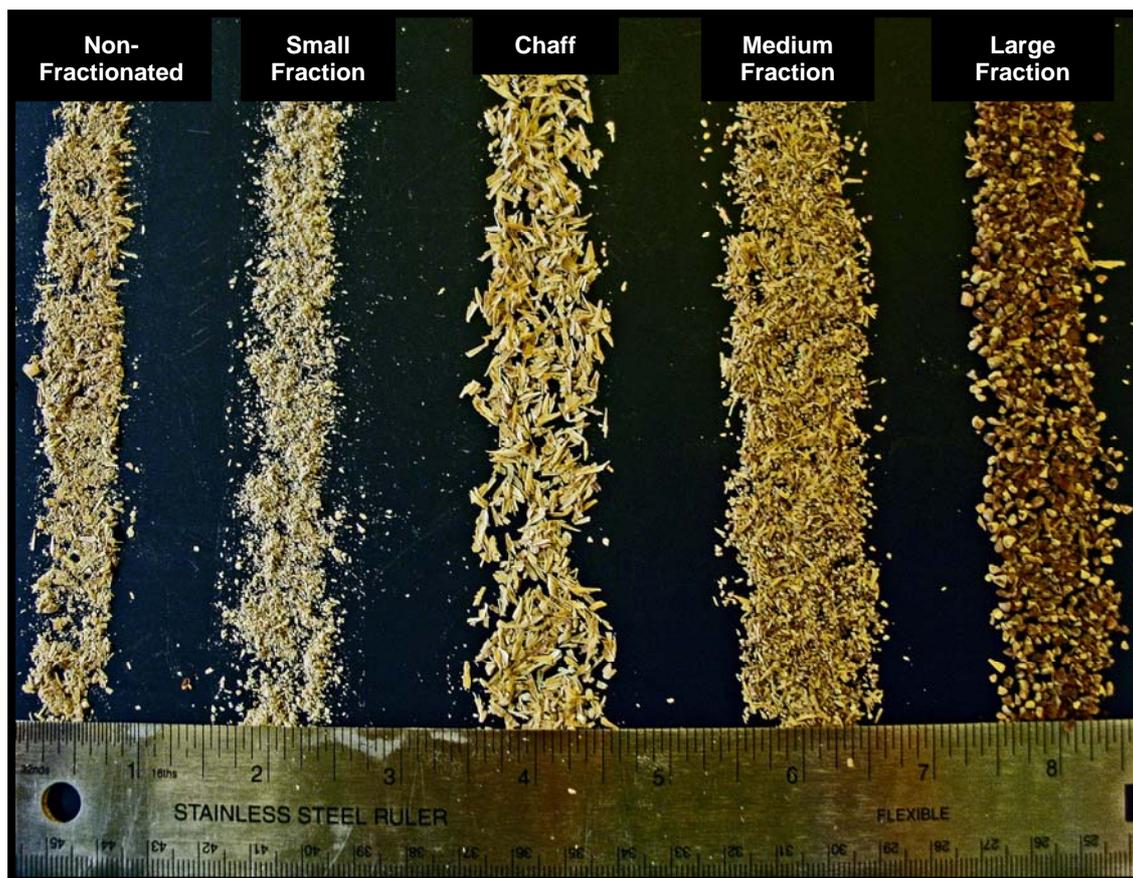


Fig. 1: The milled barley was fractionated into the small fraction, the medium fraction, the large fraction, and the chaff, which is the hull of the barley without any seed attached.

Greenhouse Pathogenicity of Fraction Sizes

The efficacy of the fraction sizes of field inoculum to cause disease was tested using 5 treatments for each year's inoculum (2009 and 2010). Each size fraction (small, medium and large), sterile barley as a negative control, and the non-fractionated 2009 or 2010 inoculum as a positive control were tested in the greenhouse. The average daytime temperature was 28°C and night temperature 19°C, with an average relative humidity of 35%. Treatments were completely randomized, blocked by genotype and replicated twice in time. There were 2 pots with 3 plants each x treatment (5) x genotype (2) x inoculum source (2) x replicate (2).

Greenhouse Pathogenicity of grain sources

The evaluation of different carbohydrate sources' effect on pathogenicity was conducted using the above procedure. The use of millet, milled wheat, milled grain sorghum, milled buckwheat, milled barley (2009 and 2010), barley chaff, and milled hull-less barley were examined. The average daytime temperature was 28°C and night time temperature 21°C, with an average relative humidity of 22%. There were 9 treatments (grain types) used, with 2010 field barley as positive control, sterilized barley (autoclaved) as negative control, and 2010 barley chaff separated from inoculum by hand. Treatments were completely randomized, blocked by genotype, and replicated twice in time. There were 2 pots with 3 plants each x treatment (9) x genotype (2) x replicate (2).

RESULTS AND DISCUSSION

To optimize the inoculation procedure, knowledge of the efficacy of the different size fractions to cause disease was crucial. The effects of different fractions using DI as a measure were tested in the greenhouse. Disease indices were analysed using linear mixed model (Proc Glimmix – SAS version 9.3). The 'slice' feature of PROC Glimmix was used to examine interaction effects of treatment and inoculum (inoculum being the year the inoculum was produced) and treatment and media (PDA and PDA with antibiotics) by testing the simple effects of treatment within inoculum and media separately.

Both the small fraction and chaff fractions were not significantly different in the number of particles that were colonized by *R. solani* at 3 days when averaged over media. The small fraction had significantly less *R. solani* colonization than the medium and large fractions (fig. 2). When averaged over inoculum the results were the same except that the large chaff had significantly more *R. solani* colonization than the small fraction.

The small fraction also exhibited the highest amounts of fungal and bacterial contaminants (fig. 2). Fungal contamination was primarily *Aspergillus* and *Penicillium*. Close examination of the chaff fractions revealed that the *R. solani* had not colonized the chaff but emanated from a minute piece of seed that remained attached. Additional chaff particles were plated without seed attached and confirmed the absence of *R. solani* colonization (data not shown). Differences are evident between the two years inoculum sources. The amount of contamination was higher in the older source and the number of *R. solani* infected particles was greater in the newer source. Also the medium and large fractions had fewer overall contaminated particles.

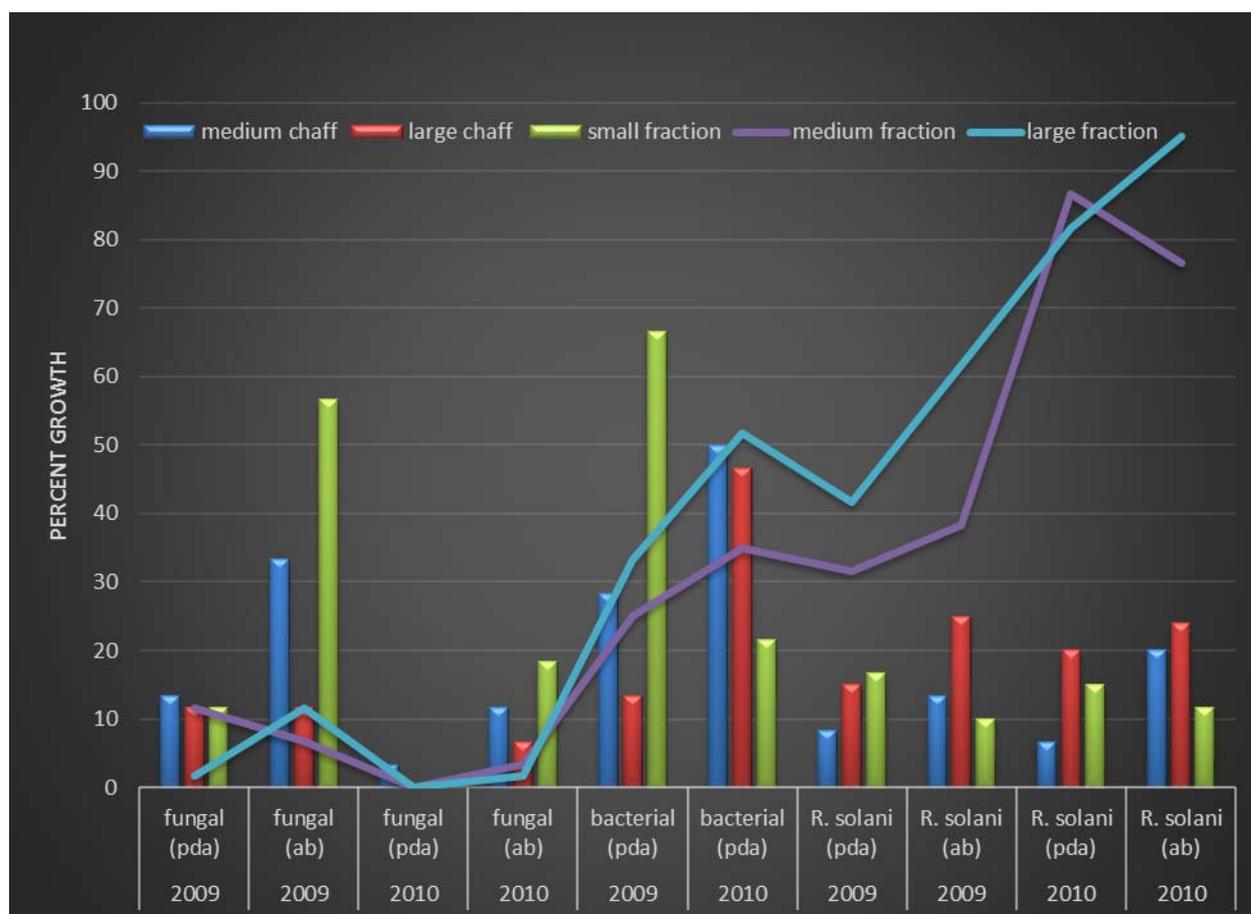


Fig. 2: Comparison of the particle infectivity of the five fractions of barley inoculum. Each fraction from two years inoculum was plated on potato dextrose agar (PDA) with and without the addition of antibiotics (AB). The fractions were analyzed to determine what each individual particle contained: fungus, bacteria, or *R. solani*. (Note: no bacteria grew on the (AB) plates and is not shown).

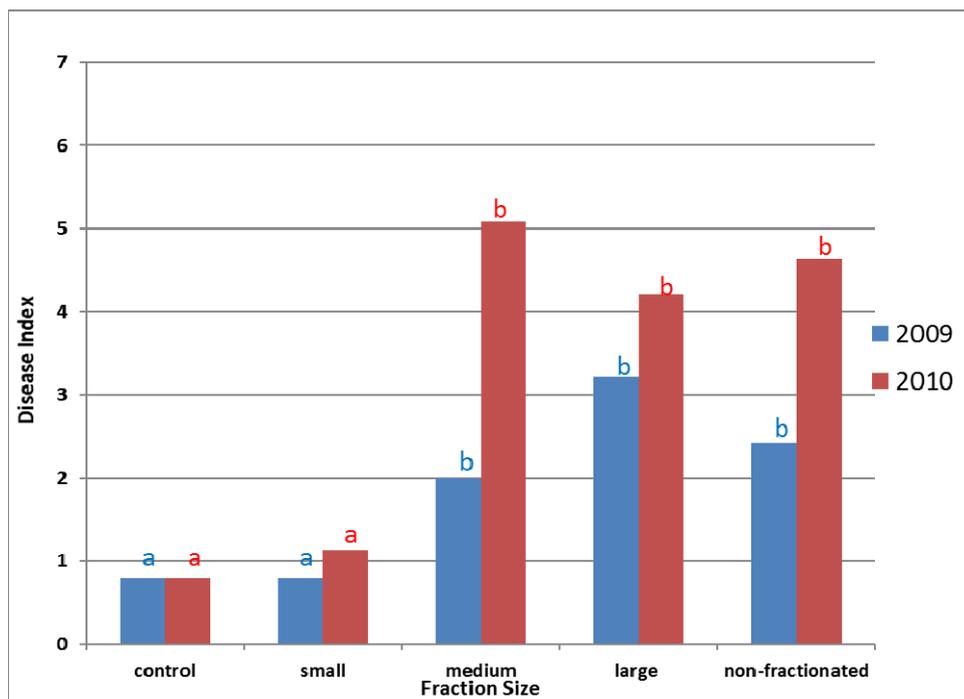


Fig. 3: Comparison of inoculum from different years of production on the fraction size's ability to cause disease symptoms on sugar beet, averaged over genotype and measured by a disease index, where 0 = healthy and 7 = dead.

The effects of different fractions using DI as a measure were tested in the greenhouse. Disease indices were analysed using linear mixed model (Proc Glimmix – SAS version 9.3). Inoculum replicates and their interactions with fraction and media types were represented as random effects in the model. The 'slice' feature of PROC Glimmix was used to examine interaction effects of treatment and year (year being the year the inoculum was produced) and treatment and genotype (resistant and susceptible lines) by testing the simple effects of treatment within year and genotype separately. Figure 3 shows comparison of the different year of inoculum production on the fraction's ability to cause disease (averaged over genotype) as measured by the DI. A DI that falls above 3 indicates disease is present. The negative control (sterile barley) was not colonized by *R. solani* and the treatments (fraction sizes) within year that have the same letter are not significantly different ($P = 0.05$). Both the medium and large fraction sizes were just as effective at causing disease as was the non-fractionated field inoculum. The small fraction was not significantly different from the control. The 2010 inoculum (more recently produced) was more effective at causing disease than the 2009 inoculum (fig. 3), which had been stored for one year at approximately -15.6°C .

Figure 4 shows the comparison of the different genotype's disease response (averaged over both years) to different fraction sizes as measured by the DI. There was no significant difference between the sterile barley control and the small fraction, neither caused disease ($P=0.05$). Again, the medium and large fraction sizes were not significantly different in their ability to cause disease from the non-fractionated inoculum ($P=0.05$). The resistant genotype (FC709-2) (PANELLA, 1999) had a lower DI than the susceptible genotype (FC901/C817) (PANELLA *et al.*, 2014). The correlation between the sizes of the fractions (i.e., carbohydrate source for *R. solani*) and infectivity of the inoculum has been noted in wheat with *Gaeumannomyces graminis* var. *tritici* (SIMON *et al.*, 1987; WILKINSON *et al.*, 1985). These researchers also showed that fungus colonized the seed surface of the inoculum as well as the interior of the seed (SIMON *et al.*, 1987).

Contamination was present more frequently in the small fraction, and it was clear that it did not contribute to the ability of the inoculum to cause disease. It also became apparent that the chaff from the barley hull was only a source of contamination, not *R. solani* inoculum. The need to focus on the medium and large fractions to optimize production of high quality inoculum for artificially inoculated field experiments was evident. This was especially useful information because the small fraction could be eliminated easily by separation at the time of milling.

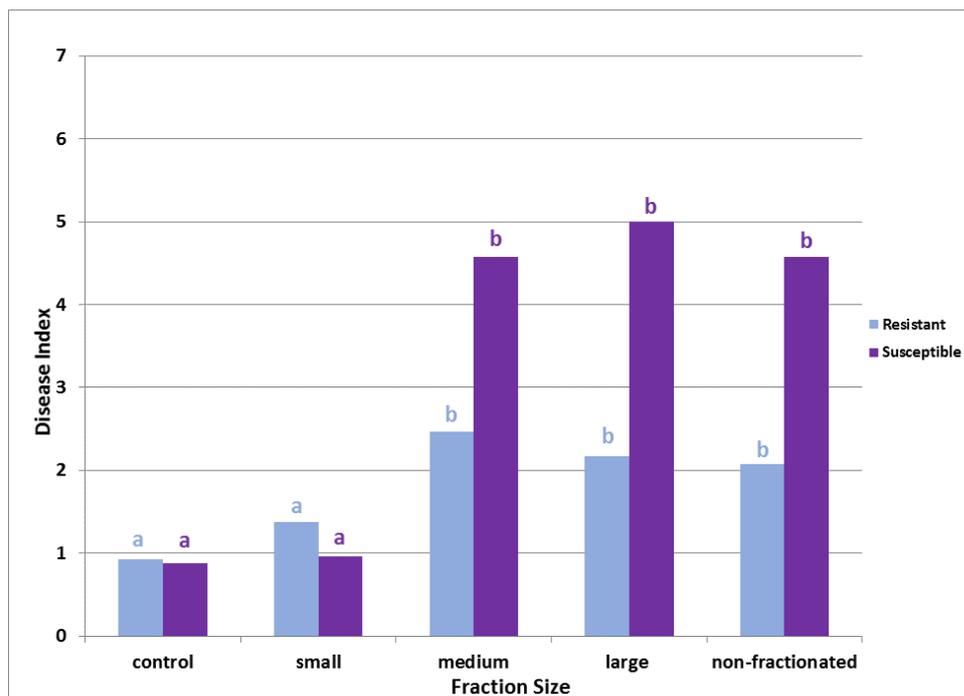


Fig. 4: Comparison of inoculum from the different fraction size's ability to cause disease symptoms on a resistant (FC709-2) and susceptible (FC901/C817) sugar beet genotype, averaged over year and measured by a disease index, where 0 = healthy and 7 = dead.

Another challenge was how to easily and efficiently remove the chaff from the inoculum. The solution chosen was to use hull-less barley. It was imperative to determine whether hull-less barley and barley performed comparably in causing disease when infested with *R. solani*. We also took this opportunity to look at other potential grain sources to examine whether they performed better than barley.

A source of food grade hull-less barley was used and found to have much lower levels of initial contamination compared to other hull-less barley sources (data not shown). Also tested were barley chaff, two different year's barley, buckwheat, millet, sorghum, and wheat. All were milled except for the millet. Millet was of interest because not every program has access to a milling device. The effects of different grain types on DI's were tested in the greenhouse. Disease indices were analysed using linear mixed model (Proc Glimmix – SAS version 9.3). Inoculum replicates and their interactions with genotype and treatment were represented as random effects in the model. The 'slice' feature of PROC Glimmix was used to examine interaction effects of treatment and genotype (resistant and susceptible lines) by testing the simple effects of treatment within genotype separately. This is because there no differences among treatments on the resistant genotype.

In table 1 the pairwise differences among carbohydrate treatments (grain types) on the susceptible genotype are presented. An asterisk signifies that the treatments in the row and column intersection are significant ($P=0.05$, with a Holm-Tukey adjustment for multiple comparisons). The sterile barley control caused significantly less disease than any of the other treatments except for the 2010 chaff. All treatments except for the sorghum were significantly different from the 2010 chaff. There were no significant differences among the other treatments (2009 barley, 2010 barley, hull-less barley, buckwheat, millet, sorghum, wheat).

CONCLUSION

The continual improvement of the methods to produce artificially inoculated field evaluations of *Rhizoctonia solani* is critical if we are to accurately evaluate the interaction between the sugar beet and the pathogen. As technologies evolve and we move from simple mass selection improved resistance to high-throughput phenotyping, to the development of molecular markers for marker aided selection, PIERSON's (1959) desire for "a high degree of uniformity in the intensity of disease exposure" becomes even more vital.

Table 1: Eight different carbohydrate sources and a sterile barley control were examined to test their ability to cause disease in sugar beet, measured by a disease index (DI), where 0 = healthy and 7 = dead. An asterisk signifies that the treatments in the row and column intersection are significant (P=0.05, with a Holm-Tukey adjustment for multiple comparisons).

Carbohydrate	Disease Index	2010 barley	2010 chaff	2009 barley	buckwheat	control	hull-less barley	millet	sorghum	wheat
2010 barley	4.79		*			*				
2010 chaff	1.92			*	*		*	*		*
2009 barley	3.88					*				
buckwheat	4.08					*				
control	0.67						*	*	*	*
hull-less barley	4.58									
millet	3.83									
sorghum	3.46									
wheat	4.25									

These preliminary experiments were performed to gather basic information about the inoculum fractions and grain types, on which to grow the *R. solani* inoculum. The small fraction and the chaff grew very little *R. solani* on the PDA with or without antibiotics. The medium and large fraction sizes contained the majority of the pathogen, and both produced a DI similar to the non-fractionated inoculum. The study comparing grain types showed that all substrates except grain sorghum caused uniform disease symptoms on sugar beet grown in a greenhouse. Chaff performed similar to the sterile barley control. Millet produced a DI not significantly different from our field inoculum. If one did not have access to a milling device, millet could be used and directly applied to the sugar beet crowns using a Gandy applicator. The round shape of the seed, however, could cause problems with inoculum dispersion, because it would have the tendency to bounce and roll more easily than an angular milled seed particle. Both laboratory and greenhouse studies showed that neither chaff nor the small fraction provided much in the way of *R. solani* inoculum and often, both were a source of contamination. We therefore recommend the small fraction (23% by weight) be removed during milling and the use of hull-less barley as a substrate eliminates chaff from the inoculum. Coupled with this knowledge and the fact that hull-less barley performed equivalent to our 2010 field inoculum we recommend the use of this substrate.

Further investigations (comparing the previous inoculation protocols against new protocols) are currently in progress to optimize the inoculum production and its application in the field. Each new method will be analyzed for efficiency of production, contamination, pathogenicity, and spatial distribution. Different techniques are being examined, including using liquid media to inoculate the hull-less barley substrate and the use of spawn bags (used by the mushroom industry) to incubate the sterile barley inoculated with the pathogen. The continued use of barley as a substrate, although hull-less, with the R-9 isolate permits the ability to compare evaluation nursery performance over time.

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