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ORIGINAL ARTICLE

Effects of inoculum density of *R. solani* AG 2-2IIIB and age of plant on root rot severity in sugar beet

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Abstract

Root rot of sugar beet (*Beta vulgaris* L.), caused by *Rhizoctonia solani* anastomosis group AG 2-2 IIIB is responsible for significant crop losses in North Dakota and Minnesota, USA. Understanding the association between plant age and inoculum density with disease severity of sugar beet cultivars is a prerequisite to properly screen for varietal resistance. Therefore, investigations were conducted to determine the responses of 4-, 6-, and 8-week-old plants in seven commercial sugar beet cultivars to inoculum densities of one, two, and three grains of *R. solani*-colonized barley in a greenhouse and with three corresponding levels of colonized barley, mycelial plugs, and sclerotia in field experiments. Under greenhouse conditions, disease severity was greatest before plants reached six weeks of age ($p = 0.05$). There was a positive linear relationship between the density of the inoculum and disease severity. All seven cultivars were equally susceptible ($p > 0.05$) to *R. solani*. Interactions between cultivars and plant age and between plant age and intensity of inoculum were not significant ($p > 0.05$). Field experiments showed that the density of inoculums was significant ($p < 0.001$), and the disease severity was highest in plants inoculated with three colonized barley seeds per plant compared to doses of other inoculum types.

Keywords: cultivar resistance, inoculum potential, sclerotia, soilborne pathogen

Introduction

Globally, sugar beet (*Beta vulgaris* L.) and sugar cane (*Saccharum officinarum L.*) are the two most important sugar-yielding crops, with the former contributing approximately 20% of global sugar production (ISO 2021). Sugarcane and sugar beets account for about 45 and 55% of US sugar production (USDA-ERS 2023). Rhizoctonia root and crown rot (RRCR) of sugar beet is a destructive fungal disease caused by *Rhizoctonia solani* (Kuhn) in North America, South America, Asia, and Europe (Buhre *et al*. 2009; Misra *et al*. 2023). *R. solani* is a soilborne facultative saprophyte on a wide range of hosts and is found in both tropical and temperate regions of the world. This fungus can exist as mycelia in soil or other organic debris and can survive for a long time as sclerotium, an asexual resting spore. *R. solani* consists of 13 different anastomosis groups (AGs), which are genetically different isolated populations, and they can be further subdivided into intra-specific groups-ISGs (Arakawa and Inagaki 2014). In North America and Europe, Rhizoctonia damping-off and RRCR of sugar beet are caused by *R. solani* AG 2-2 IIIB, AG 2-2 IV, and AG 4 on sugar beets (Strausbaugh *et al.* 2011; Misra *et al*. 2023). There are also reports of other AGs of *R. solani* and binucleate *Rhizoctonia* as the causal agents of RRCR (Misra *et al.* 2023). The most virulent isolates of *R. solani* belong

to AG 2-2 IIIB and IV with isolates from the former group being more aggressive (Bolton *et al*. 2010). In the US and Europe, AG 2-2 IIIB is widely considered to be the most significant sugar beet pathogen (Strausbaugh *et al*. 2011). Economic yield losses of sugar beet were estimated at 24% or even > 60% in the US while 5 to 10% in Europe and severe disease conditions resulted in complete crop failure (Buhre *et al*. 2009).

Effective management of damping-off and RRCR relies on several strategies, including cultivation of resistant varieties, tillage operations, crop rotations with non-host crops (wheat, barley, corn) seed treatments, and timely application of fungicides (Carlson *et al*. 2012; Bartholomäus *et al*. 2017). RRCR's severity depends on the type (e.g., mycelial fragments or sclerotia) and density of inoculum in the soil, heavy rainfall, organic-rich soil that is moderately wet and temperature ranges from 15° to 18°C (59–64°F), and cultivar resistance (Goswami *et al*. 2011; Brantner and Chanda 2021). Properly characterizing the reaction of sugar beet cultivars to RRCR under natural environmental conditions is difficult due to the patchy pattern occurrence of this fungus in the field (Anees *et al*. 2010). Thus, for Rhizoctonia disease management assessment with an effective and uniform density of inoculum under uniform environmental conditions in the greenhouse and further corroboration in the cultivation field is desirable for germplasm screening and utilization of resistant varieties are important. Disease severity trials under greenhouse conditions demonstrates uniformity of disease development in replicated experiments, capable of developing characteristics of root rot and seedling damping-off symptoms in sugar beet, are useful for accurately screening sugar beet varieties for assessing disease resistance. However, field trials may produce variable pressure due to the patchy patterns of the disease and unpredictable environmental conditions which can hamper accurate disease severity rating of breeding materials.

The age of plants often affects the pathogen-host relationship. When a pathogen challenges a host plant, the plant's reaction is frequently influenced by the host's developmental stage (Hu and Yang 2019). The chronological (e.g., days or weeks after sowing or planting), as well as physiological (e.g., the appearance of certain morphological or physiological features) age of a plant often determine the outcome of plant-pathogen interactions. This age-related resistance is commonly observed in other pathosystems from diverse pathogenic groups such as fungi, bacteria, viruses, and insect predators (Hu and Yang 2019). Young sugar beet seedlings possess a lower level of resistance to *R. solani* than mature plants (Bolton *et al*. 2010). Liu *et al*. (2019) reported that plants were highly susceptible to *R. solani* before they reached the sixth to eighth leaf growth stage after planting, regardless of assigned ratings of varietal resistance to *R. solani*. However, the authors did not investigate how various inoculum types and densities affect disease severity on a temporal scale, and their investigation was limited to the greenhouse environment only. Exceptions to age-related resistance are also known, as in rice sheath blight caused by *R. solani* AG1-IA, where disease severity and incidence increase with plant age.

R. solani characteristically affects sugar beet plants at two stages of the crop's growth phase: at the seedling stage, causing pre- and post-emergence damping-off which represents water-soaked sunken lesion at ground level which is seen as discolored to grayish or brown root decay of stems, stunted growth of young seedlings accompanied by wilting, and later in mature plants, causing RRCR (Neher and Gallian 2011). Disease ratings of commercial cultivars are primarily based on RRCR resistance of adult plants and the reaction of seedlings to the disease has often been overlooked. With the introduction of Roundup Ready® sugar beet (RRSB) cultivars, the frequency of weed control and its associated involuntary mechanical crown inoculation has been significantly reduced (Khan 2014; Morishita 2016). Concomitantly, the incidence of *Rhizoctonia*-induced crown rot decreased and the root rot incidence in sugar beet has increased in commercial Rhizoctonia-resistant RRSB varieties (Bhuiyan *et al*. 2022), necessitating reevaluation of commercial sugar beet cultivars for seedling resistance.

Understanding the effect of inoculum density and age of plants is essential to elucidate the overall interaction of the host and pathogen for better management decisions to protect sugar beet crops. The aim of the present study was to elucidate how inoculum density and growth stages of sugar beet plants affect varietal response to root rot severity under both greenhouse and field conditions. The specific objectives were: (i) to define the range during which plant age affects Rhizoctonia root rot severity; and (ii) the type and amount of inoculum needed per seedling to create maximum disease pressure for evaluating seedling resistance.

Materials and Methods

Greenhouse experiment conditions and sugar beet varieties

Experiments were conducted at the North Dakota State University Sugar beet Pathology Laboratory and the Jack Dalrymple Agricultural Research Complex, in Fargo, ND, USA in 2021. The greenhouse was set to allow a 12 h photoperiod and maintained a temperature of 25 ± 2°C (Argus Control Systems Ltd.; British Columbia, Canada) conducive for RRCR development during the experiment. Seven commercial sugar beet

varieties belonging to four companies were used. The varieties labeled as A (Maribo MA504; Maribo Seed International, Denmark), B (Hilleshog 4302; Hilleshog Seed LLC, CO, USA), C (Crystal 101RR), D (Crystal 467RR) (American Crystal Sugar, Moorhead, MN), E (BTS 8500), F (BTS 8606RR), G (BTS 80RR) (Beta® Seed, Moorhead, Minnesota) have a wide range of reactions to *R. solani* (Table 1). The resistance ratings and reactions based on the field evaluation were conducted by the American Crystal Sugar Company (ACSC). A rating score >4.4 of sugar beet variety is considered as susceptible to *R. solani*, a score of 4.3 to 4.4 is moderately resistant and <4.3 is considered to be resistant to *R. solani*. Seeds were commercially treated with Kabina ST (FRAC 7 fungicide), and Poncho® Beta (Bayer Crop Science, USA), a systemic insecticide which acts against damaging soil and foliar insect pressure and provides seed pest protection from injury caused by leafhopper, root maggot, flea beetles etc. Plants were grown in plastic pots measuring 10 × 7 × 12 cm (T.O. Plastics Inc., Clearwater, Minnesota, U.S.A.), filled with a peat mix (Sunshine mix 1, Sun Gro Horticulture Ltd.; Alberta, Canada) added with slow-release fertilizer (N-P-K) (Osmocote 15-9-12; 20 g · pot^{-1}, Scotts-Sierra Horticultural Products Company, Marysville, Ohio, USA). Plants were regularly watered and monitored until they reached various designated growth stages for root inoculation.

Table 1. Resistance rating and the reactions of sugar beet varieties to *Rhizoctonia solani* AG 2-2 IIIB

Sugar beet Variety	Ratings	Reaction
Maribo MA504	4.5	S
Hilleshog 4302	3.6	R
Crystal 101 RR	4.8	S
Crystal 467 RR	4.4	MR
BTS8500	4.5	S
BTS 8606RR	4.7	S
BTS 80RR52	4.3	MR

S – susceptible, MR – moderately resistant, and R – resistant. Ratings and reactions based on the field evaluation conducted by American Crystal Sugar Company (ACSC)

Inoculum preparation

For the greenhouse experiment, the *R. solani* AG 2-2IIIB isolate RSKZ-1 was selected for its high level of aggressiveness to sugar beet varieties in all inoculations (Liu *et al.* 2019). The isolate was grown in potato dextrose agar (PDA) medium (Sigma Aldrich, Missouri, USA) amended with streptomycin sulfate at 200 mg · l^{-1} for 10 days. For the study, PDA and clarified V8 agar (CV8) were used to obtain mycelial plugs and sclerotia for root inoculation. To prepare colonized-barely grain inoculum, sterilized barley grains were inoculated with agar plugs containing *R. solani* hyphal tips from pure cultures as described by Noor and Khan (2014).

Root inoculation and disease severity evaluation in the greenhouse

Seeds were grown each week in the greenhouse and the experiment was conducted at the same time when all plants reached the desirable growth stages (4, 6 and 8 weeks). In the greenhouse study, the reaction of 4-, 6-, and 8-week-old sugar beet plants to inoculation with one, two, or three *R. solani*-colonized barley grains was evaluated in a completely randomized design with four replications. Inoculations were achieved by placing the inoculum at 2.5 cm (1 inch) below the soil line and adjacent to the taproot of each of the four plants per variety. Four additional plants were inoculated with sterile, non-colonized barley grains (mock--inoculated) to serve as control checks. This study was conducted twice. The disease severity was evaluated at 28 days post-inoculation (dpi). Tap roots were carefully pulled by hand and washed with tap water. Disease severity was evaluated and rated using a 0 to 7 root rot severity scale where 0 – no visible disease symptoms, 1 – 1–5% root surface with visible lesions, 2 – 6–10% root surface with visible lesions, 3 – 11– 25% root infection, 4 – 26–50% root infection, 5 – 51–75% root infection, 6 – >75% root infection, and 7 – entire root completely deteriorated or dead plant (Hecker and Ruppel 1977; Torres *et al.* 2016).

Root inoculation under field conditions

Field trials were conducted at Hickson, North Dakota, USA. Field plots comprised of six 10-feet (3 m) long rows spaced 22 inches (55 cm) apart. Plots were planted in mid May with commercial sugar beet variety Crystal M-572 which is susceptible to *R. solani*. Seed spacing within the row was 4.7 inches (12 cm). Fertilization and other cultural practices, such as weeding were done according to guidelines in the NDSU Extension Sugar Beet production guide (Khan 2018). Weeds were controlled with herbicide applications as well as by hand weeding throughout the summer. The average temperature of the experimental location was 42° (5.5°C) to 43°F (6°C) and the average precipitation was 13 to 16 inches (30 to 40 cm), respectively during the growing season in 2020 and 2021 as recorded by the North Dakota Agricultural Weather Network (NDAWN). Plots were inoculated on July 12 with three different types of *R. solani* AG 2-2 IIIB inoculum. Root rot inoculation was done manually

on individual plants. Twelve to 14 representative roots from each plot, not including the roots at the ends of the plot, were selected at harvest to evaluate the root rot severity. The experiment layout was a randomized completely block design (RCBD), with 10 treatments, one to three infested barley grains, one to three agar plugs with hyphal tips, one to three *R. solani* sclerotia, and one mock inoculated check (control). The study was conducted in 2020 and 2021, each time with four replications. Disease severity was evaluated at harvest following a 0 to 7 scale where 0 – no visible disease symptoms, 1 – 1–5% root surface with visible lesions, 2 – 6–10% root surface with visible lesions, 3 – 11–25% root infection, 4 – 26–50% root infection, 5 – 51–75% root infection, 6 – greater than 75% root infection, and 7 – root completely deteriorated or dead plant (Torres *et al.* 2016).

Data analysis

Non-parametric statistical analyses were used for the categorical disease severity scale (0–7) which relies on numbers but as a type of ranking. Mean relative treatment effects and their 95% confidence intervals were calculated using the severity mean ranks. Replicate median disease severity and mean rank of disease severity scores were calculated for each treatment in greenhouse and field trials. Non-parametric Levene's test for homogeneity of variances of disease severity was conducted to determine whether the variances of trials within greenhouse and field environments were homogeneous. For greenhouse experiments, the data was analyzed as a split-split plot where whole plots were cultivars, split plots were the amount of inoculum (number of barley seeds), and the split-split plot was the age of plants when inoculated. For field experiments, a combined analysis of variance for the ranks of the response variables of each treatment would be conducted using the generalized linear mixed model procedure (Proc Glimmix) of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) if the Levene's test was non-significant ($p = 0.05$). Otherwise, field trials were analyzed separately. In addition, the relative effects of root rot disease severity for each treatment and their 95% confidence intervals were determined and compared using Brunner's LD_CI SAS macro as described by Shah and Madden (2004). The mock-inoculated control was included in the analysis for greenhouse data; however, the data from the non-inoculated check (control) in the field trials was not included in the analysis since they were all healthy and free from infection.

Results

Root rot severity under greenhouse conditions

All inoculated plants developed characteristic root rot symptoms (Fig. 1). Since variances of greenhouse trials were significantly ($p < 0.001$) different from each other, each trial was analyzed separately. Sugar beet varieties inoculated with *R. solani*-colonized barley grains produced typical *R. solani* root rot symptoms. Since interactions between cultivars and plant age, and between plant age and amount of inoculum were not significant ($p > 0.05$), the main effects of cultivar, plant age, and inoculum density were presented. All cultivars evaluated were equally susceptible ($p > 0.05$) to *R. solani* AG2-2 IIIB (Fig. 2). In general, younger plants were more susceptible ($p < 0.05$) to the disease, but the pathogen could cause infection when inoculated on 8-week-old plants (Fig. 3). There was a significant

Fig. 1. Root rot severity scoring scale (0–7) was done following Torres et al. (2016) in this experiment where 0 (no visible disease symptoms), 1 (1–5% root surface with visible lesions), 2 (6–10% root surface with visible lesions), 3 (11–25% root infection), 4 (26–50% root infection), 5 (51–75% root infection), 6 (>75% root infection), and 7 (the root completely deteriorated or dead plant), respectively

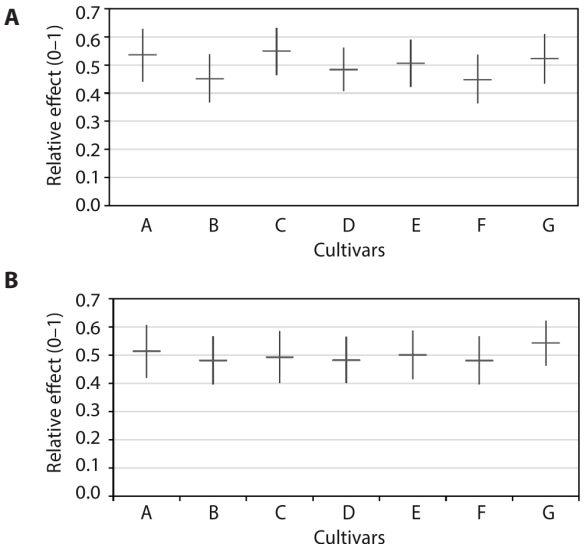

Fig. 2. Reaction of seven sugar beet cultivars to inoculation with Rhizoctonia solani AG 2-2IIIB in two trials (A and B) conducted under greenhouse conditions. Disease severity was assessed using a 0–7 categorical severity scale where 0 represents no disease and 7 represents tap root completely deteriorated and/or plant dead. Mean relative treatment effects (represented by horizontal bars) and their 95% confidence intervals (represented by vertical bars) were calculated using the severity mean ranks. Relative effects closer to zero denote lower disease severity. Letters A through G represent varieties M-504, H-4302, C-101, C-467, BT-85, BT-86, and BT-80, respectively

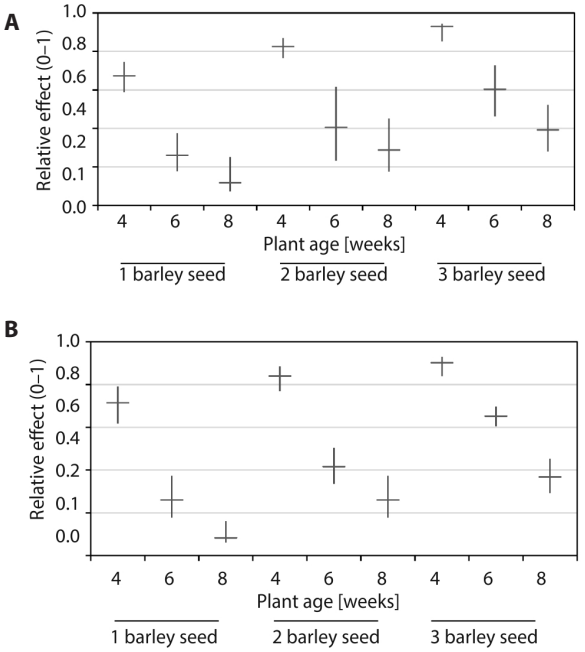

Fig. 3. Effect of three plant ages and inoculum density on the reaction of sugar beet plants to inoculation with Rhizoctonia solani AG 2-2IIIB in two trials (A and B) conducted under greenhouse conditions. Disease severity was assessed using a 0–7 categorical severity scale where 0 represents no disease and 7 represents tap root completely deteriorated and/or plant dead. Mean relative treatment effects (represented by horizontal bars) and their 95% confidence intervals (represented by vertical bars) were calculated using the severity mean ranks. Relative effects closer to zero denote lower disease severity

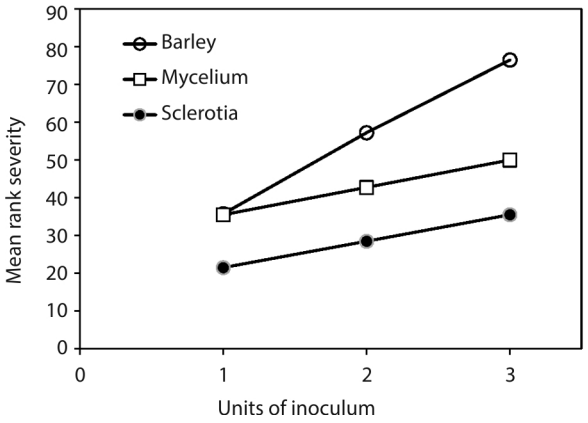

Fig. 4. Interaction between three types of Rhizoctonia solani inoculum and the number of inoculum units used on sugar beet plants. Plants were inoculated at planting time and disease severity was measured at harvest. Disease severity for each type by unit treatment was ranked from lowest to highest

(p = 0.05) and positive linear relationship between the amount of inoculum (i.e., number of *R. solani*-colonized barley seed) and disease severity (Fig. 4).

Evaluation of root rot severity in field trials

Sugar beet plants developed characteristic disease symptoms in all inoculated plants whereas non-inoculated plants (control check) did not develop root rot disease symptoms. Levene's test for homogeneity of variances indicated that variances of field trials were statistically similar (p = 0.989) and thus, a combined analysis was performed. Relative effects closer to zero denote lower disease severity. The combined analysis revealed a significant interaction between type and amount of inoculum (p = 0.04). This interaction was one of magnitude rather than direction (Fig. 5). As the number of inoculum units used increased, the mean rank severity of all types of inoculum also increased; however, the change in severity was almost twice as large when going from one colonized barley seed to three compared to going from one mycelial plug or one sclerotium to three of the same type. The relative effects reflected a similar trend (Fig. 5).

Discussion

We investigated the role of inoculum density and growth stages of sugar beet seedlings for inoculation on disease severity under greenhouse and field conditions. We observed that 4-week-old sugar beet seedlings are more susceptible than 6- and 8-week-old seedlings to *R. solani* infection. Moreover, three

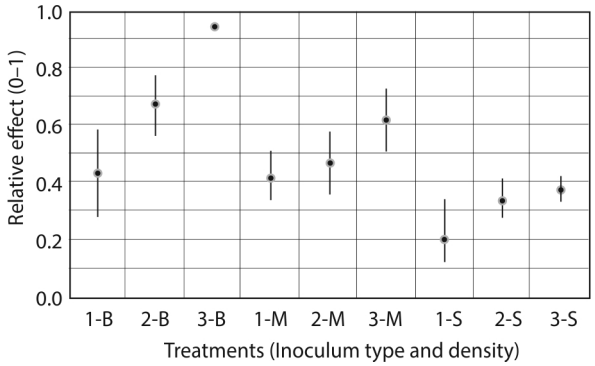

Fig. 5. Effect of inoculum types and density on disease rating in field condition as measured by relative effect (solid circle). The bars represent the range of 95% confidence interval of the estimated relative effects. The relative treatment effects for each treatment and their confidence intervals were estimated and compared using Brunner's LD_CI SAS macro. Combinations of letters B, M, and S and numbers 1, 2, and 3 represent the number of Rhisoctonia solani-colonized barley grains, mycelial plug, and sclerotia inoculated per plant

R. solani-colonized barley grains per seedling demonstrated higher inoculum potentials than corresponding one or two grains in the greenhouse. Previously, it was established that colonized barley grains were more effective per unit than mycelial plugs or sclerotia under greenhouse conditions (Bhuiyan *et al*. 2023).

Colonized barley grains had the highest ability to cause damping off incidence. They caused the expected root rot severity that better matched the known rating evaluated in the field by seed companies and proven for generating maximum disease pressure for germplasm resistance screening. Barley inoculums are effective, with ease of production, preparation, and handling. Furthermore, they are low-cost, readily available, and feasible for commercial use in the field (Bhuiyan *et al*. 2022). On the other hand, resting spore secrotia were slow-growing in media, and less effective in causing disease symptoms when inoculated on seeds in vitro and under greenhouse conditions than the other two forms of inoculm mycelial plugs and colonized barley grains. When compared in the field with the three inoculum doses of mycelial plugs and sclerotia in two cultivation seasons (2020 and 2021), the aforesaid dose of three colonized grains of barley per seedling also showed the highest inoculum potential. Inoculum density influenced the reaction of germplasm materials to various plant pathogens.

It is quite inefficient to use mycelia and sclerotia for large-scale research iunder field conditions because of the inconvenience of *in vitro* preparation, handling, and storage. Although sclerotia are natural overwintering propagules, it is cumbersome to recover sclerotia of *R. solani* from infested soils for large-scale inoculation experiments. It has been previously reported that colonized barley grains and mycelia are effective inoculums for disease rating under greenhouse conditions (Behn *et al*. 2012; Wigg and Goldman 2020). In the present laboratory it was previously reported that the colonized barley grains inoculum consistently produced higher median root rot severity and varieties responded significantly differently ($p < 0.001$) when different forms of inoculums were used. Sugar beet varieties inoculated with sclerotia developed lower disease severity ($p < 0.05$) than colonized barley grains and mycelial plugs (Bhuiyan *et al*. 2023). The effect of different inoculums was statistically significant ($p < 0.05$). Mycelial plug inoculation to all sugar beet varieties showed comparatively lowered disease severity than colonized barley grains.

Perhaps its clearest influence is on the severity of the diseases they cause. Knowing and determining the level of pathogen inoculum in soil are thus essential for developing control strategies. Muthukumar and Venkatesh (2013) reported that a 5% load inoculum of *Sclerotium rolfsii* caused maximum disease incidence. Higher inoculum density resulted in higher disease incidence in Sclerotium wilt of potato and Sclerotinia disease in lettuce (Kulkarni 2007; Chitrampalam *et al.* 2010). The nature and quantification of the infective inoculum under field conditions and the variation of spatial and temporal patterns of propagules play a significant role in disease development. Previously, it was reported that an exponential reduction in the incubation period was due to increasing *Fusarium oxysporum* inoculum intensity in chickpeas.

In the present study, a significant ($p = 0.05$) and positive linear relationship was observed between the amount of inoculum (i.e., the number of colonized barley seeds per plant) and disease severity. This observation agrees with other researchers who reported a positive correlation between disease incidence of Rhizoctonia root rot in sugar beet and the number of viable sclerotia. Furthermore, Brantner *et al*. (2014) observed that inoculum density was negatively associated with sucrose yield in sugar beet. In addition to influencing the severity of the disease, increased plant pathogen inoculum densities may alter the physiology of their hosts' reactions. For example, *R. solani* AG-8 and *R. oryzae* stimulated the early production of crown roots in barley seedlings (Schroeder and Paulitz 2008), while higher concentrations of *R. solani* inhibited lettuce growth. Moreover, the incidence and severity of root rot increased in snap beans due to increased *R. solani* inoculum densities. High disease ratings in the field were usually associated with high populations of the fungus *Fusarium solani* f. sp. *phaseoli* in the soil causing Fusarium root rot of white beans, and earlier disease onset by *Verticillium dahlia*on cabbage.

The reaction of sugar beet to *R. solani* may be influenced by plant age and inoculum concentration.

Liu *et al*. (2019) noted that age-related resistance of sugar beet cultivars kicked in at the four-leaf stage growth stage. Their observation was made on plants inoculated with a single *R. solani*-colonized barley seed per plant. In the current study, 4 to 6-week-old plants remained susceptible when inoculated with two or three times that of inoculum concentrations. The present observation found that younger plants at 4 to 6 weeks post planting were more susceptible ($p < 0.05$) to the disease which supported a previous report that older plants showed symptoms upon inoculation but recovered later and grew to maturity. In the present research, all inoculated plants produced characteristic disease symptoms under greenhouse conditions. Similar findings of inoculum density and plant age on disease severity were observed in other host-pathogen systems (Paugh and Gordon 2019; Scott *et al*. 2012), except for rice sheath blight caused by *R. solani* AG1-IA, where the disease severity and incidence increase with plant age.

Conclusions

This research showed that the relationship between varietal response resistance, inoculum density, and growth stage is critical for determining the resistance response of sugar beet cultivars against *R. solani* under field conditions. Consequently, it is logical to assume the inoculation methods that cause maximum disease pressure may be better suited to identifying resistance germplasm and properly evaluating disease management practices for *R. solani* in sugar beet. The present experiment demonstrated that 4-week-old sugar beet seedlings are more susceptible than 6- and 8-week-old seedlings to *R. solani* infection. Moreover, three *R. solani*-colonized barley grains per seedling had higher inoculum potentials than corresponding one or two grains of barley and one, two, and three units of mycelial plug or sclerotium. It would be wise to initiate early application of fungicides to protect sugar beet seedlings at early stages against damping-off and root rot caused by *R. solani* AG 2-2IIIB.

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