Evaluating Inoculation Methods to Infect Sugar Beet with *Fusarium oxysporum* f. *betae* and *F. secorum*

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Abstract

Minnesota and North Dakota combined contain 55% of the sugar beet production area in the United States, contributing to 49% of the nation's sugar beet production in 2018. *Fusarium* diseases caused by *Fusarium oxysporum* f. *betae* and *F. secorum* on sugar beet can cause significant reduction in both root yield and sucrose concentration and purity. The objective of this research was to identify an alternative artificial inoculation method to induce *Fusarium* diseases on sugar beet leaves and roots caused by both *Fusarium* spp. in greenhouse conditions to better aid in research efforts. We tested four inoculation methods, including barley to seed, barley to root, drenching, and cutting, and compared them with the conventional root-dipping inoculation method. The inoculation

method of placing *Fusarium*-colonized barley seed close to sugar beet seed (barley to seed) caused levels of symptom severities on both leaves and roots similar to the root-dipping method. Because the traditional root-dipping method involves a laborious transplant process, use of infected barley seed as inoculum may serve as an alternative method in the evaluation of host resistance and pathogen virulence among *Fusarium* diseases by *Fusarium* spp. on sugar beet at the seed or seedling stage.

Keywords: disease management, *F. oxysporum* f. *betae*, fungi, *Fusarium*-colonized barley seed, *Fusarium secorum*, root-dipping, sugar beet, techniques, vegetables

Sugar beet (*Beta vulgaris* L.) is a major source of global sucrose production, especially in temperate regions. The United States was the second-largest sugar beet producer in the world in 2017 (FAO 2017). In 2018, Minnesota and North Dakota accounted for 55% of the sugar beet growing area and contributed 49% of the total sugar beet production in the United States (USDA-ERS 2019). Diseases caused by *Fusarium* spp. on sugar beet can reduce root yield and extractable sucrose (Hanson and Jacobsen 2009).

In the Red River Valley of North Dakota and Minnesota, *Fusarium oxysporum* f. *betae* (D. Stewart) W. C. Snyder & H. N. Hansen and *F. secorum* are the pathogens most consistently associated with *Fusarium* diseases on sugar beet (Khan et al. 2009). *Fusarium* yellows, caused by *F. oxysporum* f. *betae*, was first reported in the Red River Valley in 2002 (Windels et al. 2005). The disease symptoms are a characteristic interveinal chlorosis, internal taproot vascular-discoloration without external appearance, and canopy wilt. In 2005, a new disease, *Fusarium* yellowing decline, caused by *F. secorum*, was first reported by Rivera et al. (2008) in Minnesota (Secor et al. 2014). Unlike *F. oxysporum* f. *betae*, only *F. secorum* causes seedling death, yellowing during early growing season, and petiole vascular discoloration (Burlakoti et al. 2012).

Effective artificial inoculation methods are necessary for the identification of sources of host resistance, host-pathogen interactions, and studies on disease control strategies (Das et al. 2015). The root-dipping inoculation method has been the standard method for evaluating *F. oxysporum* infection, which affects several plant species, including chickpea, tomato, cotton, and cucumber (Dowd et al. 2004; Maitlo et al. 2016; Rowe 1980; Vakalounakis 1996).

The root-dipping method has also been used to evaluate *F. oxysporum* f. *betae* on sugar beet (Hanson 2006). This same inoculation method has been used to study the effect of *F. secorum* on sugar beet (Burlakoti 2007; Rivera et al. 2008). The root-dipping inoculation method involves damaging the roots, allowing the pathogen to invade through wounds, avoiding a natural barrier at the epidermis (Eynck et al. 2009). Alternative inoculation methods which do not result in artificial wounding of the root such as the standard root-dipping method would be of value because they better simulate natural conditions during pathogen attempts at establishment. In this work, we tested four alternative inoculation methods to identify a more effective inoculation method for *Fusarium* disease evaluations.

Materials and Methods

Fungal isolates. Known pathogenic isolates *F. oxysporum* f. *betae* F-19, isolated from Salem, OR in 2001, and provided by the United States Department of Agriculture-Agricultural Research Service Sugarbeet Research Unit, Fort Collins, CO, and *F. secorum* 784-12-4, isolated from Sabin, Minnesota in 2007, and provided by Dr. G. A. Secor, North Dakota State University, Fargo, ND were used for this study.

Inoculum preparation. Liquid cultures were prepared using carboxymethylcellulose (CMC) medium. One liter of CMC medium contains 15 g of CMC sodium salt (Sigma-Aldrich), 1 g of ammonium nitrate (ACS reagent, ≥98%; Sigma-Aldrich), 1 g of potassium phosphate monobasic (Sigma-Aldrich), 0.5 g of magnesium sulfate heptahydrate (ACS reagent, ≥98%; Sigma-Aldrich), and 1 g of yeast extract (Sigma-Aldrich). All chemicals were dissolved in 1 liter of distilled water and autoclaved at 170 kPa and 120°C for 20 min. Fungal cultures were prepared by transferring hyphae from a long-term storage vial into 100-by-15-mm petri dishes (Falcon) containing full-strength potato dextrose agar (PDA) (Sigma-Aldrich) and incubating them under fluorescent light at room temperature (24°C) for 1 week. Erlenmeyer flasks containing 200 ml of CMC medium were inoculated with 20 pieces of 5-mm² plugs containing actively growing hyphae. The inoculated CMC medium was placed in a rotary shaker (MaxQ Shakers; Thermo Scientific) and incubated at 210 rpm under soft white fluorescent light at 25°C. After 7 days, the CMC medium was passed through two layers of Miracloth

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(Calbiochem, EMD Millipore Corporation) to collect spores. A hemocytometer (Propper Manufacturing Co., Inc.) was used to estimate the concentration. The spore suspension was adjusted to 5×10^4 spores/ml with distilled water and used immediately.

Barley seed (nontreated) were used as a solid substrate. *Fusarium*-infested barley inoculum were produced following the same method used for producing *Rhizoctonia solani*-infested barley grains (Kirk et al. 2008; Noor and Khan 2014). Mixtures of 4.8 g of potato dextrose broth (Sigma-Aldrich), 200 ml of barley, and 120 ml of distilled water (5:3 barley/distilled water [vol/vol] ratio) were placed into 500-ml flasks (Pyrex) and autoclaved at 170 kPa and 120°C for 30 min, then left to cool to room temperature overnight. The initial inoculum was grown on PDA as described above, cut into 3-mm² plugs, and transferred into autoclaved flasks containing barley. One flask of barley was inoculated with plugs from one petri dish. Inoculated flasks were sealed, mixed every 2 days by hand shaking, incubated at room temperature for 2 weeks, and then air dried under a laminar flow hood for 2 days. The air-dried barley grains were stored at 4°C until used. CFU were calculated for each isolate by grinding 50 grains in 100 ml of autoclaved distilled water for 5 min using a blender. Three serial 1/10 dilutions were prepared and a total volume of 100 µl from each dilution was plated onto 100-by-15-mm PDA plates with three replicates. The number of CFU was estimated after 24 h of incubation at room temperature.

Sugar beet plants and seed. This study was conducted in a greenhouse (Argus Control Systems, Ltd.) of the Agricultural Experiment Station of North Dakota State University in Fargo, ND, U.S.A. Three seeds of *Fusarium* spp.-susceptible variety Maribo 409 (Niehaus 2016) were planted in a plastic pot (10 × 10 × 12 cm; T. O. Plastic Inc.) filled with Sunshine Mix number 1 peat (Sun Gro Horticulture Ltd.). One teaspoon of Osmocote 15-9-12 fertilizer (3 to 4 months' formula) (Everris NA Inc.) was added and mixed to each pot before seeding. One week after planting, seedlings were thinned to one plant per pot. Greenhouse conditions were set to an average temperature of 24°C and 16-h photoperiod. Plants were watered as needed. Three-week-old sugar beet plants (at the four-leaf stage) were used for all inoculations except the barley to seed method, where sugar beet seed were used and which is described below.

To identify the most effective alternative inoculation method, five inoculation methods were evaluated: the conventional standard method (root-dipping), drench without injury (drenching), drench with injury (cutting), *Fusarium*-colonized barley seed placed next to sugar beet plants (barley seed to root), and *Fusarium*-colonized barley seed placed next to sugar beet seed at planting (barley to seed). After inoculation, all plants were kept in the greenhouse environment, set at a temperature of 24°C and a 16-h photoperiod, and watered as needed. Six replicates of individual plants and trays with 10 seeds each for each isolate were used. This experiment was performed using a completely randomized design two times.

Inoculation methods. Root-dipping (root-dipping). Three-week-old plants were carefully removed from their pots. Roots were washed with distilled water, dried with tissue paper, and soaked in a *Fusarium* spore suspension (5×10^4 spores/ml) for 8 min (Hanson and Hill 2004). A 1% CMC medium in distilled water was used as a control. After inoculation, plants were transplanted into wet plastic pots as described above. Old or yellow leaves were removed 3 days after inoculation (Hanson and Hill 2004).

Drenching without injury (drenching). Inoculation was conducted by pouring 20 ml of *Fusarium* spore suspension (5×10^4 spores/ml) uniformly across the soil surface of pots containing one 3-week-old plant each (Maitlo et al. 2016). Control pots had 1% CMC medium in distilled water poured instead of spore suspension.

Drenching with injury (cutting). To injure 3-week-old sugar beet roots, two longitudinal cuts about 10 cm deep were made about 1.3 cm away from opposite sides of each root using a knife sterilized in 75% ethanol. These two cuts were parallel to each other. Inoculation was performed the same way as drench inoculation without injury. The control plants were inoculated with 1% CMC medium in distilled water.

***Fusarium*-colonized barley seed placed next to sugar beet plants (barley to root).** Inoculation was conducted by placing one

Fusarium-colonized barley seed 1 cm away from the root and 2 cm below the soil surface, then covering the seed with Sunshine Mix number 1 peat for each sugar beet plant (Liu and Khan 2016). A sterilized barley seed without *Fusarium* infection was placed beside each plant in the control.

***Fusarium*-colonized barley seed placed next to sugar beet seed at planting (barley to seed).** For this inoculation method, plastic trays (28 × 12 × 12 cm) were used, and fertilizer was added to potting soil as previously described. Ten sugar beet seeds were planted in 2-cm-deep furrows and one *Fusarium*-colonized barley seed was placed 1 cm to the side of each sugar beet seed, then covered with Sunshine Mix number 1 peat (Liu and Khan 2016). A sterilized barley seed that was not inoculated with the pathogen was placed beside each seed in the control.

Foliar and root disease symptom evaluation. Disease evaluation was based on *Fusarium* yellows and *Fusarium* yellowing decline symptoms. The severity scale used to assess foliar disease symptoms in the study was as follows: 0 = no disease; 1 = leaves wilted, small chlorotic areas on lower leaves, most of leaf green; 2 = leaves showing interveinal yellowing; 3 = leaves with small areas of necrosis or becoming necrotic and dying, less than half of the leaves affected; 4 = more than half of leaves dead, plant stunted, most living leaves showing symptoms; and 5 = plant death (Hanson et al. 2009). Disease severity ratings were taken every week for 5 weeks after inoculation.

Five weeks after inoculation, plants were carefully removed from pots and washed under tap water, and roots were longitudinally cut to check for discoloration within the vascular system. The severity scale used for root symptom rating was as follows: 0 = no internal browning; 1 = slight internal browning, usually at the tip of the tap root; 2 = moderate to severe internal browning of the entire tap root; and 3 = severe internal browning extending from the tap root into the lower stem above the soil line (Rowe 1980).

Data analysis. *Analysis of variance-type statistic test.* Levene's test was first used to determine whether the data sets for disease severity had homogeneous variances and could be combined for analyses. Then, data were analyzed by a nonparametric method, using the rank and mixed procedures of SAS (version 9.4; SAS Institute Inc.). Data from foliar and root symptoms were ranked separately using the rank procedure and analysis of variance (ANOVA)-type statistics

Table 1. Test statistics for the effect of five different inoculation methods and two *Fusarium* spp. on foliar disease severity of sugar beet at 7, 14, 21, 28, and 35 days after inoculation

Effect	Analysis of variance-type statistics (ATS) ^a			
	<i>df_N</i>	<i>df_D</i>	ATS	<i>P</i> value
Isolate	1	1	28.78	<0.0001
Inoculation method	3.35	1	27.98	<0.0001
Isolate × inoculation method	3.35	4.09	5.54	0.0005
Time	2.85	1	400.14	<0.0001
Isolate × time	2.85	1	11.03	<0.0001
Inoculation method × time	7.52	1	9.15	<0.0001
Isolate × inoculation method × time	7.52	1	3.02	0.0028

^a *df_N* = numerator degrees of freedom and *df_D* = denominator degrees of freedom.

Table 2. Test statistics for the effect of five different inoculation methods and two *Fusarium* spp. on disease severity of sugar beet root at 35 days after inoculation

Effect	Analysis of variance-type statistics (ATS) ^a			
	<i>df_N</i>	<i>df_D</i>	ATS	<i>P</i> value
Isolate	1	47.9	49.54	<0.0001
Inoculation method	2.24	47.9	24.6	<0.0001
Isolate × inoculation method	2.24	47.9	10.85	<0.0001

^a *df_N* = numerator degrees of freedom and *df_D* = denominator degrees of freedom.

(ATS) analysis was conducted using the mixed procedure. For foliar data, the significance of the main effects of isolate, inoculation method, timing of observation, and their interactions were evaluated. For root data, the main effects of isolate, inoculation method, and their interactions were evaluated. SAS macros F2_LD_F1 and LD_CI were used to calculate relative treatment effects and their 95% confidence intervals (Shah and Madden 2004).

Data transformation. To assess the rate of foliar symptom severity progress through time after inoculation, a foliar (leaf) symptom severity scale at each observation point was normalized to the maximum scale of 5 expressed as a percent. We called this transformed symptom severity the normalized symptom severity. For example, if a plant was evaluated with a leaf symptom scale of 2, the normalized leaf symptom severity (NS_L%) was $(2 \div 5) \times 100$, which came to be 40%.

The root symptom severity was assessed at the end of the experiments and the maximum root score of 3 was used to normalize the data. So, if the root symptom was scored 2 for a plant, the normalized root symptom severity expressed as a percent (NS_R%) was $(2 \div 3) \times 100$, which came to be 66.67%.

The normalized symptom severity values of NS_L% and NS_R% observed at the end of the experiment were subjected to ANOVA. Mean normalized symptom severity value of each alternative inoculation was tested against the mean value of the standard root-dipping method as a control using Dunnett's method.

Fitting the Gompertz equation. The mean NS_L% value from each observation time point under each inoculation method in two experiments was fitted to the Gompertz model expressed as the following form (Tjørve and Tjørve 2017):

$$y = A \times \exp\{-\exp[-b \times (DAI \times T_i)]\} \quad (1)$$

in which y is the NS_L value expressed as a percent (i.e., NS_L%), DAI is the days after inoculation, A is the asymptotic value (i.e., the

maximum relative disease severity), b is the slope curvature parameter controlling the rate at which the disease severity progresses with time, and T_i is the infection point of days after inoculation at which the slope is steepest (i.e., the rate of increase in disease is the highest). This model was fitted separately for the two isolates. Parameters were obtained using the nonlinear least squares method with the NLIN procedure of SAS (version 9.4; SAS Institute Inc.).

Results

The two runs of data for this study were combined because their homogeneity test for variance was not significantly different ($P > 0.67$). The negative controls for each inoculation method were without foliar or root symptoms of *Fusarium* yellows or *Fusarium* yellowing decline and were not included in data analysis.

ATS for sugar beet disease severity based on foliar symptom observation is shown in Table 1. Inoculation methods and timing of observations resulted in significantly different foliage disease severity ($P < 0.001$). Isolates differed significantly in foliage disease symptom severity ($P < 0.001$). *F. oxysporum* f. *betae* F-19 caused a higher severity score than *F. secorum* 784-12-4. Importantly, the two-way and three-way interactions were all significant. Thus, an appropriate time point was chosen to assess the foliage disease severity between isolates among different inoculation methods. The ATS for root symptom severity was given in Table 2. Again, the root symptom severity significantly differed between the two isolates and among the five inoculation methods. Interaction of isolate with inoculation method on root symptom scores was also present ($P < 0.001$).

Foliar disease severity for all treatments of inoculated sugar beet at 7, 14, 21, 28, and 35 days after inoculation (DAI) is shown in Table 3. The Gompertz model describing progress of normalized foliage symptom severity is shown in Figure 1 while the

Table 3. Effect of five different inoculation methods and two *Fusarium* spp. on foliar disease severity of sugar beet at 7, 14, 21, 28, and 35 days after inoculation (DAI)^a

Method	DAI	MDS ^b		MR ^c		REDS ^d		95% CI ^e	
		<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>
Dipping	7	0.00	0.00	122.50	122.50	0.203	0.203	0.187–0.220	0.187–0.220
Dipping	14	4.00	3.00	373.33	332.66	0.621	0.554	0.562–0.677	0.510–0.596
Dipping	21	5.00	4.50	487.08	452.99	0.811	0.754	0.752–0.858	0.693–0.806
Dipping	28	5.00	5.00	501.75	482.24	0.835	0.803	0.801–0.865	0.750–0.846
Dipping	35	5.00	5.00	501.75	491.98	0.835	0.819	0.801–0.865	0.774–0.857
Drenching	7	0.00	0.00	122.50	122.50	0.203	0.203	0.187–0.220	0.187–0.220
Drenching	14	0.00	0.00	146.63	134.60	0.244	0.223	0.196–0.300	0.186–0.266
Drenching	21	1.00	0.00	240.40	210.57	0.400	0.350	0.295–0.516	0.257–0.456
Drenching	28	2.00	1.50	328.01	272.01	0.546	0.452	0.403–0.681	0.366–0.542
Drenching	35	3.50	3.00	402.30	308.44	0.670	0.513	0.575–0.753	0.424–0.601
Cutting	7	0.00	0.00	122.50	122.50	0.203	0.203	0.187–0.220	0.187–0.220
Cutting	14	0.00	0.00	134.59	149.15	0.223	0.248	0.186–0.267	0.195–0.309
Cutting	21	3.50	1.00	323.36	257.28	0.538	0.428	0.396–0.674	0.336–0.526
Cutting	28	5.00	3.00	367.30	280.68	0.611	0.467	0.434–0.762	0.378–0.558
Cutting	35	5.00	3.00	438.55	307.42	0.730	0.512	0.590–0.834	0.414–0.608
Barley to root	7	0.00	0.00	122.50	122.50	0.203	0.203	0.187–0.220	0.187–0.220
Barley to root	14	2.50	0.00	304.56	122.50	0.507	0.203	0.394–0.619	0.187–0.220
Barley to root	21	5.00	0.00	435.87	176.48	0.726	0.293	0.613–0.814	0.223–0.376
Barley to root	28	5.00	1.00	467.65	236.03	0.778	0.393	0.715–0.830	0.313–0.479
Barley to root	35	5.00	3.00	496.93	334.72	0.827	0.557	0.775–0.869	0.518–0.596
Barley to seed	7	0.00	0.00	122.50	122.50	0.203	0.203	0.187–0.220	0.187–0.220
Barley to seed	14	0.50	2.00	241.33	280.38	0.401	0.466	0.275–0.543	0.333–0.604
Barley to seed	21	5.00	4.50	454.67	357.61	0.757	0.595	0.629–0.850	0.425–0.744
Barley to seed	28	5.00	5.00	511.50	450.06	0.852	0.749	0.834–0.867	0.632–0.837
Barley to seed	35	5.00	5.00	511.50	493.79	0.852	0.822	0.834–0.867	0.758–0.871

^a *Fusarium oxysporum* (*F. oxy*) and *F. secorum* (*F. sec*).

^b Median disease rating. Disease severity was evaluated every week for 5 weeks based a 0-to-5 scale, where 0 = no disease; 1 = leaves wilted, small chlorotic areas on lower leaves, most of leaf green; 2 = leaves showing interveinal yellowing; 3 = leaves with small areas of necrosis or becoming necrotic and dying, less than half of the leaves affected; 4 = more than half of leaves dead, plant stunted, most living leaves showing symptoms; and 5 = plant death.

^c Mean rank.

^d Relative effect of disease severity.

^e Upper and lower values of 95% confidence interval (CI) of relative effect.

respective parameter estimates are given in Table 4. With majority of the inoculation methods, leaf symptoms caused by two *Fusarium* spp. were first observed at 14 DAI, except for the treatments with *F. secorum* using the barley to root method, where symptoms were first observed at 21 DAI. Root-dipping, barley to root, and barley to seed had a similar high rate of increase in foliar symptoms. For the barley to root inoculation method, the use of *F. secorum* resulted in significantly lower disease development than *F. oxysporum* f. *betae*.

Fig. 1. Normalized foliage symptom severity progress through time post inoculation with five inoculation methods with A, *Fusarium secorum* 784-12-4 and B, *F. oxysporum* f. *betae* F-19.

Table 4. Effect of five different inoculation methods and two *Fusarium* spp. on root disease severity of sugar beet at 35 days after inoculation (DAI)^a

Method	MDS ^b		MR ^c		REDS ^d		95% CI ^e	
	<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>	<i>F. oxy</i>	<i>F. sec</i>
Dipping	3	3	80.50	80.50	0.667	0.667	0.641–0.691	0.641–0.691
Drenching	3	2	56.04	32.50	0.463	0.267	0.337–0.595	0.177–0.391
Cutting	3	2	63.63	27.04	0.526	0.221	0.392–0.656	0.149–0.327
Barley to root	3	1	80.50	23.29	0.667	0.190	0.641–0.691	0.122–0.302
Barley to seed	3	3	80.50	80.50	0.667	0.667	0.641–0.691	0.641–0.691

^a *Fusarium oxysporum* (*F. oxy*) and *F. secorum* (*F. sec*).

^b Median disease rating. Sugar beet plants were hand harvested at 35 DAI and root disease severity was rated with a 0-to-3 scale, where 0 = no internal browning, 1 = slight internal browning, usually at the tip of the tap root, 2 = moderate to severe internal browning of the entire tap root, and 3 = severe internal browning extending from the tap root into the lower stem above the soil line.

^c Mean rank.

^d Relative effect of disease severity.

^e Upper and lower values of 95% confidence interval (CI) of relative effect.

Root disease severity for all the treatments of inoculated plants at 35 DAI is shown in Table 5. Among all of the treatments, root-dipping and barley to seed with both species and barley to root with *F. oxysporum* f. *betae* resulted in the highest similar disease severities. However, *F. secorum* 784-12-4, via barley to root inoculation, did not induce root symptoms similar to *F. oxysporum* f. *betae*. Cutting with *F. oxysporum* f. *betae* was not significantly different from root-dipping and barley to seed methods, with a lower infection on sugar beet roots, but the disease severity was low with *F. secorum* 784-12-4. Drenching induced root symptoms but was inconsistent.

NS_L% and NS_R% caused by each isolate at 35 DAI using the standard root-dipping inoculation method were compared with those using each of the four alternative inoculation methods from ANOVA. Regarding foliage symptom severity by *F. secorum* 784-12-4, barley to seed inoculation did not differ significantly from the standard root-dipping inoculation ($P > 0.05$) whereas the standard root-dipping method resulted in significantly higher NS_L% than the other three alternative methods (Table 6). However, regarding foliage symptom severity by *F. oxysporum* f. *betae* F-19, the standard root-dipping method did not cause significantly higher NS_L% than any of the four alternative inoculation methods ($P > 0.05$) (Table 6). Similarly, the NS_R expressed as a percent (i.e., NS_R%) was not significantly different between root-dipping and barley to seed inoculation methods with *F. secorum* 784-12-4, or significantly different between root-dipping and all four alternative inoculation methods (Table 7).

Discussion

The standard root-dipping method was the most effective inoculation method for both *Fusarium* spp. inoculation on sugar beet. The root-dipping method included soaking seedlings in spore suspension followed by transplanting. During this process, spores could directly get in contact with the damaged root system and lead to pathogens entering the vascular system through wounds. Therefore, root-dipping allowed the pathogens to avoid resistance mechanisms at the root epidermal level (Eynck et al. 2009; Michielse and Rep 2009). Studies showed that *F. oxysporum* f. *betae* could directly penetrate the root epidermis after forming and accumulating net-like hyphae on the surface of root tips, then colonize tissues intracellularly and intercellularly (Bishop and Cooper 1983; Czymbek et al. 2007; Mendgen et al. 1996; Van Peer and Schippers 1992). This also explains why drench inoculation without injury (drenching) and with injury (cutting) caused the same level of disease symptom severity. However, these two inoculation methods caused significantly lower foliage disease severity than the standard root-dipping method with *F. secorum* 784-12-4. Spore distribution in soil is limited by spore morphology and electrical charge, and by soil physical features (Hepple 1960; Wallace 1978). The study by Gracia-Garza and Fravel (1998) showed that spores were unevenly distributed in soil, and CFU at a depth of 0 to 2 cm were 10 times higher than at 8 to 10 cm. The low disease severity observed in drench treatments in our study may be due to the fact that the majority of the spores applied in the drench treatment remained on the surface and in the top 2 cm of the soil, which reduced the chance of spores getting in

contact with sugar beet roots and, thus, resulted in low disease severity.

Barley-based inoculum has been used to study the effect soilborne pathogens such as *R. solani* on sugar beet before (Gaskill 1968; Kirk et al. 2008; Noor and Khan 2014). In our study, *Fusarium*-colonized barley inoculum placed by the sugar beet seed at planting time caused the highest disease severity, with symptoms being observed as early as 7 DAI compared with the 14 DAI for standard root-dipping

Table 5. Estimate values for parameters in the Gompertz equation (i.e., equation 1) for each combination of two isolates with five inoculation methods

Isolate, inoculation method	A ^a	b ^b	T _i ^c	R ²
<i>Fusarium secorum</i> 784-12-4				
Dipping	95.992	0.314	11.984	1.000
Drenching	55.084	0.148	20.738	0.974
Cutting	45.739	0.267	16.892	0.998
Barley to root	100.000	0.115	30.243	0.984
Barley to seed	100.000	0.090	15.815	0.974
<i>F. oxysporum</i> f. <i>betae</i> F-19				
Dipping	98.038	0.388	11.213	1.000
Drenching	93.697	0.115	23.710	0.999
Cutting	72.668	0.334	17.474	0.986
Barley to root	95.478	0.209	12.578	0.993
Barley to seed	100.000	0.152	13.226	0.999

^a Asymptotic value (i.e., the maximum relative disease severity).

^b Slope curvature parameter controlling the rate at which the disease severity progresses with time.

^c Infection point of days after inoculation at which the slope is steepest.

Table 6. Normalized foliage disease symptom severity (i.e., NS_L%) from root-dipping method in comparison with four alternative inoculation methods at 35 days after inoculation (DAI)^a

Isolate, root dipping versus	Severity difference	Significant at 5% level
<i>Fusarium secorum</i> 784-12-4		
Barley to seed	15.840	ns
Barley to root	38.330	*
Drenching	46.670	*
Cutting	50.000	*
<i>F. oxysporum</i> f. <i>betae</i> F-19		
Barley to seed	1.230	ns
Barley to root	1.670	ns
Drenching	26.670	ns
Cutting	20.000	ns

^a Mean NS_L% with root-dipping method at 35 DAI was 96.67% with *F. secorum* 784-12-4 and 98.33% with *F. oxysporum* f. *betae* F-19; ns = not significant.

Table 7. Normalized root disease symptom severity (i.e., NS_R%) from root-dipping method in comparison with four alternative inoculation methods at 35 days after inoculation (DAI)^a

Isolate, root dipping versus	Severity difference	Significant at 5% level
<i>Fusarium secorum</i> 784-12-4		
Barley to seed	12.256	ns
Barley to root	55.556	*
Drenching	44.444	*
Cutting	50.000	*
<i>F. oxysporum</i> f. <i>betae</i> F-19		
Barley to seed	1.320	ns
Barley to root	0.000	ns
Drenching	0.000	ns
Cutting	19.440	ns

^a Mean NS_R% with root-dipping method at 35 DAI was 100% with *F. secorum* 784-12-4 and 100% with *F. oxysporum* f. *betae* F-19; ns = not significant.

method, and did not allow for distinction between isolates. However, when placing the *Fusarium*-colonized barley seed by the roots of sugar beet plants, *F. secorum* caused significantly lower disease severity with delayed onset of symptoms (21 DAI) compared with *F. oxysporum* f. *betae* (14 DAI). Also, during this investigation, *F. oxysporum* f. *betae* (F-19) grew faster and more abundantly on barley than *F. secorum* (784-12-4). Number of CFU for *F. oxysporum* f. *betae* (F-19) was 4.8×10^5 CFU/barley, which was 2.6 times higher than the number of CFU for *F. secorum* (784-12-4) (data not shown). Plant stage also had an effect on sugar beet disease severity, and younger plants were more susceptible than older plants.

Burlakoti et al. (2012) reported that different *Fusarium* spp. could have similar foliar symptoms at 60 DAI but, when evaluating the diseased roots, the more-virulent isolates resulted in more vascular discoloration than the less-virulent ones. In this study, foliar symptoms were evaluated by using a scale to record the yellowing response at 0, 7, 14, 21, 28, and 35 DAI contributing to disease severities for each *Fusarium* isolate. This evaluation method for foliar symptoms caused by *Fusarium* spp. could be reliable, because both foliar (Table 3) and root (Table 4) evaluations indicated that *F. oxysporum* f. *betae* (F-19) induced significantly higher disease severity than *F. secorum* isolate (784-12-4). Burlakoti et al. (2012) reported that *F. secorum* was more aggressive than *F. oxysporum* f. *betae* on sugar beet. However, the specific isolate number of *F. secorum* was unknown. Because *F. secorum* was a relatively new species (Rivera et al. 2008), the differentiation in pathogenicity and virulence among its isolates was still unclear. Given the fact that *F. oxysporum* f. *betae* (F-19) was evaluated by Hill et al. (2011) as highly pathogenic to sugar beet, *F. oxysporum* f. *betae* (F-19) could be more aggressive than the specific *F. secorum* isolate 784-12-4.

In conclusion, this study evaluated artificial inoculation methods to induce *Fusarium* diseases on sugar beet in greenhouse conditions. The results showed that both root-dipping and barley to seed were effective inoculation methods with both isolates when symptoms were assessed at 35 DAI that could be used for *Fusarium* study on sugar beet. However, for large-scale sugar beet germplasm-resistant selection, the root-dipping method is time consuming, labor intensive, and impractical for field study because this method requires transplanting after inoculation. Therefore, barley to seed can be an alternative inoculation method that could be used for *Fusarium* study on sugar beet.

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