Leuconostoc spp. Associated with Root Rot in Sugar Beet and Their Interaction with *Rhizoctonia solani*

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ABSTRACT

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Rhizoctonia root and crown rot is an important disease problem in sugar beet caused by *Rhizoctonia solani* and also shown to be associated with *Leuconostoc* spp. Initial *Leuconostoc* studies were conducted with only a few isolates and the relationship of *Leuconostoc* with *R. solani* is poorly understood; therefore, a more thorough investigation was conducted. In total, 203 *Leuconostoc* isolates were collected from recently harvested sugar beet roots in southern Idaho and southeastern Oregon during 2010 and 2012: 88 and 85% *Leuconostoc mesenteroides*, 6 and 15% *L. pseudomesenteroides*, 2 and 0% *L. kimchi*, and 4 and 0% unrecognized *Leuconostoc* spp., respectively. Based on 16S ribosomal RNA sequencing, haplotype 11 (*L. mesenteroides* isolates) comprised 68 to 70% of the isolates in both years. In pathogenicity field studies with commercial sugar beet 'B-7', all *Leuconostoc* isolates caused more rot ($P < 0.0001$; $\alpha = 0.05$) when combined with *R. solani* than when inoculated alone in both years. Also, 46 of the 52 combination treatments over the 2 years had significantly more rot ($P < 0.0001$; $\alpha = 0.05$) than the fungal check. The data support the conclusion that a synergistic interaction leads to more rot when both *Leuconostoc* spp. and *R. solani* are present in sugar beet roots.

Rhizoctonia root and crown rot (RRCR) in sugar beet (*Beta vulgaris* L.) caused by *Rhizoctonia solani* Kühn is an important disease problem in production areas worldwide, which can lead to yield losses of 50% or more (Büttner et al. 2004; Führer Ithurrart et al. 2004; Kiewnick et al. 2001; Ohkura et al. 2009; Strausbaugh et al. 2011a). In Treasure Valley, Idaho, RRCR has been observed to lead to almost total crop loss at times due to poor furrow irrigation (taking longer than 24 h to get water to the end of the field), stress from other diseases such as curly top and rhizomania, and a warmer, longer growing season than the rest of the Idaho production area (Strausbaugh and Gillen 2009; Strausbaugh et al. 2011a). Previous investigations into RRCR with sugar beet roots have identified *R. solani* AG-2-2 IIIB strains to be of primary importance in Idaho and other production areas (Bolton et al. 2010; Buhre et al. 2009; Führer Ithurrart et al. 2004; Kluth et al. 2010; Pfähler and Petersen 2004; Strausbaugh et al. 2011a; Taheri and Tarighi 2012; Windels and Brantner 2005).

Investigations in Idaho established that internal sugar beet root tissue invaded by *R. solani* is frequently associated with bacteria and yeast (Strausbaugh and Gillen 2008). In particular, *Leuconostoc mesenteroides* subsp. *dextranicum* (Beijerinck) Garvie has been established as being associated with root rot in sugar beet and can lead to some root rot when inoculated alone (Strausbaugh and Gillen 2008; Strausbaugh et al. 2010). Thus, microbial invasion associated with RRCR leads to loss of tonnage and reduced sucrose levels in roots in the field. Additional losses can occur in storage piles and factory processing because of extracellular polysaccharides from bacteria and increased impurities in roots (Cescutti et al. 2005; Cogan and Jordan 1994; Strausbaugh et al. 2011b; Tallgren et al. 1999).

Leuconostoc van Tieghem is a gram-positive heterofermentative lactic acid bacterium widely distributed in the environment in locations such as such as soil, plant surfaces, fermented vegetables, dairy products, manure, and wine (Benkerroum et al. 1993; Chen et al. 2005; Cogan and Jordan 1994; Conn et al. 1995; Gardner et al. 2001; Hemme 2012; Holt et al. 1994; Orberg and Sandine 1984; Server-Busson et al. 1999; Zarazaga et al. 1999). Thus, finding *Leuconostoc* spp. associated with rotted, fermented sugar beet root tissue should not be surprising. *Leuconostoc* spp. are known to be important in the early stages of fermentation but usually are superseded by other bacteria and yeast at some point during fermentation (Adesogan et al. 2003; Amoa-Awua et al. 2007; Gardner et al. 2001; Jung et al. 2012).

In sugar beet roots, the dry black rot associated with *R. solani* invasion is typically restricted to the surface of the root and the immediate underlying tissue, while the wet, fermented-smelling rot extends into the root and tends to be associated with a range of bacteria and yeast (Strausbaugh and Gillen 2008; Strausbaugh et al. 2011b). A number of the bacteria and yeast associated with rotted sugar beet root tissue can slow or inhibit the growth of *R. solani*, which could explain why *R. solani* is largely restricted to surface-related root tissues (Lovic et al. 1993; Strausbaugh and Gillen 2008, 2009). When these bacteria and yeast were inoculated individually back into sugar beet root tissue, *L. mesenteroides* subsp. *dextranicum* was determined to be associated with the most rot (Strausbaugh and Gillen 2008). However, these previous *Leuconostoc* investigations included a limited number of strains and were conducted with whole roots removed from the ground or in the laboratory with root slices, which led to data that were correlated with storage rather than the field (Strausbaugh and Gillen 2008; Strausbaugh et al. 2013a).

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*The *e-X*tra logo stands for "electronic extra" and indicates that one supplementary table is published online.

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Thus, a more thorough investigation focused on *Leuconostoc* spp. needed to be conducted to determine the *Leuconostoc* sp. and distribution of these species in commercial sugar beet production areas. Also, the association of the genus *Leuconostoc* with *R. solani* in sugar beet root tissue needs to be investigated. To achieve these goals, isolates of *Leuconostoc* were collected from recently harvested roots originating from commercial sugar beet fields in southern Idaho and southeastern Oregon in The Amalgamated Sugar Company LLC (TASCO) production area to establish the species and diversity in *Leuconostoc* associated with RRCR. Genetically diverse representative isolates were then utilized in pathogenicity studies conducted in the field to improve our knowledge of the role of genus *Leuconostoc* in sugar beet root rots and how it may interact with *R. solani*.

MATERIALS AND METHODS

Survey of sugar beet roots with rot. From 27 October to 3 November 2010, 53 storage piles at receiving stations from Declo, ID to Vale, OR were visually evaluated for RRCR. From these piles, 451 symptomatic roots were arbitrarily collected. From this collection, 120 roots were arbitrarily selected for isolation to give representation to all areas. In 2012, the survey was repeated from 30 October to 15 November by collecting roots from 56 storage piles covering the same production area covered in 2010. From these piles, 619 symptomatic roots were collected and 200 were arbitrarily selected for isolation to give representation to all areas.

TABLE 1. GenBank accessions included in the phylogenetic analyses for the 16S ribosomal RNA locus of *Leuconostoc* spp.

Leuconostoc spp.	Strain identification	GenBank accession	Haplotype
This study			
Leuconostoc kimchii	L12177 (NRRL B-65330)	KT952367	5
L. kimchii	L12540 (NRRL B-65337)	KT952368	6
L. mesenteroides subsp. *suionicum*	L12056 (NRRL B-65328)	KT952369	9
L. mesenteroides	L12611 (NRRL B-65332)	KT952370	11
L. mesenteroides	L10262	KT952371	12
L. mesenteroides	L12203	KT952372	13
L. mesenteroides	L10380	KT952373	14
L. mesenteroides	L12132 (NRRL B-65329)	KT952374	15
L. mesenteroides	L10219	KT952375	16
L. mesenteroides	L12099 (NRRL B-65335)	KT952376	18
Leuconostoc sp.	L10134	KT952378	20
L. pseudomesenteroides	L12001 (NRRL B-65333)	KT952379	21
L. pseudomesenteroides	L12198 (NRRL B-65331)	KT952380	23
L. pseudomesenteroides	L12036	KT952381	24
L. pseudomesenteroides	L12028 (NRRL B-65334)	KT952382	25
L. pseudomesenteroides	L10138	KT952383	26
L. pseudomesenteroides	L12434	KT952384	27
L. pseudomesenteroides	L10154	KT952385	28
L. pseudomesenteroides	L10157	KT952386	29
L. pseudomesenteroides	L10142	KT952387	30
Leuconostoc sp.	L10190	KT952388	31
Leuconostoc sp.	L10127	KT952389	32
Leuconostoc sp.	L10156	KT952390	33
Other studies			
L. carnosum	NRIC1722	AB022925	8
L. carnosum	JB16	NR_102781	43
L. citreum	NRIC1776	AB022923	36
L. citreum	KM20	NR_074694	39
L. citreum	NRRL B-742	CCNG01000016	39
L. citreum	B-1299	NZ_CCNH01000010	39
L. citreum	LBAE C11	NZ_CAGF01000044	39
L. citreum	LBAE E16	NZ_CAGG01000030	39
L. citreum	LBAE C10	NZ_CAGE01000034	44
L. fallax	DSM20189	NR_041830	45
L. gasicomitatum	LMG18811	AF231131	2
L. gelidum	NRIC1778	AB022921	1
L. gelidum	KCTC 3527	NZ_AEMI01000043	1
L. gelidum	JB7	NC_018631	1
L. holzapfelii	BFE7000	NR_042620	35
L. inhae	KCTC3774	AF439560	3
L. kimchii	KCTC2386	AF173986	7
L. kimchii	C2	CP002898	5
L. kimchii	IMSNU 11154	NR_075014	42
L. lactis	JCM6123	AB023968	34
L. mesenteroides	P45	JRGZ01000005	38
L. mesenteroides	KFRI-MG	CP000574	40
L. mesenteroides subsp. *cremoris*	NCFB543	AB023247	17
L. mesenteroides subsp. *dextranicum*	NCFB529	AB023244	11
L. mesenteroides subsp. *dextranicum*	DSM 20484	NZ_CP012009	11
L. mesenteroides subsp. *dextranicum*	B322 (NRRL B-65327)	KT952377	19
L. mesenteroides subsp. *mesenteroides*	ATCC8293	NR_074957	11
L. mesenteroides subsp. *mesenteroides*	J18	CP003101	11
L. mesenteroides subsp. *suionicum*	LMG8159	HM443957	10
L. miyukkimchii	M2	NR_109072	4
L. palmae	TMW2.694	NR_042695	37
L. pseudomesenteroides	NRIC1777	AB023237	22

Isolations. To collect *Leuconostoc* spp., isolations from the leading edge of rotted areas in sugar beet root tissue were conducted on glucose-yeast extract-peptone agar (GYP) (glucose, 10.0 g; yeast extract, 5.0 g; peptone, 5.0 g; sodium acetate 2.0 g; Tween 80, 0.25 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 4H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 5.0 g; $CaCO_3$, 5.0 g; agar, 20 g; and reverse osmosis water, 1,000 ml; adjusted to pH 6.8), with bromocresol purple (0.04 g/liter) amended with tetracycline (0.2 mg/liter) and vancomycin (0.03 g/liter) to make it semiselective for *Leuconostoc* spp. (Benkerroum et al. 1993; Cai et al. 1999).

TABLE 2. Root rot in sugar beet roots of the commercial cultivar B-7 inoculated with 26 *Leuconostoc* isolates with and without *Rhizoctonia solani* AG-2-2 IIIB strain F517 along with fungal and water checks in 2014 and 2015 Kimberly, ID field studies[v]

Treatment[x]	Haplotype[y]	Root rot (mm)[w] 2014	2015
L12177 + F517	5	12 j–o	40 a
L12174 + F517	21	19 d–k	38 ab
L12311 + F517	11	26 b–g	37 a–c
L12431 + F517	30	14 h–l	35 a–d
L12388 + F517	11	23 c–h	35 a–d
L12487 + F517	23	16 g–k	33 a–d
L12028 + F517	25	21 d–j	32 a–f
L12113 + F517	23	24 c–g	31 a–f
L12099 + F517	18	27 b–e	31 a–f
L12040 + F517	23	29 a–d	31 a–f
L12203 + F517	13	18 e–k	31 a–f
L12048 + F517	11	38 a	30 b–f
L12198 + F517	23	23 c–h	30 b–f
L12347 + F517	11	16 g–k	29 b–f
L12384 + F517	15	31 a–c	29 c–f
L12001 + F517	21	12 j–n	28 c–f
L12199 + F517	21	20 d–j	28 c–f
L12544 + F517	15	23 d–i	28 d–f
L12434 + F517	27	16 f–k	27 d–f
L12443 + F517	21	16 g–k	27 d–f
L12036 + F517	24	23 d–i	26 d–f
L12056 + F517	9	17 e–k	26 d–f
L12540 + F517	6	20 d–j	25 e–g
B322 + F517	19	27 b–f	25 e–g
L12132 + F517	15	35 ab	25 e–g
L12611 + F517	11	25 b–g	23 f–h
Water + F517	NA	2 m–p	16 g–i
L12311	11	4 l–p	14 h–j
L12099	18	1 p	13 i–k
L12113	23	0 p	11 i–l
L12443	21	0 p	11 i–l
L12132	15	10 k–p	10 i–l
L12434	27	1 p	10 i–l
L12048	11	1 p	10 i–l
L12040	23	0 p	9 i–m
L12203	13	5 l–p	9 i–m
L12388	11	4 l–p	9 i–m
B322	19	2 op	9 i–m
L12036	24	0 p	9 i–m
L12384	15	4 l–p	9 i–m
L12431	30	0 p	8 i–m
L12611	11	13 i–m	8 i–m
L12028	25	0 p	8 i–m
L12199	21	0 p	8 i–m
L12347	11	0 p	8 i–m
L12544	15	5 l–p	8 i–m
L12174	21	1 p	8 i–m
L12487	23	1 p	8 i–m
L12177	5	0 p	6 j–m
L12001	21	0 p	5 j–m
L12540	6	0 p	4 k–m
L12056	9	2 n–p	4 lm
L12198	23	2 op	4 lm
Water	NA	0 p	0 m
$P > F$[z]	NA	<0.0001	<0.0001

[v] Means followed by the same letter within a column did not differ significantly based on least square means ($\alpha = 0.05$). NA = not applicable.
[w] Internal root rot measured inside a bisected sugar beet.
[x] Twenty-six strains of *Leuconostoc* were evaluated with and without *Rhizoctonia solani* AG-2-2 IIIB strain F517 along with F517 alone (in bold) and non-inoculated water check (in bold).
[y] Haplotype was based on sequencing (1,437 to 1,440 bp) from the 16S ribosomal RNA region.
[z] $P > F$ was the probability associated with the F value.

Molecular characterization of *Leuconostoc* isolates. To further characterize the 203 *Leuconostoc* isolates, these isolates (Supplementary Table S1) along with 2 isolates from other studies—B322 (Strausbaugh and Gillen 2008) and B853 (from the 2012 Rhizoctonia Root Rot Nursery in Kimberly, ID)—were investigated by sequencing 1,437 to 1,440 bp of the 16S ribosomal RNA (rRNA) locus. To obtain DNA, the isolates were grown in de Man, Rogosa, and Sharpe (MRS) broth (EMD Chemicals Inc., Gibbstown, NJ) in shake culture at 140 rpm and 30°C for 48 h. The bacteria were then pelleted at 8,000 rpm, resuspended in molecular-grade water (5 Prime, Gaithersburg, MD), and stored at −80°C. The polymerase chain reactions (PCR) were performed in volumes of 30 µl in accordance with the manufacturer's instructions: 15.35 µl of molecular-grade water (5 Prime Inc.), 6 µl of 5× PCR GoTaq buffer (Promega Corp., Madison, WI), 0.8 µl of 25 mM $MgCl_2$ (Applied Biosystems, Forster City, CA), 0.6 µl of 10 mM dNTP (Promega Corp.), 2.5 µl of 3 µM each primer (Integrated DNA Technologies, Coralville, IA), 0.25 µl of GoTaq *Taq* DNA polymerase, and 2 µl (approximately 10^8 cells/ml) of target DNA. The amplification cycle consisted of 3 min at 95°C followed by 35 cycles of 95°C for 30 s, a 66°C annealing temperature for 30 s, and 72°C for 1 min. The final cycle was followed by 72°C for 5 min and then held at 4°C. The primer sequence pairs were E8F (AGAGTTTGATCCTGGCTCAG) and E939R (CTTGTGCGGGCCCCCGTCAATTC) plus E786F (GAT TAGATACCCTGGTAG) and U1510R (GGTTACCTTGTTACGA CTT) (Baker et al. 2003; Coloqhoun 1997; Lopez-Garcia et al.

TABLE 3. Survey summary of 203 *Leuconostoc* isolates collected at the end of the 2010 and 2012 growing seasons from sugar beet roots with the *Rhizoctonia*–bacteria root rot complex located in the Amalgamated Sugar Company's production area in southern Idaho and southeastern Oregon

Leuconostoc spp.[y]	Haplotype[z]	Isolates (%)[x] 2010	2012
Leuconostoc mesenteroides	9	0	2
	11	70	66
	12	1	0
	13	0	1
	14	1	0
	15	11	15
	16	4	0
	18	1	1
Total	…	88	85
L. pseudomesenteroides	21	0	4
	23	1	5
	24	0	1
	25	0	3
	26	1	0
	27	0	1
	28	1	0
	29	1	0
	30	1	1
Total	…	6	15
L. kimchii	5	1	0
	6	1	0
Total	…	2	0
Leuconostoc sp.	20	1	0
	31	1	0
	32	1	0
	33	1	0
Total	…	4	0

[x] Number of *Leuconostoc* isolates that fell in the different haplotypes based on 72 and 131 isolates collected in 2010 and 2012, respectively.
[y] Haplotype association with *Leuconostoc* spp. identified on the phylogram based on sequencing from the 16S ribosomal RNA locus.
[z] Haplotypes identified based on sequencing from the 16S ribosomal RNA locus.

2001; Martínez-Murcia et al. 1995; Reysenbach and Pace 1995; Reysenbach et al. 1994; Rudi et al. 1997; Tajima et al. 2001). Amplification products were electrophoresed through agarose gels (1.8% wt/vol) supplemented with ethidium bromide (0.01 mg/ml) in Tris-borate EDTA buffer (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). Amplicons were sent to TACGen (Richmond, CA) for PCR cleanup to remove any excess dNTP and unincorporated primers, and for sequencing in both directions. Sequences were evaluated using BioEdit, version 7.1.3.0 (Hall 1999) and representative haplotypes were submitted to GenBank (accessions KT952367 to KT952390). DNA sequences were aligned using ClustalX, version 2.0 (Larkin et al. 2007).

Phylogenetic analysis. The phylogenetic analyses were conducted using the GenBank accessions found in Table 1. The maximum parsimony analysis was performed using PAUP (version 4.0b10; Sinauer Associates, Inc., Sunderland, MA) with the heuristic search, simple taxon addition sequences, tree bisection-reconnection branch swapping, and MaxTrees = 100. Statistical support was determined for the analyses by bootstrap values for 1,000 replicates. MEGA 6.05 (Tamura et al. 2011) was used to determine the substitution model that best fit the data according to the Akaike Information Criterion. The T92 (Tamura three-parameter model) + G + I model was selected. Maximum-likelihood analyses were conducted with MEGA, with an initial search (two replicates) used

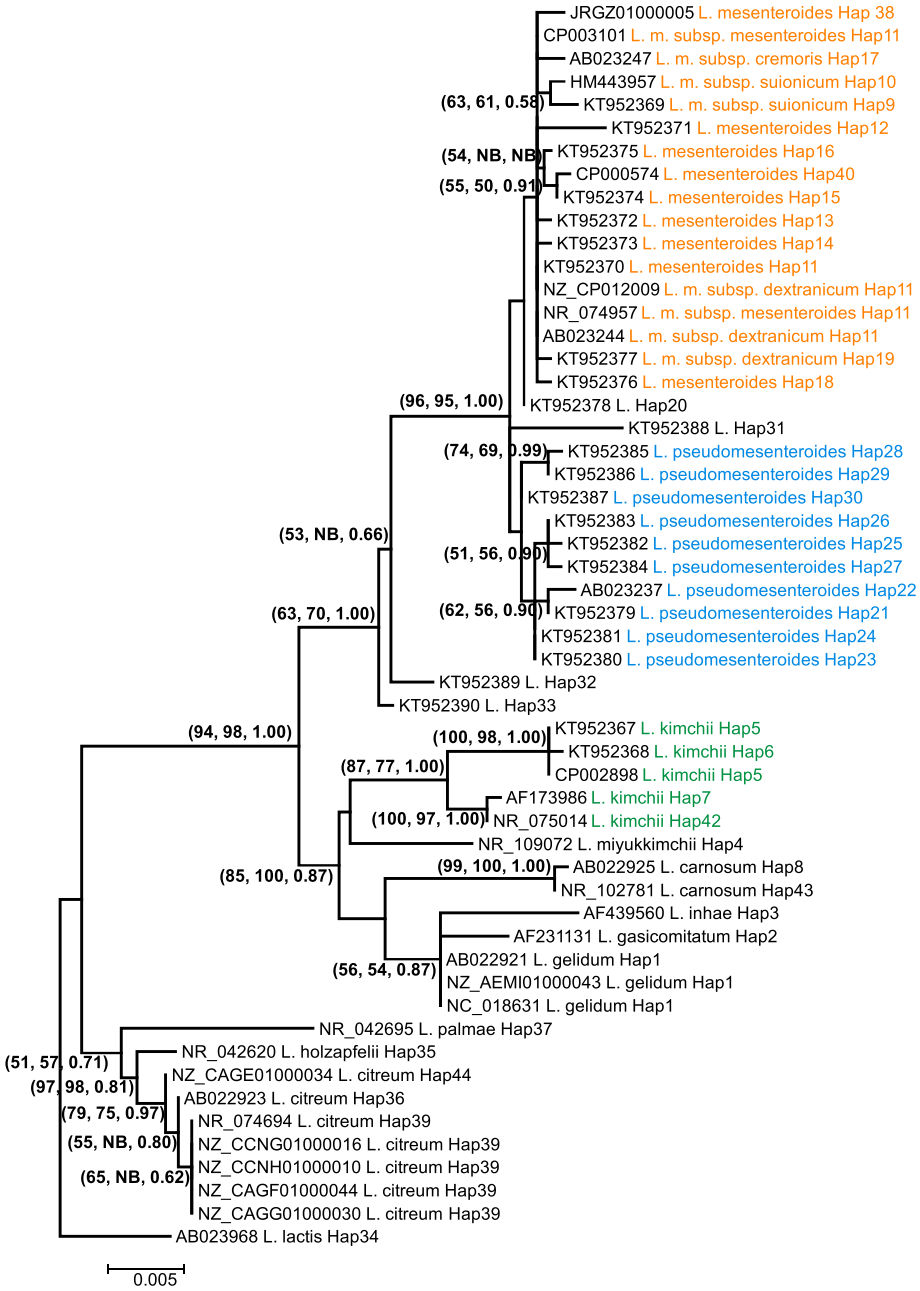

Fig. 1. Phylogenetic relationships among 205 *Leuconostoc* isolates collected from rot in sugar beet roots in Idaho and Oregon based on sequences (1,437 to 1,440 bp) from the 16S ribosomal RNA region. Numbers on nodes of the maximum-likelihood (ML) phylogram represent the statistical support for ML (1,000 replicates, left number), maximum parsimony (1,000 bootstrap replicates, middle number), and Bayesian method (posterior probabilities, right number). NB = no branch in that analysis. *Leuconostoc* strains in the phylogram are designated by GenBank accession number followed by name and haplotype. *Leuconostoc fallax* (very distantly related to the other *Leuconostoc* spp.) was excluded from the analysis to avoid compressing the phylogram. Most isolates (86%) fall in the *Leuconostoc mesenteroides* group (orange), while the *Leuconostoc pseudomesenteroides* group (blue) and *Leuconostoc kimchi* group (green) represent 10 and 1% of the isolates, respectively. Haplotype 11 was most prevalent, representing 67% of the isolates.

to estimate the model parameters. The parameters were then fixed for a bootstrap analysis of 1,000 replicates. The Bayesian phylogenetic analyses were conducted with MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003), with two searches run simultaneously until the standard deviation of split frequencies fell below 0.01. The analysis was conducted using the default priors. The majority-rule consensus was then calculated after removing the first 25% of generations as burn-in. The trees were visualized using FigTree (version 1.4.0; Institute of Evolutionary Biology, University of Edinburgh, Edinburgh, UK).

Carbon source utilization. Representative isolates for the 12 most common haplotypes were evaluated for carbon source utilization using GenIII MicroPlates (Biolog Inc., Hayward, CA). The protocols suggested by the manufacturer were followed and the assays were repeated once.

2014 Field study. The 2014 field study was planted in a field located in Twin Falls County on the United States Department of Agriculture–Agricultural Research Service North Farm (42°33.172′N, 114°21.525′W, elevation 1,190 m) near Kimberly, ID, which has Portneuf silt loam soil and had been in barley the previous year. The field was disked and plowed in fall 2013. Fertilizer (N at 100.8 kg/ha and P_2O_5 at 123.3 kg/ha) was applied on 11 April and incorporated with a roller harrow. The commercial sugar beet 'B-7' (Betaseed Inc., Kimberly, ID), susceptible to RRCR (C. A. Strausbaugh, unpublished data), was planted 21 April to a density of 352,272 seeds/ha and thinned to 117,424 plants/ha on 31 May. There were 56 cm between rows. Irrigation water was applied through hand lines as needed to replace evapotranspiration, based on data from the Twin Falls AgriMet station (station TWFI; elevation 1,197 m, 42°32.746′N, 114°20.762′W, 1.32 km from plots). The field was managed using standard cultural practices for Idaho as mentioned in the 2014 Sugarbeet grower's guide book (TASCO, Boise, ID). The experiment was arranged in a randomized complete block design with six replications. In total, there were 54 treatments: a water check; a fungal check, *R. solani* AG-2-2 IIIB strain F517 (Strausbaugh et al. 2011a) inoculated with water; 26 *Leuconostoc* isolates (Table 2) inoculated individually; and the same 26 *Leuconostoc* isolates inoculated with *R. solani* strain F517. These bacterial isolates represented a cross section of the haplotypes from the TASCO production area affected by RRCR. Each individual root served as an experimental unit. A cork borer plug (8 mm in diameter by 24 mm deep) on the shoulder of the root was pulled on 13 August to allow for inoculation. For the water check, 0.2 ml of sterile well water was placed in the hole. For the fungal check, 0.06 g of dried barley inoculum, prepared as described by Strausbaugh et al. (2013a), of *R. solani* AG-2-2 IIIB strain F517 was placed in the hole with 0.2 ml of sterile well water. For the bacterial inoculations, 0.2 ml of a *Leuconostoc* strain suspension (10^8 CFU/ml), prepared

TABLE 4. Carbon source utilization assays for 12 *Leuconostoc* isolates from different haplotypes

	Leuconostoc haplotypes[y]											
Carbon sources[z]	5	6	9	11	15	18	19	21	23	24	25	30
Acetic acid (H8)	+	+	D	+	+	+	D	+	+	+	+	+
Acetoacetic acid (H6)	+	+	+	+	+	+	+	+	+	+	−	+
L-alanine (E3)	−	−	−	−	−	−	−	−	−	−	−	−
D-arabitol (D3)	−	−	−	−	−	−	−	−	−	−	−	−
L-arginine (E4)	−	−	−	−	−	−	−	−	−	−	−	−
D-aspartic acid (D8)	−	−	−	−	−	−	−	−	−	−	−	−
L-aspartic acid (E5)	−	−	−	−	−	−	−	−	−	−	−	−
γ-Amino-butyric acid (H2)	−	−	−	−	−	−	−	−	−	−	−	−
α-Hydroxy-butyric acid (H3)	−	−	−	−	−	−	−	−	−	−	−	−
β-Hydroxy-D,L-butyric acid (H4)	−	−	−	−	−	−	−	−	−	−	−	−
α-Keto-butyric acid (H5)	−	−	D	+	−	+	−	−	+	−	+	+
D-cellobiose (A5)	+	+	+	+	+	+	+	+	+	+	+	+
Citric acid (G5)	−	−	−	−	−	−	−	−	−	−	−	−
Dextrin (A2)	+	+	+	+	+	+	+	+	+	+	+	+
Formic acid (H9)	−	−	−	−	−	−	−	−	−	−	−	−
D-fructose (C3)	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose-6-PO4 (D7)	−	−	−	−	−	−	−	D	−	−	−	D
D-fucose (C6)	+	+	−	D	D	+	−	+	+	+	+	+
L-fusose (C7)	−	−	−	−	−	−	−	−	−	−	−	−
N-acetyl-D-galactosamine (B8)	−	−	−	−	−	D	D	D	−	D	+	D
D-galactose (C4)	−	−	−	+	+	+	−	+	+	+	+	+
D-galacturonic acid (F2)	−	−	−	−	−	−	−	−	−	−	−	−
L-galacturonic acid lactone (F3)	−	−	−	−	−	−	−	−	−	−	−	−
Gelatin (E1)	−	−	−	−	−	−	−	−	−	−	−	−
Gentiobiose (A6)	+	+	+	+	+	+	+	+	+	+	+	+
D-gluconic acid (F4)	+	+	−	+	+	+	+	+	+	+	+	+
N-acetyl-D-glucosamine (B6)	+	+	+	+	+	+	+	+	+	+	+	+
α-D-glucose (C1)	+	+	+	+	+	+	+	+	+	+	+	+
3-Methyl-glucose (C5)	−	−	−	−	−	D	−	D	D	D	+	D
β-Methyl-D-glucoside (B4)	+	+	+	+	+	+	+	+	+	+	+	+
Glucuronamide (F6)	−	−	−	−	−	−	−	−	−	−	−	−
D-glucuronic acid (F5)	−	−	−	−	−	−	−	−	−	−	−	−
L-glutamic acid (E6)	−	−	−	−	−	−	−	−	−	−	−	−
α-Keto-glutaric acid (G6)	−	−	−	−	−	−	−	−	−	−	−	−
Glycerol (D5)	−	−	−	−	−	−	−	−	−	−	−	−
L-histidine (E7)	−	−	−	−	−	−	−	−	−	−	−	−

(*continued on next page*)

[y] Haplotypes were established using sequencing from the 16S ribosomal RNA locus, as demonstrated in the phylogenetic analysis shown in Figure 1. In the carbon source utilization, the haplotypes were represented by the following *Leuconostoc* isolates: 5 = L12177, 6 = L12540, 9 = L12056, 11 = L12611, 15 = L12132, 18 = L12099, 19 = B322, 21 = L12001, 23 = L12198, 24 = L12036, 25 = L12028, and 30 = L12431. Symbols: + = positive, D = delayed response, and − = negative.

[z] Carbon source utilization for the *Leuconostoc* haplotypes came from GenIII MicroPlates (Biolog Inc., Hayward, CA). The designation in parentheses following the carbon source name is the microplate location.

as described by Strausbaugh et al. (2013a), was placed in the hole. For the *Leuconostoc* + *R. solani* combination inoculation, both the bacterial and fungal isolates or strains were placed in the hole using the same amount as the individual inoculations. The plug was then replaced following inoculation and sealed with petroleum jelly (UNILIVER, Greenwich, CT). On 16 September, roots were dug by hand and bisected through the inoculation site to measure the rot with a ruler perpendicular to the plug. In all, 32 isolations (6 from fungal checks, 6 from bacterial-only treatments, and 20 from the combination treatments) from the leading edge of the rot from arbitrarily selected roots were conducted on fungal and bacterial media. The fungal isolations were conducted on potato dextrose agar (PDA) amended with streptomycin sulfate (200 mg liter^{-1}) and the bacterial isolations were conducted on GYP amended with tetracycline (0.2 mg/liter), and vancomycin (0.03 g/liter), as described above.

2015 Field study. The 2015 field study was planted in a different section of the same field as the 2014 field study using the same methods. This section of the field had been in barley in 2014 and was fertilized (N at 100.8 kg/ha and P_2O_5 at 123.3 kg/ha) on 9 April 2015. The field was planted on 20 April and thinned on 29 May. The roots were inoculated on 12 August and ratings and isolations were conducted on 10 September.

Data analysis. The SAS univariate procedure (SAS version 9.2; SAS Institute Inc., Cary, NC) was used to test for normality of the data. Data were also subjected to analysis of variance using the SAS generalized linear mixed-models procedure (Proc GLIMMIX). In the model statement, the fixed effect was treatment and the random effect was block. In the model statement, the denominator degrees of freedom were calculated using the DDFM = KENWARDRODGER option. Mean comparisons were conducted using least square means ($\alpha = 0.05$).

RESULTS

Survey isolations. In 2010, a collection of 72 *Leuconostoc* isolates was obtained from 120 root isolations. In 2012, a collection of 131 *Leuconostoc* isolates was obtained from 200 root isolations.

16S rRNA sequencing. The 205 isolates (203 isolates from survey along with B-322 and B-853 from other studies) were further characterized by sequencing 1,437 to 1,440 bp of the 16S rRNA locus (GenBank accessions KT952367 to KT952390). The isolates fell into 23 haplotypes and were associated with the following *Leuconostoc* spp. during 2010 and 2012: 88 and 85% *L. mesenteroides*, 6 and 15% *L. pseudomesenteroides* Farrow, 2 and 0% *L. kimchi* Kim, and 4 and 0% unrecognized *Leuconostoc* sp., respectively (Table 3). The most dominant haplotype found was *L. mesenteroides* Hap11, which accounted for 70% of the isolates in 2010 and 66% of the isolates in 2012. *L. mesenteroides* Hap15 was the second most common haplotype but represented only 11 and 15% of the isolates in 2010 and 2012, respectively. The other 21 haplotypes had a frequency of only 0 to 5%, depending on haplotype and year.

Phylogenetic analyses. Phylogenetic analyses were conducted to compare these 23 survey haplotypes with those associated with currently recognized *Leuconostoc* spp. However, the phylogram was compressed by *L. fallax* Martinez-Murcia & Collins (data not shown). Thus, *L. fallax* was dropped from the analyses and not included in the results presented in Figure 1. The isolates in Figure 1 congregated primarily into three clusters associated with these *Leuconostoc* spp.: *L. mesenteroides*, *L. pseudomesenteroides*, and *L. kimchii*. The isolates associated with Hap20, Hap31, Hap32, and Hap33 may represent previously unrecognized *Leuconostoc* spp. or subspecies.

TABLE 4. (*continued from preceding page*)

Carbon sources[z]	*Leuconostoc* haplotypes[y]											
	5	6	9	11	15	18	19	21	23	24	25	30
Inosine (C9)	–	D	–	–	–	+	–	+	+	+	+	+
myo-Inositol (D4)	–	–	–	–	–	–	–	–	–	–	–	–
D-lactic acid methyl ester (G3)	–	–	–	–	–	–	–	–	–	–	–	–
L-lactic acid (G4)	–	–	–	–	–	–	–	–	–	–	–	–
α-D-lactose (B2)	–	–	–	–	D	+	D	+	D	+	+	+
N-acetyl-neuraminic acid (B9)	–	–	–	–	–	–	–	–	–	–	–	–
D-malic acid (G7)	–	–	–	–	–	–	–	–	–	–	–	–
L-malic acid (G8)	–	–	–	–	–	–	–	–	–	–	–	–
D-maltose (A3)	+	+	D	+	+	+	+	+	+	+	+	+
D-mannitol (D2)	+	+	+	+	D	–	+	+	–	+	–	–
N-acetyl-β-D-mannosamine (B7)	–	–	–	–	–	–	D	D	–	+	+	D
D-mannose (C2)	+	+	+	+	+	+	+	+	+	+	+	+
D-melibiose (B3)	–	–	D	+	+	+	+	+	+	+	+	+
Mucic acid (F7)	–	–	–	–	–	–	–	–	–	–	–	–
Pectin (F1)	+	+	+	+	+	+	+	+	+	+	+	+
p-Hydroxy-phenylacetic acid (G1)	–	–	–	–	–	–	–	–	–	–	–	–
Glycyl-L-proline (E2)	–	–	–	–	–	–	–	–	–	–	–	–
Propionic acid (H7)	–	–	–	–	–	–	–	–	–	–	–	–
L-pyroglutamic acid (E8)	–	–	–	–	–	–	–	–	–	–	–	–
Methyl pyruvate (G2)	–	–	–	–	–	–	–	–	–	–	–	–
Quinic acid (F8)	–	–	–	–	–	–	–	–	–	–	–	–
D-raffinose (B1)	–	–	–	+	+	+	+	+	+	+	+	+
D-rhamnose (C8)	–	D	–	–	–	D	–	D	–	+	–	–
D-saccharic acid (F9)	–	–	–	–	–	–	–	–	–	–	–	–
D-salicin (B5)	+	+	+	+	+	+	+	+	+	+	+	+
D-serine (D9)	–	–	–	–	–	–	–	–	–	–	–	–
L-serine (E9)	–	–	–	–	–	–	–	–	–	–	–	–
D-sorbitol (D1)	D	–	–	–	–	–	–	–	–	–	–	+
Stachyose (A9)	–	–	–	+	+	+	+	+	+	+	+	+
Bromo-succinic acid (G9)	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose (A7)	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose (A4)	+	+	+	+	+	+	+	+	+	+	+	+
D-turanose (A8)	+	+	+	+	+	+	+	+	+	+	+	+
Tween 40 (H1)	+	+	+	+	+	+	+	+	+	+	+	+

Carbon source utilization. The 12 *Leuconostoc* isolates representing different haplotypes were all positive for utilizing acetic acid, D-cellobiose, dextrin, D-fructose, gentiobiose, N-acetyl-D-glucosamine, α-D-glucose, β-methyl-D-glucoside, D-maltose, D-mannose, pectin, D-salicin, sucrose, D-trehalose, D-turanose, and Tween 40 (Table 4). Most of the 12 isolates were also positive for acetoacetic acid (except L12028 = Hap25), D-frucose (except L12056 = Hap9 and B322 = Hap19), D-gluconic acid (except L12056 = Hap9), D-melibiose (except L12177 = Hap5 and L12540 = Hap6), D-raffinose (except L12177 = Hap5, L12540 = Hap6, and L12056 = Hap9), and stachyose (except L12177 = Hap5, L12540 = Hap 6, and L12056 = Hap9). *L. pseudomesenteroides* isolates representing haplotypes 21, 23, 24, 25, and 30 were also positive for D-galactose, inosine, and α-D-lactose.

Pathogenicity tests. In both 2014 and 2015, the *Leuconostoc* isolates always led to more rot ($P < 0.0001$) than the water check when combined with *R. solani* strain F517 (Table 2). In 2014, 15 *Leuconostoc* isolates inoculated individually had measurable rot while, in 2015, all *Leuconostoc* isolates had measurable rot. However, in 2014, isolate L12611 was the only *Leuconostoc* isolate that caused significantly more rot than the water check when inoculated without *R. solani*. In 2015, all *Leuconostoc* isolates had measurable rot and seven *Leuconostoc* isolates caused significantly more rot than the water check when inoculated without *R. solani*. In 2014, the fungal check had measurable rot but it was not significantly different from the water check. In 2015, the fungal check caused rot that was significantly more than the water check. In 2014, 24 of 26 treatments with *Leuconostoc* combined with *R. solani* had significantly more rot than the fungal check. In 2015, 22 of 26 of the combination treatments induced more rot than the fungal check. In both years, all *Leuconostoc* isolates caused more rot when combined with *R. solani* than when inoculated alone. Therefore, the data for both years supported the conclusion that a synergistic interaction occurred between *Leuconostoc* and *R. solani*.

When the *Leuconostoc* treatments were combined together by haplotype in both 2014 and 2015, the haplotypes always led to more rot ($P < 0.0001$) than the water check when combined with *R. solani* (Table 5). In the majority of the comparisons (13 of 14 in 2014 and 12 of 14 in 2015), the *Leuconostoc* haplotype + *R. solani* combination also lead to significantly more rot than the fungal check. Inoculated individually, the *Leuconostoc* haplotypes led to measurable rot at times but the rot was not different from water check in 2014 and not different in 2015 in 11 of 14 comparisons.

When the *Leuconostoc* treatments were combined together by species in both 2014 and 2015, the *Leuconostoc* spp. always led to more rot ($P < 0.0001$) than both the water and fungal checks when combined with *R. solani* strain F517 (Table 6). Only *L. mesenteroides* caused measurable rot when inoculated individually in 2014 but all three species caused rot in 2015. However, the only rot significantly different from the water check was that caused by *L. mesenteroides* and *L. pseudomesenteroides* in 2015.

In 2014, isolations from 20 rot samples inoculated with both *Leuconostoc* and *R. solani* were all positive for *Leuconostoc* but only one sample was positive for *R. solani*. In 2015, isolations from 20 rot samples inoculated with both *Leuconostoc* and *R. solani* were all positive for *Leuconostoc* and none were positive for *R. solani*. In both 2014 and 2015, isolations from six samples inoculated with only *Leuconostoc* spp. were positive for *Leuconostoc* and contained no *R. solani*. In both 2014 and 2015, the isolations from the fungal check indicated that *R. solani* could be confirmed 67% of the time but *Leuconostoc* spp. were also present (not unexpected because inoculations were conducted in the field and *Leuconostoc* is common in the environment). No rot ever occurred in the water checks; therefore, isolations were not conducted from the water checks.

TABLE 5. Root rot in sugar beet roots of the commercial cultivar B-7 inoculated with 26 *Leuconostoc* isolates with and without *Rhizoctonia solani* AG-2-2 IIIB strain F517 and analyzed by haplotype along with fungal and water checks in 2014 and 2015 Kimberly, ID field studies

	Root rot (mm)[x]	
Treatment[y]	2014	2015
5 + F517	12 c–e	40 a
30 + F517	14 cd	35 ab
25 + F517	21 bc	32 a–c
23 + F517	23 ab	31 a–c
18 + F517	27 ab	31 a–c
13 + F517	18 bc	31 a–c
11 + F517	26 ab	31 bc
21 + F517	17 bc	30 bc
15 + F517	30 a	27 bc
27 + F517	17 bc	27 bc
24 + F517	23 a–c	26 bc
9 + F517	17 bc	26 bc
6 + F517	20 bc	25 cd
19 + F517	27 ab	25 cd
Water + F517	2 ef	16 de
18	1 f	13 ef
11	4 ef	10 e–g
27	1 f	10 e–g
13	5 d–f	9 e–h
15	6 d–f	9 e–h
19	2 ef	9 e–h
24	0 f	9 e–h
30	0 f	8 e–h
25	0 f	8 e–h
21	0 f	8 f–h
23	1 f	8 f–h
5	0 f	6 f–h
6	0 f	4 f–h
9	2 ef	4 gh
Water	0 f	0 h
$P > F$[z]	<0.0001	<0.0001

[x] Internal root rot measured inside a bisected sugar beet. Means followed by the same letter within a column did not differ significantly based on least square means ($\alpha = 0.05$).

[y] Treatment = *Leuconostoc* haplotype with and without *Rhizoctonia solani* AG-2-2 IIIB strain F517. The haplotype was based on sequencing (1,437 to 1,440 bp) from the 16S ribosomal RNA region. Fungal and water checks are in bold.

[z] $P > F$ was the probability associated with the F value.

DISCUSSION

Based on 203 *Leuconostoc* isolates collected from rot in recently harvested sugar beet roots in southern Idaho and southeastern Oregon, the following *Leuconostoc* spp. were found (during 2010 and 2012): *L. mesenteroides* (88 and 85% of isolates, respectively), *L. pseudomesenteroides* (6 and 15%), *L. kimchi* (2 and 0%), and an unrecognized *Leuconostoc* sp. (4 and 0%). Based on sequencing

TABLE 6. Root rot in sugar beet roots of the commercial cultivar B-7 inoculated with 26 *Leuconostoc* isolates with and without *Rhizoctonia solani* AG-2-2 IIIB strain F517 and analyzed by *Leuconostoc* sp. along with fungal and water checks in 2014 and 2015 Kimberly, ID field studies

	Root rot (mm)[x]	
Treatment[y]	2014	2015
L. kimchi + F517	16 b	33 a
L. pseudomesenteroides + F517	19 b	31 a
L. mesenteroides + F517	26 a	29 a
Water + F517	2 c	16 b
L. mesenteroides	4 c	9 bc
L. pseudomesenteroides	0 c	8 c
L. kimchii	0 c	5 cd
Water	0 c	0 d
$P > F$[z]	<0.0001	<0.0001

[x] Internal root rot measured inside a bisected sugar beet. Means followed by the same letter within a column did not differ significantly based on least square means ($\alpha = 0.05$).

[y] Treatment = *Leuconostoc* sp. with and without *Rhizoctonia solani* strain F517. The *Leuconostoc* sp. was based on sequencing (1,437 to 1,440 bp) from the 16S ribosomal RNA region. Water and fungal checks are in bold print.

[z] $P > F$ was the probability associated with the F value.

from the 16S rRNA region, 23 haplotypes were found but haplotype 11 (*L. mesenteroides* isolates) comprised 68 to 70% of the isolates both years. Pathogenicity tests revealed that representative isolates from 14 of these haplotypes led to more rot through a synergistic interaction with *R. solani*, because all *Leuconostoc* isolates induced more rot ($P < 0.0001$; $\alpha = 0.05$) when combined with *R. solani* than when inoculated alone in both years. Also, 46 of the 52 combination treatments over the 2 years had significantly more rot ($P < 0.0001$; $\alpha = 0.05$) than the fungal check. Therefore, the data support the conclusion that a synergistic interaction leading to more root rot can occur between *Leuconostoc* and *R. solani*. However, the interaction was only evaluated against *R. solani* strain F517 under Idaho conditions. To ensure that the response is widespread, the synergistic response should be evaluated in other growing areas and additional strains of *R. solani* should be evaluated versus the *Leuconostoc* strains.

Bacterial root rot in sugar beet has traditionally been studied in association with *Pectobacterium betavasculorum* (Thomson et al.) Gardan et al. (syn. *Erwinia carotovora* (Jones) Bergey et al. subsp. *betavasculorum* Thomson et al.) and *Pseudomonas* spp. (Dutta et al. 2014; Gardan et al. 2003; Jacobsen 2009; Ruppel et al. 1975; Thomson et al. 1977). However, in 2008, *L. mesenteroides* subsp. *dextranicum* was also established to be associated with root rot in sugar beet and frequently associated with recently harvested roots infested with *R. solani* (Strausbaugh and Gillen 2008). A recent study in Germany confirms these earlier observations, having found the genus *Leuconostoc* to be associated with 92% of the recently harvested sugar beet roots with root rot (Liebe and Varrelmann 2016). The present study builds on the knowledge from these earlier studies to show that *L. mesenteroides* haplotype 11 is the primary *Leuconostoc* sp. associated rot in sugar beet in southern Idaho and southeastern Oregon, because it comprised 68 to 70% of the isolates. In addition, *L. pseudomesenteroides* and *L. kimchi* isolates were also shown to be associated with sugar beet root rot for the first time, although less frequently than *L. mesenteroides*. When individual isolates from these *Leuconostoc* spp. were inoculated with *R. solani*, they led to more rot than when they were inoculated individually. A previous study with recently harvested roots also indicated that there may be an interaction between *L. mesenteroides* and *R. solani* but the results were similar to a storage response rather than field results (Strausbaugh et al. 2013a).

The results in the current study represent the first field study to show that a synergistic interaction can exist between *Leuconostoc* spp. and *R. solani*. Other investigations studying *R. solani* on sugar beet and other crops have been focused on investigating bacteria in an effort to develop a biocontrol to limit disease development by the fungus (De Curtis et al. 2010; Gkarmiri et al. 2015; Heydari and Misaghi 2003; Hua and Höfte 2015; Lovic et al. 1993; Mendes et al. 2011; Olorunleke et al. 2015; Postma and Schilder 2015; Postma et al. 2010; Schillinger and Paulitz 2014; Solanki et al. 2012; Weller et al. 2002; Yin et al. 2013; Zachow et al. 2011).

Leuconostoc is a heterofermentative bacterium known to be important in the initial phase of fermentation but it usually is superseded by other bacteria and yeast (Adesogan et al. 2003; Amoa-Awua et al. 2007; Breidt 2004; Gardner et al. 2001; Jung et al. 2012). A number of these other bacteria and yeast associated with root rot in sugar beet roots have been shown to slow down rot by *L. mesenteroides* and inhibit *R. solani* (Lovic et al. 1993; Strausbaugh and Gillen 2008). The competition provided by these bacteria and yeast may help explain why *R. solani* is largely restricted to tissue near the root surface (Strausbaugh and Eujayl 2012; Strausbaugh and Gillen 2008; Strausbaugh et al. 2013a,b). This competition also makes it problematic to cleanly isolate *R. solani* and *Leuconostoc* spp. from root tissue even when using semiselective media (Strausbaugh et al. 2013a). Because of competition from fast-growing contaminants, our success rate for *Leuconostoc* isolations from recently harvested commercial roots was 63%, while isolations from the fungal checks in the field study was 67%. An *R. solani* reisolation success rate of 66% was found in another study when roots were taken from the field and inoculated with *R. solani* (Strausbaugh et al. 2013a). If collecting isolates is not a necessary part of the study, than utilizing a recently developed microarray may be a more reliable way to prove that *Leuconostoc* spp. and *R. solani* are associated with the rotting sugar beet roots (Liebe et al. 2016). Another advantage of using a DNA-based microarray approach may be to limit one's exposure to *Leuconostoc* spp. *Leuconostoc* spp., including *L. mesenteroides* and *L. pseudomesenteroides*, are emerging as human pathogens that can lead to severe infections, particularly in immunocompromised individuals (Albanese et al. 2006; Bou et al. 2008; Deng et al. 2012; Kumudhan and Mars 2004; Shin et al. 2011; Taneja et al. 2005; Taşkapilioğlu et al. 2011; Tholpady et al. 2010; Wong et al. 2012; Yang et al. 2015). However, considering the rarity of *Leuconostoc* infections in people and the large presence of *Leuconostoc* spp. in foods and the environment, they are generally recognized as safe (Hemme 2012).

RRCR appears to be on the rise in a number of growing areas worldwide; therefore, developing management options is a primary concern (Buddemeyer et al. 2004; Buhre et al. 2009; Führer Ithurrart et al. 2004; Ohkura et al. 2009). The management of RRCR through host resistance would be desirable and likely the most cost-effective control measure (Panella 2005). However, most commercial sugar beet cultivars provide only low to intermediate levels of resistance (Strausbaugh et al. 2013a). In addition, cultivars that do contain good resistance typically do not have the yield and resistance to other diseases necessary to make it through the cultivar approval process (Strausbaugh et al. 2013b). Developing resistant cultivars is problematic, because resistance is quantitative and the cultivars frequently suffer from yield drag (Hecker and Ruppel 1975; Lein et al. 2008; Panella 2005). Also complicating the screening process for resistance in sugar beet are the different strains of *R. solani* and their interactions with different *Leuconostoc* spp. (Strausbaugh et al. 2013b). The use of crop rotation (Buddemeyer et al. 2004; Buhre et al. 2009; Engelkes and Windels 1996; Kluth and Varrelmann 2010; Ruppel 1985; Rush and Winter 1990) and fungicides (Bolton et al. 2010; Kiewnick et al. 2001; Kirk et al. 2008; Stump et al. 2004; Windels and Brantner 2005) can also help limit RRCR; however, unacceptable levels of rot still frequently occur. Perhaps investigations into what leads to the synergistic interaction between *R. solani* and *Leuconostoc* spp. may allow for a better understanding of the rot process and additional control measures.

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