Research

Identification of Sources of Resistance to Blackleg (*Leptosphaeria maculans*) in a *Brassica napus* Germplasm Collection

Syedmojtaba Mansouripour and Luis E. del Río Mendoza, Department of Plant Pathology, North Dakota State University, Fargo 58108

Accepted for publication 27 April 2017.

Abstract

The increased prevalence of strains of pathogenicity group 4 of *Leptosphaeria maculans*, causal agent of blackleg, represents a serious threat to the canola (*Brassica napus* L.) industry in North Dakota, a state that contributes >85% of U.S. canola production. The objective of this study was to identify sources of resistance to PG-4 in a collection of 559 *B. napus* plant introduction materials (PIs). Replicated trials were conducted twice in the greenhouse using a mixture of five PG-4 isolates to evaluate the reaction at seedling stage; then,

an elite group of PIs was evaluated in replicated field trials between 2014 and 2016 at Langdon, ND, using a combination of lab-produced inoculum and blackleg-infested canola residues. Combined analysis of field data indicated three PIs had on average 73 to 80% less ($P < 0.05$) internal stem tissue discoloration than the commercial hybrids used as the controls. These PIs could be used in canola breeding programs as good sources of resistance against PG-4; in addition, efforts should be made to characterize the resistance they carry.

Blackleg, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [syn. = *Phoma lingam* (Tode: Fr.) Desm.], has become the most important disease affecting canola production in the United States (Fig. 1). In North Dakota, the largest canola producer in the country (USDA-NASS 2015), the disease was first observed in the early 1990s (Lamey 1995). At that time, the most prevalent *L. maculans* strains were classified as pathogenicity group (PG) 2; strains belonging to this group are incapable of affecting plants carrying resistance genes *Rlm1*, *Rlm2*, and/or *Rlm3* (Ansan-Melayah et al. 1998; Balesdent et al. 2002). The introduction of cultivars carrying these resistance genes reduced the severity and importance of this disease in the following decade (Bradley and Lamey 2005). Since then, however, the disease has steadily increased in severity and prevalence powered by the spread of strains belonging to PG-4 (del Río Mendoza et al. 2012).

Identifying sources of resistance against PG-4 is necessary and urgent. A study published in 2010 suggested that most canola cultivars currently in use in North Dakota are susceptible to strains of PG-4. (Marino and del Río 2010), which can overcome resistance genes *Rlm1*, *Rlm2*, and *Rlm3*. As PG-4 strains become more ubiquitous in North Dakota, the possibilities of severe blackleg outbreaks increase. An end-of-season survey conducted in 2014 on 114 canola fields in North Dakota revealed that blackleg-symptomatic plants were present in 96% of fields, with several having incidences greater than 30% (del Río Mendoza, unpublished data). The objective of this study was to identify *B. napus* germplasm that could be used as sources of resistance against PG-4 strains of *L. maculans*.

Greenhouse Screenings

Greenhouse screenings were intended to characterize the reaction of 559 *B. napus* plant introductions (PI) at the seedling stage. Seeds,

obtained from the North Central Regional PI Station of the U.S. National Plant Germplasm System located in Ames, IA, were planted in batches containing 29 entries at a time, with cv. Westar serving as the susceptible control. Westar is no longer a commercial cultivar and has been used as standard susceptible control because it doesn't carry known blackleg-resistance genes (Balesdent et al. 2005). Entries in each batch were evaluated using a randomized complete block design with three replications. Seeds were placed in individual plastic cells in trays (44 × 57 × 50 mm) filled with soilless potting mix (PRO-MIX BX, Premier Tech Horticulture, Quakertown, PA) and kept in greenhouse rooms at 20 ± 2°C with 16 h of light daily supplemented with 600-watt high pressure sodium lamps (P.L. Light Systems, Inc., Beamsville, Ontario, Canada). Seedlings were inoculated, as described below, 10 days after planting when they were at the cotyledon stage. A spore suspension containing equal amounts of spores from five PG-4 isolates was used as inoculum. These five *L. maculans* isolates were collected in North Dakota and selected for their aggressiveness (Franceschi and del Río 2014; Franceschi 2015).

Inoculum of each *L. maculans* isolate was produced by culturing them in separate dishes containing V8-agar medium as described by Nepal et al. (2014). After harvest, spore concentrations of each isolate were estimated with the help of a hemocytometer, adjusted to 10⁷ spores/ml, and equal volumes of each suspension were combined for inoculations. During inoculation, the center of each cotyledon leaf was lightly pricked once with sharp forceps and a 10-μl droplet of the spore suspension was deposited on the wound. The inoculated seedlings were incubated in cool mist chambers at 20°C and 98% humidity in darkness for 24 h and then returned to the greenhouse room.

The reaction of cotyledon leaves was recorded 10 days after inoculation based on the 0-to-9 scale of Williams and Delwiche (1979), where 0 to 3 represents a resistant reaction and 7 to 9 shows susceptibility (Fig. 2A). Levene's test for homogeneity of variances conducted on the reaction of Westar across batches indicated these batches could be combined ($P > 0.05$) for analysis. The median severity per replication for each PI was calculated using PROC MEANS of SAS v. 9.4 (SAS Institute Inc., Cary, NC) and then

Corresponding author: Luis E. del Río Mendoza;
E-mail: Luis.delRio-Mendoza@ndsu.edu

FIGURE 1

Typical blackleg symptoms caused by *Leptosphaeria maculans* on canola (*Brassica napus*) plants with lesions on (A) leaves (photo courtesy K. Chittem) and (B) stems.

ranked with respect to other accessions using PROC RANK. Ranks closest to one are associated with most resistant reactions. ANOVA-type statistic analysis was then conducted on the ranks using PROC

FIGURE 2

Blackleg severity scales used to evaluate damage (A) at seedling stage on cotyledon leaves and (B) at adult plant stage as percentage of internal tissues in the crown region of the stems.

FIGURE 3

Frequency distribution of the reaction of 559 *Brassica napus* plant introduction materials to inoculations with a mixture of five pathogenicity group 4 strains of *Leptosphaeria maculans* in greenhouse trials. Reactions evaluated at seedling stage using the 0-to-9 severity scale of Williams and Delwiche (1979) where 0 to 3 are resistant reactions and 7 to 9 are susceptible reactions.

MIXED as described by Shah and Madden (2004). To calculate each PI's relative effect and 95% confidence interval, the ranks were analyzed using the SAS macro LD_CI.sas from Brunner et al. (2002). The relative effects range from 0 to 1 and the values closest to zero are associated with the most resistant reactions. As a group, the PIs reaction to inoculation followed a normal distribution (Fig. 3) with an overall mean severity of 4.83 and a median severity of

five. Approximately 30% of accessions were considered resistant, among which 29 PIs had medians <3, an indication of strong hypersensitive reactions (Table 1).

Field Evaluations

Field plots were established in 2014, 2015, and 2016 at the Langdon Research Extension Center of North Dakota State University in Langdon, ND. A total of 24 entries including 16 PG-4-resistant, five PG-4-susceptible PIs, Westar as the standard susceptible control, and two commercial hybrids were planted by hand on 15, 24, and 19 May, respectively. Entries were planted in single 3-ft-long rows following a randomized complete block design with four replications each year. Each block consisted of six tiers of four entries and two border rows. Rows were separated by 18 cm. At least four canker-bearing canola stems collected from commercial fields in the previous growing season were deposited in each tier to provide inoculum for the plants. In addition, seedlings were sprayed at least three times between the cotyledon and three-leaf stages with a 10^7 spores/ml spore suspension of the five isolates used in greenhouse screenings. The spore suspensions were prepared as described earlier

and were delivered using a backpack sprayer until runoff. Disease severity was recorded when plants reached physiological maturity by cutting the stems at the crown and estimating the percentage of internal tissue discoloration (Fig. 2B). Levene's test of homogeneity of variances was conducted using PROC GLM of SAS to determine whether data could be combined for analysis. Upon confirmation of the homogeneity of variances ($P > 0.05$), a combined analysis was performed using PROC MIXED with years and replications considered random variables and entries considered fixed variables. Pairwise comparisons between least square means of PIs were estimated using the option "pdiff" in the lsmeans statement and its output analyzed using the SAS macro "pdmix800.sas" (Saxton 1998) to separate PIs using a letter system. Field trials showed significant differences ($P \leq 0.05$) among PIs (Table 2) but not all entries performed as expected from their behavior in greenhouse trials. Thirteen of the 16 PIs considered resistant in greenhouse trials had significantly less ($P = 0.05$) disease severity than Westar, the susceptible control; however, the remaining three, Colt, Gido and Eckendorfer Mali, behaved statistically as susceptible as Westar and more susceptible than the PIs Liglandor and CR167/65a. The latter two were considered susceptible ($P = 0.05$) in the greenhouse trials.

TABLE 1
Response of 29 *Brassica napus* plant introduction materials to a mixture of PG-4 isolates of *Leptosphaeria maculans* inoculated on seedlings in greenhouse trials

Accession name	Median ^a	Mean ranks	Treatment relative effect ^b	
			Mean	95% confidence interval
Mokpo 2	1	11	0.02	0.01 - 0.05
Iwao natane	1	16	0.03	0.02 - 0.06
Gebr Dippes	1	56	0.11	0.06 - 0.22
Giant rape	1.5	8	0.04	0.02 - 0.10
Gylle	1.5	148	0.30	0.15 - 0.51
Wira	2	32	0.07	0.05 - 0.10
Kinki 21	2	55	0.10	0.07 - 0.13
Wichita	2	48	0.10	0.05 - 0.17
Dong Hae 16	2	54	0.11	0.07 - 0.17
Mokpo #3	2	55	0.11	0.05 - 0.22
Su weon cheg	2	54	0.11	0.06 - 0.22
Aomori-1	2	60	0.12	0.06 - 0.22
Hobson	2	64	0.12	0.06 - 0.22
Jet Neuf	2	80	0.15	0.09 - 0.24
Liropa	2	70	0.15	0.09 - 0.25
PI 169080	2	87	0.16	0.06 - 0.39
Armander	2	71	0.16	0.06 - 0.37
Abilene	2	80	0.18	0.04 - 0.55
France 8	2	94	0.20	0.06 - 0.49
KS 1701	2	137	0.26	0.08 - 0.60
Dong Hae 20	2	161	0.30	0.14 - 0.54
Norin 35	2	155	0.33	0.15 - 0.59
Synra	2	152	0.33	0.11 - 0.67
Ames 6073	2	162	0.38	0.05 - 0.88
Kuju 40	2.5	74	0.13	0.12 - 0.14
77-71	2.5	74	0.15	0.08 - 0.25
Hamburg	2.5	86	0.18	0.08 - 0.35
Iwashiro-natane	2.5	150	0.30	0.11 - 0.61
Dong Buk	2.5	150	0.33	0.12 - 0.63

^a Medians based on the reactions on two cotyledons per plant; 10 plants per replication; three replications per trial and two trials. Reactions rated according to the 0 to 9 scale of Williams and Delwiche.

^b Mean relative effects closer to 0 are considered more resistant.

TABLE 2
Reaction of elite^a *Brassica napus* plant introduction materials to *Leptosphaeria maculans* evaluated in field trials conducted at Langdon, ND, in 2014 to 2016

Accessions	Median	Treatment relative effect ^b		Severity (%) ^c		Incidence (%)	
		Mean	95% confidence interval	Mean	Letter group	Mean	Letter group
Colt	3	0.19	0.14 - 0.26	68	A	94	A
Westar	7.2	0.82	0.80 - 0.84	61	AB	92	AB
Ujfertodi	5	0.48	0.21 - 0.75	55	ABC	84	ABCDE
Ames26653	7	0.72	0.50 - 0.87	47	BCD	86	AB
Gido	3	0.14	0.07 - 0.25	47	BCDE	88	AB
Titus	7	0.68	0.46 - 0.83	46	BCDEF	82	ABCD
Eckendorfer Mali	3	0.27	0.12 - 0.48	43	BCDEF	81	ABC
Rico	3	0.18	0.13 - 0.26	42	CDEFG	84	AB
Laura	3	0.22	0.18 - 0.26	40	CDEFG	73	BCDEF
Commercial hybrid #1	—	—	—	37	CDEFG	72	BCDE
Legend	3	0.24	0.13 - 0.39	34	CDEFGH	82	ABC
Target	3	0.24	0.23 - 0.25	31	CDEFGHIJKL	83	ABCDE
Su weon cheg	2	0.11	0.06 - 0.22	31	CDEFGHI	63	CDEF
N001-28-246-5-4	3	0.18	0.13 - 0.26	28	EFGHIJ	56	DEFG
Commercial hybrid #2	—	—	—	27	FGHIJ	61	CDEF
77-71	2.5	0.15	0.08 - 0.25	27	DEFGHIJK	55	EFGH
Nabo	3	0.24	0.13 - 0.39	24	FGHIJKL	63	DEFG
Liglandor	9	0.94	0.87 - 0.97	22	GHIJKL	59	DEFG
CR 167/65a	7	0.77	0.67 - 0.85	17	HIJKL	51	FGH
Oleifera	3	0.23	0.12 - 0.40	14	IJKL	54	EFG
Bolko	3	0.17	0.12 - 0.24	11	JKL	35	GH
Sumner	3	0.28	0.14 - 0.48	8	KL	36	GH
Aomori-1	2	0.12	0.06 - 0.22	8	KL	42	GH
CR 165/76a	3	0.38	0.20 - 0.60	6	L	31	H

^a Westar and commercial hybrids #1 and #2 were used as controls.

^b Treatment relative effects calculated for plant introductions but not for commercial hybrids (—) in greenhouse screenings.

^c Severity expressed as percentage of discolored internal stem tissues at crown. Means in a column with the same letter are not significantly different ($P = 0.05$) per Fisher's protected least significant difference test.

These inconsistencies in performance could be explained in part by the nature of the resistance present in them. Resistance to blackleg can be qualitative or quantitative and the genes controlling them are “generally distinct” (Delourme et al. 2006). The former, also known as race-specific or vertical resistance, is typically evaluated on seedlings in greenhouse conditions while the latter, also known as polygenic or partial resistance, is evaluated on adult plants under field conditions. By supplementing the fields with blackleg-infested canola residues, we exposed these plants to strains other than the ones used in the greenhouse screenings. While no efforts were made to identify the strains of blackleg present on the residues or in infected PIs, previous studies determined that approximately 22% of isolates retrieved from commercial canola residues do not fit the profile of any PG (Nepal et al. 2014). Among the PIs whose reaction was consistent in greenhouse and field trials, CR 165/76a, Aomori-1, and Sumner had on average 73 to 80% less disease severity and 40 to 49% less incidence than either commercial control, while Bolko and Oleifera had on average 66% less severity and 38% less incidence than one of the commercial controls and had a reaction statistically similar to the other.

Conclusions

The sources of resistance identified in this study could be a valuable tool for canola breeding programs. The top three PIs presented a consistent resistant reaction to exposure to lab- and

field-produced inoculum. They were collected from different countries, which increases the possibility that the genetic bases of their resistance to blackleg may be different from one another. Also, they do not require vernalization to flower, which will facilitate the development of breeding populations adapted to the growing conditions of the region.

Acknowledgments

This work was supported by the USDA/NIFA, Supplemental and Alternative Crops Grant Program, grants 2015-38624-24214 and 2011-38624-30812, and NIFA Hatch Project ND002227.

Literature Cited

- Ansan-Melayah, D., Balesdent, M. H., Delourme, R., Pilet, M. L., Tanguy, X., Renard, M., and Rouxel, T. 1998. Genes for race-specific resistance against blackleg disease in *Brassica napus* L. *Plant Breed.* 117:373-378.
- Balesdent, M. H., Attard, A., Kühn, M. L., and Rouxel, T. 2002. New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. *Phytopathology* 92:1122-1133.
- Balesdent, M. H., Barbetti, M. J., Li, H., Sivasithamparam, K., Gout, L., and Rouxel, T. 2005. Analysis of *Leptosphaeria maculans* race structure in a worldwide collection of isolates. *Phytopathology* 95:1061-1071.
- Bradley, C. A., and Lamey, H. A. 2005. Canola disease situation in North Dakota, U.S.A., 1993-2004. *Proc. 14th. Aust. Res. Assembly on Brassicas*, Port Lincoln, Australia.
- Brunner, E., Domhof, S., and Langer, F. 2002. *Nonparametric Analysis of Longitudinal Data in Factorial Designs*. Wiley, New York.

- del Rio Mendoza, L. E., Nepal, A., and Markell, S. 2012. Outbreak of blackleg in canola in North Dakota is caused by new pathogenicity groups. *Plant Health Prog.* 10.1094/PHP-2012-0410-01-RS
- Delourme, R., Chèvre, A. M., Brun, H., Rouxel, T., Balesdent, M. H., Dias, J. S., Salisbury, P., Renard, M., and Rimmer, R. S. 2006. Major polygenic resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus*). *Eur. J. Plant Pathol.* 114:41-52.
- Franceschi, J. 2015. Phenotypic characterization of *Leptosphaeria maculans* pathogenicity groups aggressiveness on *Brassica napus*. M.S. thesis. North Dakota State University, Fargo, ND.
- Franceschi, J., and L.del Río. 2014. Characterization of virulence of different pathogenicity groups of *Leptosphaeria maculans* on *Brassica napus* L. *Phytopathology* 104:S3.42.
- Lamey, H. A. 1995. Survey of blackleg and Sclerotinia stem rot of canola in North Dakota in 1991 and 1993. *Plant Dis.* 79:322-324.
- Marino, D., and del Rio, L. E. 2010. Screening of plant introduction materials from *Brassica* species for resistance against PG3 and PG4 isolates of blackleg. *Phytopathology* 100:S77.
- Nepal, A., Markell, S., Knodel, J., Bradley, C. A., and del Río Mendoza, L. E. 2014. Prevalence of blackleg and pathogenicity groups of *Leptosphaeria maculans* in North Dakota. *Plant Dis.* 98:328-335.
- Saxton, A. M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. Pages 1243-1246 in: Proc. 23rd SAS Users Group Intl., SAS Institute Inc., Cary, NC.
- Shah, D. A., and Madden, L. V. 2004. Nonparametric analysis of ordinal data in designed factorial experiments. *Phytopathology* 94: 33-43.
- Williams, P. H., and Delwiche, P. A. 1979. Screening for resistance to blackleg of crucifers at the seedling stage. Pages 164-170 in: Proc. Eucarpia Cruciferae Conference, Wageningen, The Netherlands.