Survival of blackleg pathogen inoculum in canola stubble under simulated flooding conditions

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Abstract

Blackleg of canola (*Brassica napus*), caused by two *Leptosphaeria* spp, is a significant constraint to canola production worldwide except in china where only the less virulent *L. biglobosa* has been reported. In China, the disease is caused by a less pathogenic species, *L. biglobosa*, and there is a concern that importing canola from Canada may introduce the virulent *L. maculans*, impacting the crop there negatively. In China, canola (or rapeseed) production is centered in several eastern and central provinces where winter rapeseed is often followed by paddy rice that normally is flooded for weeks during late spring and summer. *L. maculans* or *L. biglobosa* in diseased canola stubbles serves as the key inoculum source to cause blackleg, and it has been questioned if the flooding practice may help suppress the inoculum. A study was initiated to determine the effect of flooding temperature (12 to 40°C) and duration (2 to 12 weeks) on survival of blackleg pathogen in canola stubbles. Experiments were set up on a Thermogradient Plate that is capable of simultaneously creating 96 independent temperature settings. Diseased stubbles with > scale-3 level of basal stem-canker symptoms used for the experiments were collected from a Westar canola plot in Melfort after 2011 harvest. Flooded stubbles were sampled every two weeks, surface sterilized, and incubated on V8-juice medium amended with antibiotics for 10 days to observe pycnidia cultures of L. *maculans* or *L. biglobosa* as the evidence of pathogen survival. Two trials were set up in RCBD with four replications, and pathogen incidence data (based on 25 stubble pieces per replicated) were subject to ANOVA. Significant reduction (*P*= 0.00) of pathogen incidence was observed at 2-week flooding treatment relative to control (non-flooded) and there was no pathogen recovery after 4weeks of flooding till 12 weeks of experiment. Lower flooding temperatures of 12^{o}C and 16^{o}C appeared to be slightly less effective than higher temperatures (20-40 ^{o}C) in reducing pathogen survival. Stubble tissues degraded sharply after 2weeks (contrast, *P*= 0.05) in response to the flooding temperature and the dry weight was reduced more substantially (40%) at higher temperatures. Virulence of any survived pathogen propagule after flooding is still intact and survival at any temperature or duration of flooding does not differentiate between *L. maculans* or *L. biglobosa.* High proportion of survived blackleg pathogen (pycnidia) from flooding were *L. maculans (*67%*)* and the rest *L. biglobosa* (33.0%) under Westar cotyledon test.

Key words: Winter rapeseed, patty rice, crop rotation, temperature,

Introduction

Black leg disease or phoma stem canker of *Brassica* spp. (canola, oilseed rape) is caused by two ascomycetous fungi which coexist on the host crop. These pathogens, *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.), *and L. biglobosa* Shoemaker and Brun (Kutcher *et al.,* 2011) is a significant constraint to canola production worldwide (West *et al.* 2001, Fitt, *et al.* 2008). *Leptosphearia maculans* is the more damaging species of the two and was reported from almost all canola and oilseed rape growing regions of the world except China (Fitt, *et al.* 2006) where the disease is caused by a less pathogenic species, *L. biglobosa*, and there is a concern that importing canola from Canada may introduce the virulent *L. maculans*, impacting the crop there negatively. This is of a major concern to Canada who exports large amount of Canola seeds to China. China is an important market for Canadian canola. In 2008-2009, China imported 2.87million tons of canola valued at $1.3 billion making her the top Canada's canola seed market (Canola Council of Canada, November, 2009). Canola is a unique Canadian success story with a $15.4 billion industry in Canada when transportation and processing are included (Canola Council of Canada, September, 2011). Out of this, $14b is obtained from Western Canada.

In China, the production of oilseed rape is centered in several eastern and central provinces where winter rapeseed is often followed by paddy rice that normally is flooded for weeks during late spring and summer (Wu *et al.* 2009). It is not very clear if crop rotation practices employed along side paddy rice flooding is responsible for none incidence of aggressive *L. maculans* in China. There is need to understand canola growing environment, management techniques and blackleg avoidance principles employed in China.

Hence the objective of this study were to: 1) Assess the effect of flooding temperature and duration on canola stubble degradation; 2) Identify optimal range of flooding conditions to minimize blackleg pathogen survival; 3) Determine whether the two *Leptosphaeria* species would survive differently under flooding conditions and the ability of the pathogens in infecting canola after flooding treatment.

Materials and methods

1.) Collection of blackleg pathogens

Diseased canola stubbles with both ends showing > the scale-3 level of damage were collected from a Westar plot in Melfort after 2011 harvest. Two of the diseased stem pieces were placed in a tea bag which is then wrapped with a layer of nylon cloth, in case of biodegradation of tea bags during flooding. To estimate the speed of stubble degradation, additional stubble pieces (1-cm, diseased or non-diseased) are dried in an oven at 60°C for 2 days, packaged similarly but at 1 g/tea bag (Fig1).

Six bags (each type) are placed in a 100-ml Mason jar (**Fig1**), covered with 40-cm^3 field soil, and then "flooded" with tap water to the 50-ml mark. Jars with flooded stubble pieces are placed into deep cells of a thermogradient plate (TGP). In pre-trial monitoring, deviations from set temperatures are < ±1°C.

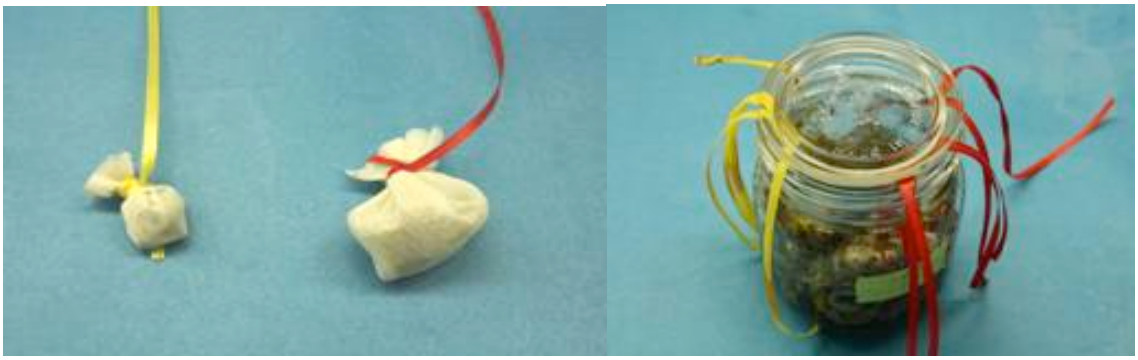

Fig 1. Two diseased stem pieces (yellow threaded pouch) and1 g/teabag canola stubble (red threaded pouch) are both placed in a 100-ml Mason jar.

A. Effect of flooding under constant temperature

Eight temperature regimes replicated 4 times as 12°C, 16°C, 20°C, 24°C, 28°C, 32°C, 36°C, and 40°C were examined. One bag each of diseased/ 1 g canola stubble was removed from each of the jars at 2, 4, 6, 8, 10, and 12 wks to determine pathogen survival and stubble tissue degradation (dry weight). Non-flooded stubbles were used as 0-wk flooding control for all flooding temperature/duration treatments. The experiment was a completely randomized design with 4 replicates for each flooding temperature/duration. Two repetitions were used for this study.

B. Determination of pathogen survival

Twenty –five (25) surface-sterilized pieces from flooded blackleg stubbles were placed randomly on V-8 juice agar in a Petri plate and replicated four times (**Fig2**). Stem pieces (on agar) were incubated at about 22°C under soft white light with a 12/12 hr photoperiod for 10 days. Incubated plates were observed for the incidence of *Leptosphaeria* sp based on the presence of pycnidia under a stereomicroscope (**Fig3**). Two trials were set up in RCBD with 4 replications, pathogen incidence data (based on 25 stubble pieces per replicate) were subject to ANOVA.

Differentiation of survived pycnidia to *L. maculans* and *L. biglobosa*

Seven survived pycnidia from different temperature regimes and 4 non- flooded control isolates were prepared for canola cotyledon inoculation. Canola seeds variety 'Westar' were sown at 0.5cm deep singly in individual flat pots of 12-pack multiport containing AAFC soil-less planting mix (pH 5.8). At 7 days after planting, seedlings were inoculated with pycnidiospores obtained from flooded blackleg canola stubble. Cotyledons of canola seedlings were wounded with a pipette tip and inoculated with 10µl of spore suspension containing $2x10^7$ spores per millilitres of water. A 10µl droplet of sterile distilled water was used as a control in experiment.

Inoculated seedlings were accessed for the degree of susceptibility to blackleg pathogen using standard disease rating scale 0-9 for blackleg (Williams, 1985; El Hadrami *et al,* 2009) at 12 days after inoculation (El Hadrami *et al,* 2009). Each isolate was replicated four times with 8 inoculation points per isolate. Two repetitions were set up for this experiment.

Data analysis

Data obtained from canola stubble degradation and pathogen recovery from trials 1 and 2 showed homogeneity of virulence on pathogen recovery (P=0.5187) and canola tissue degradation (P=0.5242) based on the Bartlett test. Therefore, data from the two trials were pooled for statistical analysis. Logarithm transformation was used to improve the normality of tissue disintegration and *Leptosphaeria* sp. recovery data using the Generalized Linear Model procedure of SAS. Effects of flooding and varied temperature on blackleg pathogen recovery as well as the interaction between flooding temperature and duration of exposure to flooding were determined based on analysis of variance (ANOVA). Means were compared using DMRT test when the ANOVA is significant. Contrast procedure of SAS was used to compare differences in tissue reduction across different temperatures. The significance of flooding temperature and duration, and their interactions were related to the outcomes of the experiment

Result

Pathogen survival

At all temperature treatments there was significant reduction in pathogen survival even after a 2-week period of flooding (**Fig 4**). Although the pathogen was still recovered from some stubble tissues at 4 weeks, it was generally with lower (12 and 16 ^{o}C) temperature treatments and at reduced incidence. By the end of the experiment (12 weeks), however, there was no evidence of pathogen survival in the diseased stubble tissues examined.

The impact of flooding temperature on the pathogen survival was significant (*P*=0.00) whereas the flooding duration or its interaction with the temperature was not (**Table 1**). There was no difference between the impact of the temperature between 16^{o}C and 40 ^{o}C, but the pathogen survival under these temperature conditions was lower relative to that at 12^{o}C (**Fig 4**).

Stubble decomposition during flooding

Under all flooding temperature and duration conditions, there was a significant decrease in dry weight matter of the stubble (*P*=0.00) relative to non-flooded controls (**Table 2**). The most significant dry-weight losses were observed in the initial 2-4 week period under all temperature treatments (**Fig 5**) whereas less substantial dry-weight changes occurred in the subsequent weeks although the weight declined further between 8th and 12th weeks. Over the course of the experiment higher temperatures generally resulted in a greater decrease in dry weights. Both the flooding temperature and duration affected the stubble degradation significantly and interactively (**Table 2**). The tissue degradation was greater when subjected to longer flooding duration under higher temperature conditions (**Fig 5**).

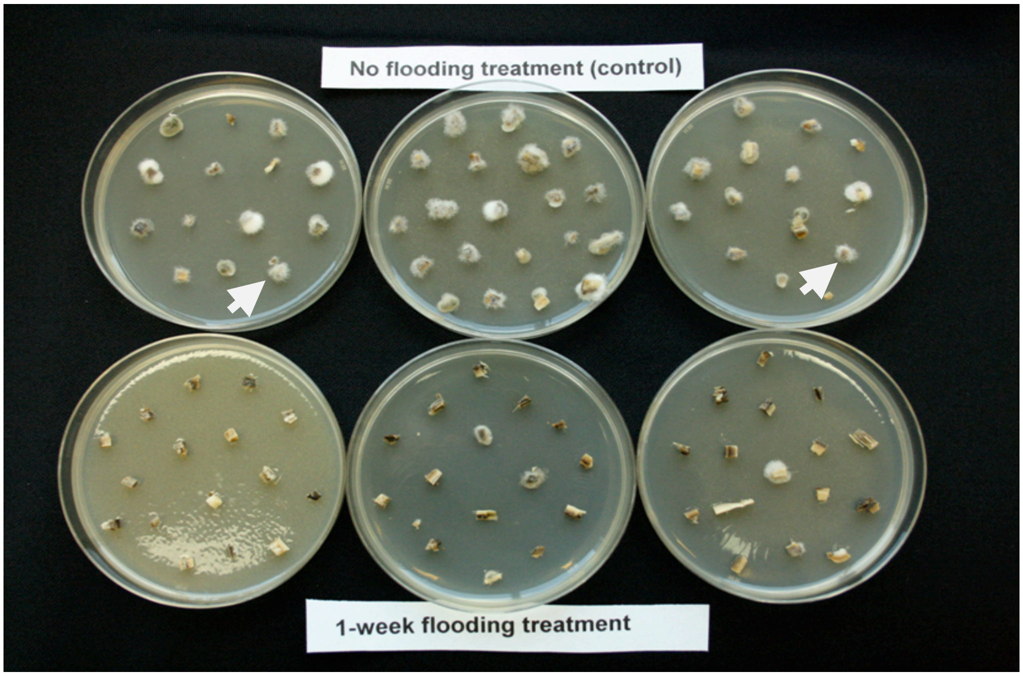

Fig 2. Recovery of *Lestophaeria* spp. (arrowed) from canola stubble tissues treated with (lower row) and without flooding.

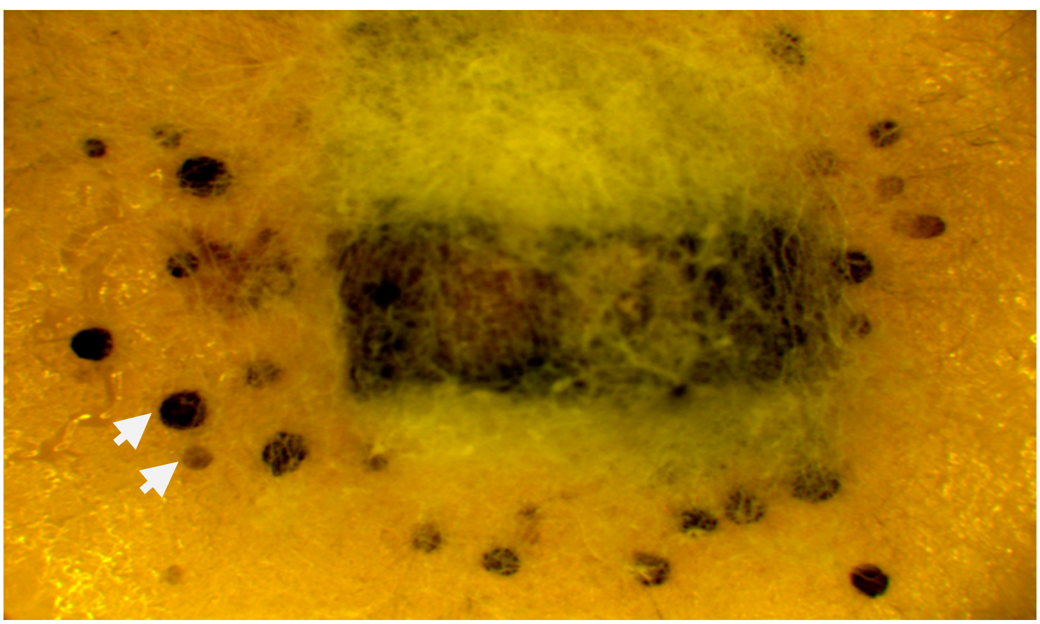

Fig 3. *Lestophaeria* sp. culture recovered from diseased canola stubble tissues treated with flooding. The pycnidia (arrowed) were positive identification of the fungus (x60).

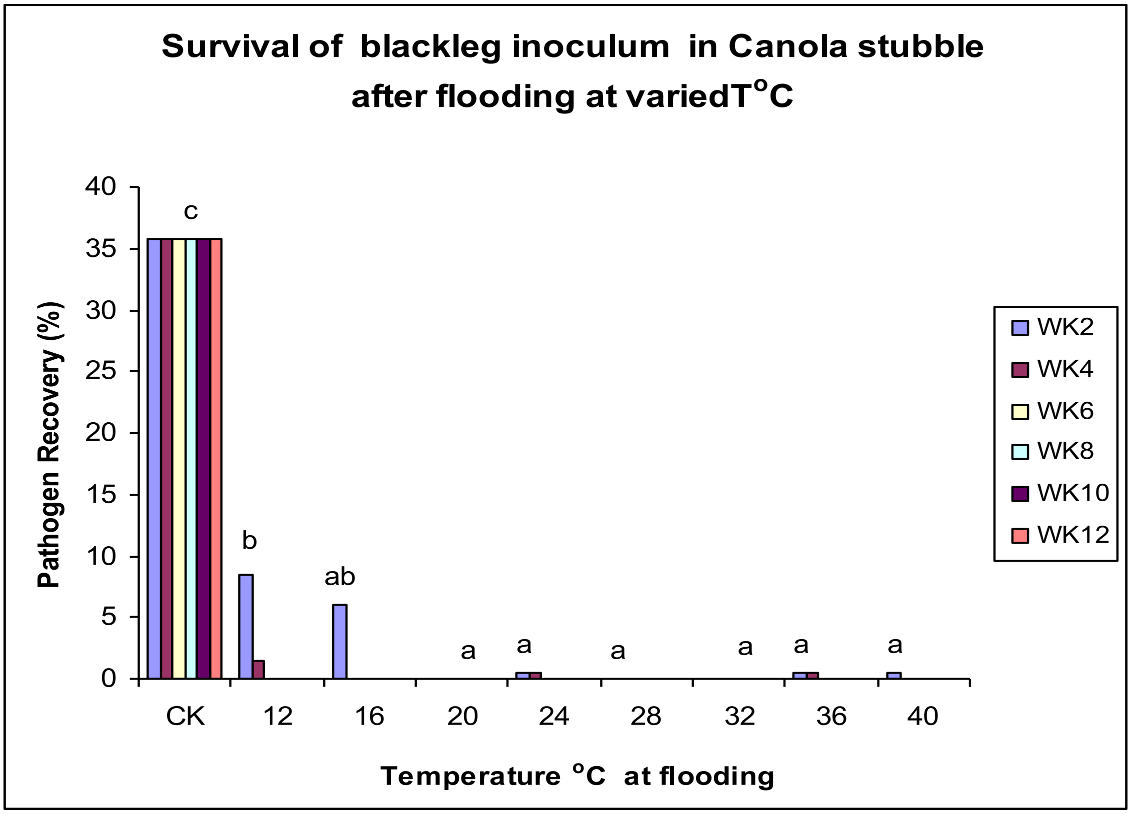

Fig 4. Survival of blackleg inoculums in canola stubble after 12 weeks of flooding at varied Temperature (°C)**.**

Table 1. ANOVA: Impact of flooding temperature and duration on the survival of *Leptosphaeria* spp. in canola stubble.

Source	Sum of Squares	df	Mean Square	F-Ratio	*P*-Value
Flooding temperature	3135.45	8	391.93	97.01	0.00
Flooding duration	12.12	5	2.43	0.60	0.70
Temperature x duration	56.02	40	1.40	0.35	0.99
Residual	1527.12	378	4.04		

Over the period of the experiment the dry weights remained lower than the initial 1.0 g in controls. The rate of decrease was slowed after the 2 week treatment (Contrast, P = 0.05) and again between the 8 and 12 week treatments (Fig 5). There was a significant overall dry weight reduction between all sampling intervals except for between the 8 and 10 weeks (Contrast, P = 0.05). In general, higher temperature conditions during flooding led to greater reduction in dry matter.

Table 2. ANOVA: Impact of flooding temperature and duration on canola stubble tissue degradation.

Source	Sum of Squares	df	Mean Square	F-Ratio	*P*-Value
Flooding temperature	4.19	8	0.52	262.61	0.00
Flooding duration	0.39	5	0.07	39.10	0.00
Temperature x duration	0.19	40	0.01	2.38	0.00
Residual	0.75	378	0.002		

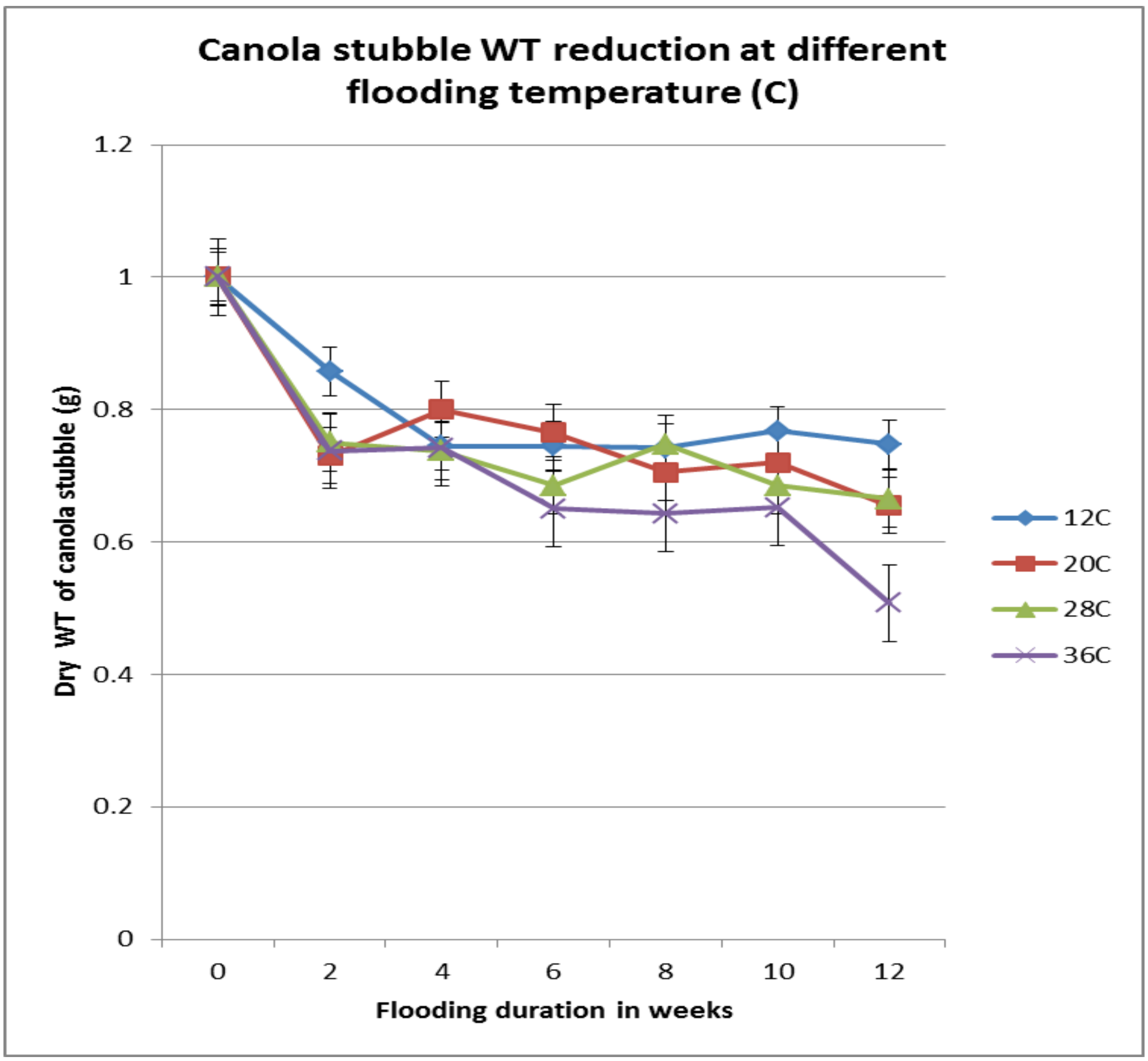

Fig 5. Reduction of canola stubble dry weight at varying flooding temperatures (^{o}C).

Pathogenicity of recovered *Leptosphaeria* spp. isolates

A total of 6 isolates were obtained from flooding-treated stubble tissues, with two of them being classified as *L. biglobosa* based on their low aggressiveness on the highly susceptible canola cultivar Westar (**Table 3**). The rest were considered *L. maculans* because of severe infection observed on the cotyledons compared to the water control. The severe symptom included tissue browning and necrosis on cotyledons.

Table 3. Infection of canola cotyledons (Westar) by *Leptosphaeria* spp. that survived a flooding treatment.

Isolate recovered	Flooding duration (wks)	Reaction (0-9) [b]	*Leptospheaeria* spp
CR1 [a]	0	1	*L biglobosa*
CR2	0	8	*L. maculans*
CR3-1	0	9	*L. maculans*
CR3-2	0	9	*L. maculans*
R1-40	2	9	*L. maculans*
R1-24	2	1	*L. biglobosa*
R2-16	2	7	*L. maculans*
R3-12	2	9	*L. maculans*
R1-12	4	9	*L. maculans*
R2-12	2	1	*L. biglobosa*
Water control	0	0	Not applicable

[a] CR= check, from non-flooded disease canola stubble.

[b] 0-9 = scale described by Williams, 1985; El hadrami *et.al.,* 2009.

Discussion

Pathogen Survival Study

Prior research has shown a preliminary relationship between flooding and *L. maculans* population; a significant reduction in spore production might happen on stubbles even after a 1-day flooding treatment compared to those left in dry soil conditions (Petrie, 1995). Likewise, our results showed that flooding reduced pathogen survival rapidly under all temperature conditions examined. Past studies also found temperatures lower than 20°C with constant water supply would be more suitable for the maturation of pseudothecia of either *L. maculans* or *L. biglobosa (*Pérès, *et al.,* 1999; Toscano-Underwood, *et al*., 2003; Naseri*, et al*, 2009; E. Lô-Pelzer *et al.,* 2009). In current study, the pathogen was observed to survive coincidentally slightly longer at lower temperatures but the link to prior observations is not clear. There appear to be paucity of information on the effect of flooding on the survival of blackleg pathogen. However, the use of flooding to control plant pathogens has been in existence for a long time. For example, Moore (1945) reported the use of flooding as a means to destroy sclerotia of *Sclerotinia sclerotiorum* in soil. Sclerotia decayed in 25-45 days (3.5-6.25 weeks) in submerged soils and no difference in decay was seen under continuous or intermittent flooding conditions. Temperature and water availability were among the factors also considered to affect the survival of other fungal pathogens under flooding (Stover, 1954). The survival of submerged *Fusariium* sp. at 13°C was 10-20 times higher than at higher temperatures of 24-36°C. This is in consonance with the present study where only 12 and 16°C allowed the survival of blackleg pathogen. The flooding duration that completely eradicates the blackleg pathogen in flooding was between 4 and 6 weeks, regardless of the temperature.

It is noteworthy that the temperature was a significant factor during flooding that contributes to pathogen mortality and the higher temperatures tend to inactive the pathogen more quickly. Although the pathogen recovery was substantially lower than non-flooded controls, the flooding at 12°C showed a higher incidence relative to the other temperature treatments within initial 4 weeks. In the eastern and eastern-central regions of China, where the acreage of winter rapeseed is the highest, paddy rice fields are flooded for much longer period during spring and summer following the harvest of winter rapeseed (Wu *et al.* 2009) and the average low and high temperature during the period range from 20-25°C to 28-33°C, respectively (World Meteorological Organization 2012). This temperature range is more than sufficient in meeting the needs of biological degradation of stubble residuals and colonizing blackleg pathogen based on the current study. Therefore, from the perspective of blackleg control, a rotation with paddy rice would be a better choice than a dry-land crop following winter rapeseed.

The pattern on stubble dry-weight reduction clearly demonstrated the effects of flooding on canola stubble decomposition. As suggested by others (West et al., 2001; Baird et al., 1999), the rate of degradation of canola stubble debris can be influenced by wetness and temperature. In current study, the stubble tissue degradation occurred rapidly under all flooding temperature conditions examined and this is consistent with the prior report (West et al. 2001). It seems that the majority of tissue degradation would have occurred shortly after the exposure to flooding.

The rapid dry-weight reduction of stubble tissues seem to coincide with the low pathogen recovery observed after 2-wk of flooding, which might be relating to poor survival of the pathogen in severely degraded stubble. Under conditions of dry summers or cold winters, stubble residues can remain a blackleg inoculum source for many years (Barbetti and Khangura, 1997). Debris that is more intact is related to the longevity of *L. maculans* and its ability to be an inoculum source. Therefore in an environment where wet soils exist the debris is more decomposed and less capable of being an inoculum source (Baird *et al.* 1999). In the winter rapeseed growing areas in China where fields are often flooded after harvested to prepare for next rice crop, we would expect that stubble decomposition occur rapidly, especially during hot summer season and the blackleg pathogen would survive poorly.

Research by Baird *et al.* (1999) also showed that that the size of stubble sections directly affects the rate of decomposition; larger stubbles remain in tact for longer periods and therefore allow for survival of *Leptosphaeria* sp. We need to keep this factor in mind when interpreting the current study results because stubble from spring canola tend to be thinner than those of winter rapeseed due to a much shorter growing season. Therefore, it may be worthwhile to use winter rapeseed stubble for more accurate assessment of flooding impact. In practice, tillage may help break up stubble and then flooding may be more effective in deteriorating inoculum populations (Baird et al., 1999)

Most *Leptosphaeria* sp. isolates recovered from flooded canola stubble caused severe infection on the susceptible canola cultivar Westar, and were considered possible *L. maculans*. This shows that although flooding can reduce the survival of the blackleg pathogen, the virulence of any survived pathogen inoculum is still intact. Additionally, the flooding effect did not seem to differentiate between *L. maculans* and *L. biglobosa*, which implies that planting paddy rice after winter rapeseed can suppress both blackleg pathogen species. A PCR method (Mahuku *et al.* 1996) may be used to differentiate the two species more definitively.

It would be interesting to further study the minimum flooding duration required to keep down the initial inoculum level of blackleg for canola or rapeseed as Petrie (1995) reported that a flooding duration shorter than 6 days would allow stubble to resume inoculum production. The constant temperatures used in current trials are not typical conditions that happen in nature, and diurnal temperature conditions may be used in further trials to better mimic the pattern of daily temperature change (Peng and Boyetchko 2006). Another issue with current trials is that the non-flooded stubble gave a relatively low pathogen incidence (< 50%). This may be due in part to stubble tissues not being fully infected under field conditions and this shortcoming may be overcome by more selective collection of diseased stubble. The data generated through the current study may be validated under various flooded paddy rice field conditions. A combination of crop rotation with paddy rice plus removal of diseased plants will help alleviate the risk of blackleg (West et al., 2001; Agriculture and Agri-Food Canada, 2010) to Chinese winter rapeseed crops.

Conclusion

Flooding facilitates the disintegration of canola stubble tissues and limits blackleg inoculum survival, especially under warmer temperature conditions. The condition in most paddy rice fields in China should be conducive to reducing the survival of both blackleg pathogen species within a season. As China remains an important canola seed trade partner with Canada, it is important to understand the challenge of blackleg to oilseed rape production in China and the measures to be taken to mitigate the risk. This information supports the choice of paddy rice (as opposed to dry-land crops) following winter rapeseed for reduction of blackleg pathogen inoculum as one of the risk mitigation strategy in crop production.

References

Agriculture and Agri-Food Canada. 2010. Canada canola sector profile. [Online]. Available: http://www.agr.gc.ca/pol/mad-dam/pubs/rmar/pdf/rmar_02_05_ 2010-11-26_ eng.pdf [February 7, 2012].

Baird, R.E., Phillips, D.V., Mullinix, B.G., and Alt, P.J. 1999. Relative longevity of Leptosphaeria maculans and associated mycobiota on canola debris. Phytoprotection. 80: 1-11.

Barbetti, M.J., and Khangura, R.K. 1997. Managing blackleg in the disease-prone environment of Western Australia. Faculty of Agriculture, University of Western Australia. Nedlands, Western Australia.

Canola Council of Canada (2009). www.canolacouncil.org/web- Canola export to China.Phytosanitary requirements for blackleg

Canola Council of Canada (2011). Canola : Western Canada‘s other oil boom. The globe and mail (Wednesday globe and mail). http://m.theglobeandmail.com/report-onbusiness/economy/canola-western-canadas-other.

El Hadrami, A., Fernando W. G. D., and Daayf, F. 2010. Variations in relative humidity modulate *Leptosphaeria* spp. pathogenicity and interfere with canola mechanisms of defence. Eur. J. Plant Pathol. 126:187-202.

E. Lô-Pelzer, J. N. Aubertot, O. David, M. H. Jeuffroy and L. Bousset(2009) Relationship between severity of blackleg *Leptosphaeria maculans/L. biglobosa*

species complex) and subsequent primary inoculum production on oilseed rape stubble *Plant Pathology* **58**, 61–70 Doi: 10.1111/j.1365-3059.2008.01931

Fitt, B.D.L., H. Brun, M.J. Barbetti, and S.R. Rimmer. 2006. Worldwide importance of phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). Eur. J. Plant Pathol. 114:3-15.

Fitt, B.D.L., Hu, B.C., Li, Z.Q., Liu, S.Y., Lange, R.M., Kharbanda, P.D., Butterworth, M.H., and White, R.P. 2008. Strategies to prevent spread of Leptosphaeria maculans (phoma stem canker) onto oilseed rape crops in China; costs and benefits. Plant Pathology. 57: 652-664.

Kharbanda PD*, (1993).* Blackleg of Canola in Alberta: Investigations on Biology, Epidemiology and Management. Vegreville, AB, Canada: AECV93–R5.

Kutcher, H.R.; W.G.D. Fernando, T.K. Turkington and D.L. McLaren (2011) Best Management Practices for Blackleg Disease of Canola. Insects and Diseases. *Prairies soils and Crops Journal* **4**:122-134 www.prairiesoilsandcrops.ca

Mahuku, G. S. R.Hall and P. H. Goodwin (1996) Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape *Physiological and Molecular Plant Pathology* (1996) ***49***, 61-72

Moore W.D. (1945). Flooding as a means of destroying the Sclerotia of *Sclerotinia sclerotiorum*. Phytopathology **39**:920-927

Peng G, and Boyetchtko (2006). Effect of variable dew temperatures on infection of green foxtail by *Pyricularia setariae*, *Drechslera gigantea*, and *Exserohilum rostratum*." Biological Control 39: 539-546.

Pérès, A., Poisson, B., Sourne, V.L., & Maisonneuve, C. (1999) *Leptosphaeria maculans:* Effect of temperature, rainfall and humidity on the formation of pseudothecia. In: *Proceedings of the 10th International Rapeseed Congress.* Canberra, Australia: 26–9

Petrie, G.A. 1995. Long-term survival and sporulation of *Leptosphaeria maculans* (blackleg) on naturally-infected rapeseed/canola stubble in Saskatchewan. Canadian Plant Disease Survey. 75: 23-24

Naseri, B., Davidson, J. A., & Scott, E. S. (2009) Maturation of pseudothecia and discharge of ascospores of *Leptosphaeria maculans* on oilseed rape stubble. European Journal of Plant Pathology **125:**523-531 doi: 10.1007/s10658-009-9500-9

Shoemaker R.A.;Brun H.(2001) The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans* Canadian Journal of Botany, Volume 79,Number 4, 412-419

Stover R.H. (1954). Flood –fallowing for eradication of *Fusarium oxysporum f. cubense*: 11. Some factors involved in fungus survival. Soil science: **77**:401-414

Toscano-Underwood C, Huang YJ, Fitt BDL, Hall AM, 2003.Effects of temperature on maturation of pseudothecia of *Leptosphaeria maculans* and *L. biglobosa* on oilseed rape stem debris. *Plant Pathology* **52**, 726–36.

West JS, Kharbanda PD, Barbetti MJ, Fitt BDL,(2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology* **50**, 10–27.

Williams, P.H. (1985). Crucifer genetics cooperative. Plant Mol. Biol. Rep 3: 129-144.

World Meteorological Organization 2012. http://www.worldweather.org/001/c00351.htm