

Phenotypic, Biochemical and Molecular Diversity of *Phoma lingam* Fungal Isolates Collected from Saskatchewan, Canada

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Abstract: The population of *Phoma lingam* associated with blackleg disease of canola (*Brassica napus*) is highly polymorphic, and infect various Brassicaceae species, including *Thlaspi arvense* (stinkweed). In order to determine the population composition and pathogenicity of *P. lingam* infecting *T. arvense*, 18 isolates recovered from *T. arvense* (designated 'Thl' isolates) from different crop fields of Saskatchewan were compared with an aggressive (IBCN 2) and eleven less/non-aggressive reference strains. No common morphological, biochemical and pathogenic characteristics were found between 'Thl' isolates and IBCN 2 strain. DNA fingerprinting and phylogenetic analysis of ITS-rRNA gene sequences revealed that the 'Thl' *P. lingam* population of Saskatchewan is highly heterogeneous and comprised of at least eight genotypes. Over 50 percentage of 'Thl' isolates clustered with different less/non aggressive reference strains belonging to *L. biglobosa*. Interestingly, a group of 'Thl' isolates clustered with an aggressive reference strain, IBCN 2. Therefore, regular monitoring is required to determine if the 'Thl' *P. lingam* population of western Canadian provinces is migrating from non-aggressive toward aggressive state.

Keywords: Blackleg disease, genetic diversity, ITS-rRNA sequence, oilseed rape, *Phoma lingam*

1. Introduction

Phoma lingam is an economically important pathogen of oilseed rape/canola (*Brassica napus* L.) and other brassica plants (e.g., *B. oleracea*, *B. rapa*) across the world [1, 2]. Populations of the fungus are highly polymorphic and thought to consist of a species complex [3, 4]. Initially, *P. lingam* isolates that cause blackleg or stem canker on *Brassica napus* or *B. oleracea* and produce the host-nonspecific toxins 'sirodesmins' were designated as 'A' group or aggressive or Tox⁺ [5, 6]. In contrast, isolates that cause no or little disease on those hosts and do not produce sirodesmins were termed 'NA', non-aggressive or Tox⁰. Subsequent molecular, biochemical and pathogenicity studies showed that isolates of the 'A' and 'NA' groups belong to two teleomorphic species of *P. lingam*, namely *Leptosphaeria maculans* and *L. biglobosa*, respectively [7, 8]. The two species occur in similar ecological niches but produce distinctive infection symptoms. *L. maculans* causes damage on lower stems while *L. biglobosa* infects upper stems of *B. napus* [6, 9]. Isolates belonging to two groups/species appeared to be comprised of multiple subgroups that often correspond to host plants and geographical origins [10, 11]. 'A' group comprised of at least two sub-groups, namely *L. maculans* 'brassicae' (isolates collected from *B. napus*) and *L. maculans* 'lepidii' (isolates from *Lepidium* sp.) [11]. In contrast, the NA group appeared to be highly heterogeneous [12] and could be divided into different sub-groups distinguished by their host plant and country origins. 'NA' isolates originating from oilseed rape and cole crops are designated 'brassicae' (NA1, Europe), 'canadensis' (NA2, Canada), NA3 (USA), and 'australiensis' (NA-Aus, Australia) and 'occiaustralensis' (Australia). The isolates from cruciferous weeds have been designated as 'erysimii' (*Erysimum* sp.), 'thlaspii' (*Thlaspi*

arvense), and 'sysimbrii' (*Sysimbrium* sp.) [8, 11, 13]. Importantly, shifts of non pathogenic populations of *P. lingam* to a highly pathogenic state have been observed in Canada and on other continents [13, 14, 15].

This research aimed at elucidating the population structure and pathogenic behaviour of *P. lingam* isolates collected from *T. arvense* from canola growing areas of Saskatchewan and other western Canadian provinces. To achieve the objectives the morphological and biochemical characteristics, aggressiveness on *B. napus* plants and genetic variability of the 'Thl' isolates were studied by comparing to a number of *P. lingam* reference strains exists in different continents. The work is expected to determine if *T. arvense* serves as an alternate host of *P. lingam* potentially harmful to canola.

2. Materials and Methods

Fungal isolates

Eighteen isolates of *P. lingam* isolated from *T. arvense*, designated 'Thl' isolates, were compared with strains from the International Blackleg of Crucifers Network (IBCN). The IBCN collection representative of 13 *P. lingam* isolates belonging to the A- and NA groups, and *L. maculans* and *L. biglobosa* (Table 1). The isolates Thl-10 to 13 were isolated from seed of *T. arvense* collected in a canola field infected with blackleg on the Agriculture and Agri-Food Canada (AAFC)-Research Farm near Melfort, Saskatchewan, Canada; isolates Thl-14 to 28 were isolated from seed of plants collected near Eastend in south-western Saskatchewan, which is outside of the traditional canola production zone.

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Table 1. Isolates of *Phoma lingam* used in the study

(a) Isolates collected from <i>Thlaspi arvense</i>			
Isolates	Location	Isolated from	Year collected
Thl-10 to 13	AAFC*-Melfort Research Farm, Saskatoon, SK**	Canola field	2000
Thl-14 to 23	Site 1, Eastend, SK, Canada	Barley field	2000
Thl-24 to 27	Site 3, Eastend, SK, Canada	Road allowance	2000
Thl-28	Site 4, Eastend, SK, Canada	Barley field	2000
(b) Reference strains compared in the study			
Strains	Group/sub-group/species	Host and country of origin	Source (year collected)
IBCN 2	A, 'aggressive'	<i>Brassica napus</i> , Germany	H.-H. Hoppe (1992)
IBCN 26	NA1, 'brassicacae'	<i>B. napus</i> , Poland	Z. Karolewski (1991)
IBCN 30	NA-Aus, 'australensis'	<i>B. napus</i> , Australia	B.J. Howlett (1991)
IBCN 64	'Thlaspii'	<i>Thlaspi arvense</i> , SK, Canada	G. Séguin-Swartz (1993)
IBCN 82	'Sisymbrii'	<i>B. juncea</i> , SK, Canada	G. Séguin-Swartz (1993)
IBCN 83	'Erysimumi'	<i>Erysimum</i> sp., SK, Canada	G. Séguin-Swartz (1993)
IBCN 84	'Lepidii'	<i>Lepidium</i> sp., SK, Canada	G. Séguin-Swartz (1993)
IBCN 91	NA3	<i>B. oleracea</i> , USA	P.H. Williams (?)
IBCN 92	NA2, 'canadensis'	<i>B. rapa</i> , Canada	P.H. Williams (?)
IBCN 65	<i>L. biglobosa</i> 'Thlaspi'	<i>T. arvense</i> , SK, Canada	Mendes-Pereira et al. 2003 (?)
O6VTJ154	<i>L. biglobosa</i>	<i>B. juncea</i> , Australia	Van de Wouw et al. 2008 (?)
UK5	<i>L. biglobosa</i> , brassicacae	<i>B. napus</i> , UK	Lui et al. 2006 (?)
UK7	<i>L. maculans</i> , brassicacae	<i>B. napus</i> , UK	Lui et al. 2006 (?)

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Phenotypic and biochemical characterization

Mycelial growth of isolates:

Mycelial growth rates of the 'Thl' isolates were compared to those of reference strains IBCN 2 (A group) and IBCN 26 (NA1, *brassicacae* sub-group) on V8-juice agar (V8A) and malt extract agar (MEA) [16]. Agar disks (4 mm diameter) bearing mycelium excised from the edge of 14-day-old cultures were placed at the centre of 9-cm diameter Petri plates. For each growth medium, there were three replicate plates per isolate and the plates were incubated at 20°C in the dark. Average daily growth rates were calculated for each isolate for the period of logarithmic growth.

Production of pigments and sirodesmins:

'Thl' isolates and strains IBCN 2 and IBCN 26 were inoculated in Czapek-Dox broth [16] amended with 2% yeast extract and 2% peptone and grown at 20°C in the dark on an orbital shaker (100 rpm). After 28 days, the cultures were filtered through Buchner funnels fitted with Whatman filter paper no. 595 into test tubes and filtrate colour was assessed visually. The fungal ability to produce sirodesmins in nutrient broth was determined by thin layer chromatography [17].

Pathogenicity tests on Canola plants

Oilseed rape cultivar 'Lirabon' (Deutsche Saatveredelung, Lippstadt, Germany) was used in the pathogenicity tests because it is susceptible to *P. lingam* [18]. The pathogenicity test was performed as two plant growth stages.

Cotyledon assay:

Seedlings were grown in a growth chamber at 22°C with a 16 h photoperiod and 65 to 75% relative humidity. Seven-day-old seedlings were transplanted to multi-cell trays containing a mixture of peat moss, compost and sand (1:1:0.1, v/v) one day before inoculation. Both lobes of each cotyledon were punctured with an entomological needle and

inoculated with a 10 µL droplet of spore suspension (10⁷ pycnidiospores/mL). Seventeen plantlets were inoculated per isolate. Disease severity was assessed 14 days post-inoculation (dpi) using a 0 to 6 rating scale [19]; each lesion was scored and mean disease severity was calculated for each isolate.

Adult plant assay:

Plants were grown in 13×13 cm pots in a greenhouse at 24°C with a 16 h photoperiod. At the 3 to 4 true leaf stage, the axil of the first true leaf was wounded with a sterile entomological needle and inoculated with a mycelium plug (2 mm diam) excised from a 10-day-old oatmeal agar culture [16]. Inoculated plants were covered with polyethylene film for 72 h. Each isolate was tested on 17 plants. Disease severity was assessed using a modification of the method of [20]. Lesion length (L) (scale = 0: no infection, 1: 1–4 mm, 2: 5–8 mm, 3: 9–12 mm, 4: 13–16 mm, 5: 17–20 mm, 6: 21–24 mm, 7: 25–28 mm, 8: 29–32 mm, 9: >32 mm), extent of stem girdling (G) and lesion depth (D) (scale = 0: no infection, 1: >0–11%, 2: 12–22%, 3: 23–33%, 4: 34–44%, 5: 45–55%, 6: 56–66%, 7: 67–77%, 8: 78–88%, 9: >88%) were assessed at 49 dpi.

The volume of diseased tissue (VDT) was calculated for each combination of plant and isolate according to the following formula: $VDT = (1 - HR^2) \times G/9 \times L$, where $HR = 1 - D/9$.

The mean VDT was then calculated for each isolate.

Analyses of genetic diversity of *P. lingam* isolates

Extraction of fungal DNA:

IBCN reference and 'Thl' isolates were grown in Czapek-Dox broth supplemented with 0.1% peptone. Three-week-old cultures were filtered using Buchner funnels fitted with 90 mm filter discs and the mycelium was lyophilized. Total genomic DNA was extracted using Qiagen's DNeasy Plant Mini Kit according to the manufacturer's instructions. The

following three genomic loci were studied for defining the genetic diversity of the *P. lingam* isolates.

Variable Number of Tandem Repeats (VNTR) fingerprinting:

PCR reactions of 25 µL final volume containing 25 ng template DNA, 0.2 mM each dNTP, 1.5 mM MgCl₂, 1 µM primer 5'-ggtgccgctct-3' [21], 1.25 units *Taq* DNA polymerase and 1X concentrated reaction buffer were run using a T-Gradient thermocycler (Biometra GmbH, Göttingen, Germany). The thermocycler was set up for 30 cycles (4 min at 94°C, 1 min at 57°C and 3 min at 72°C). Amplified fragments were separated on 1.5% agarose gels and examined the banding patterns with the Bio-Rad Video Gel Documentation System and images were taken.

Enterobacterial Repetitive Intergenic Consensus (ERIC) fingerprinting:

The PCR reaction setup and concentration of components were identical to that used for VNTR-PCR. Primers ERIC1R (5'-atgtaagctcctggggattacc-3') and ERIC2 (5'-aagtaagtactgggtgagcg-3') [22, 23] were used. DNA amplification was performed for 36 cycles (2 min at 95°C, 1 min at 52°C and 3 min at 65°C). Amplified fragments were separated on 5 to 10% polyacrylamide gradient gels and stained and documented as for VNTR-PCR.

ITS-rRNA gene sequence comparisons

The ITS region of the rRNA of the 'Thl' and reference isolates was amplified using primers ITS4 (5'-tctcgcctattgatatgc-3') and ITS5 (5'-ggaagtaaaagtcgtaacaagg-3') [24]. The PCR reaction components and concentration were similar to those used for VNTR- and ERIC-PCR, except that Vent- polymerase was used instead of *Taq*-polymerase. Amplification was performed for 90 cycles (3 min at 94°C, 1 min at 54°C and 1 min at 72°C). Aliquot three microliters of amplicon was run on 0.8% agarose gel and visualized the size polymorphisms of ITS-rRNA fragments of *P. lingam* isolates. The PCR products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research Corporation), blunt ended and ligated into linearized pMiniT™ Vector using a NEB® PCR cloning kit and protocols. The inserted fragments were PCR amplified using the plasmid embedded primers L30350F (acctgccaaccaagcgagaac) and L71-1R (tcagggttattgtctcatgagcg) and sequenced. The sequence

similarity was searched using NCBI database. Reference strains that were not sequenced in the study were: *L. biglobosa* brassicae UK5 (Q133890), *L. maculans* brassicae UK7 (DQ133891), *L. biglobosa* China (KJ574214), *L. biglobosa* erysimii (AJ550872) and *L. biglobosa* Thlaspii (AJ550891). Phylogenetic diversity among and between 'Thl' and IBCN reference strains was demonstrated by constructing a dendrogram based on Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm using MEGA 6.0 software [25].

3. Results and Discussion

Morphological and biochemical characteristics of *P. lingam* isolates

The exponential growth on MEA of most 'Thl' isolates appeared to be similar to non-aggressive reference strain IBCN 26 (*L. biglobosa*) (4.9 mm/day) (Figure 1a). Thl-21, Thl-22 and Thl-27 grew slightly faster (6.6–7.1 mm/day) than IBCN 26. In contrast, Thl-11, Thl-18, Thl-19 and Thl-24 had significantly ($p < 0.05$) slower growth rates similar to aggressive reference strain IBCN 2 (2.3 mm/day). Similarly, most 'Thl' isolates had growth rates on V8A similar to that of IBCN 26 (9.0 mm/day) and greater than ($p \leq 0.05$) that of IBCN 2 (6.0 mm/day) (Figure 1b). Thl-18 had a similar growth rate to IBCN 2 and Thl-11 was intermediate (7.3 mm/day) between IBCN 2 and IBCN 26. Isolates of *P. lingam* non-aggressive on brassica crops appeared to be generally faster growing than aggressive isolates on culture media [12], suggesting that the 'Thl' isolates would be non-aggressive to oilseed rape and related crops.

The culture filtrates of non-aggressive isolates typically contain coloured pigments in liquid culture, e.g., phomaligin a [26], while culture filtrates of aggressive isolates are typically colourless [12]. Non-aggressive isolates are unable to produce the host nonspecific sirodesmin toxins that are characteristic of aggressive isolates and may be involved in pathogenicity [12, 27]. The culture filtrates of all 'Thl' isolates and the non-pathogenic reference strain (IBCN 26) were coloured and did not contain sirodesmins (Figure 2 and 3). In contrast, the pathogenic reference strain (IBCN 2) produced colorless pigments and synthesized sirodesmins (Figure 2). This suggests that the 'Thl' isolates were non-aggressive to canola.

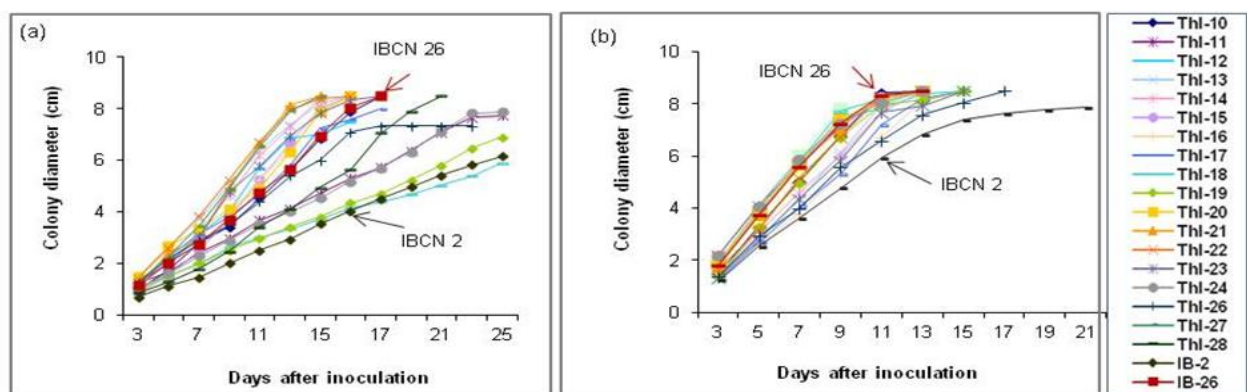


Figure 1: Colony growth of the 'Thl' isolates compared to *P. lingam* reference strains from IBCN reference collections. (a) and (b) show the fungal growth patterns on malt extract and V8 agar media, respectively. IBCN 2 and IBCN 26 denotes aggressive and non-aggressive reference strains.

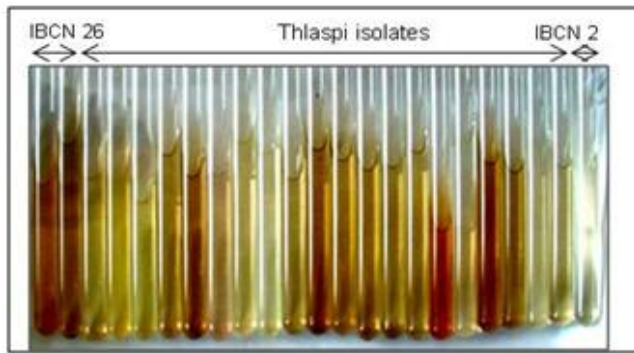


Figure 2: Production of coloured pigments by *P. lingam* isolates. IBCNC 2 and IBCNC 26 denote aggressive and non-aggressive reference strains, respectively.

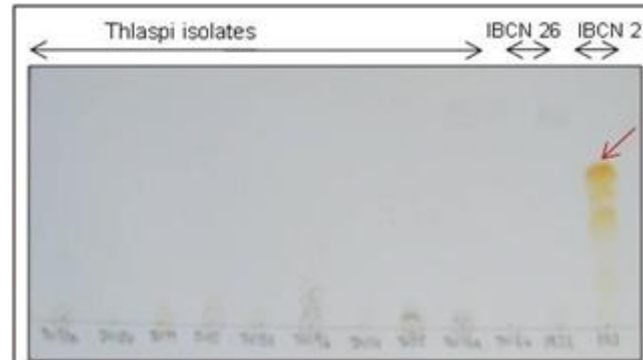


Figure 3: *In vitro* sirodesmins production by the *P. lingam* isolates. IBCNC 2 and IBCNC 26 indicate aggressive and non-aggressive reference strains, respectively.

Pathogenicity of *P. lingam* isolates

The aggressiveness of the 'Thl' isolates was assessed on cotyledons and stem tissue of older plants since resistance of oilseed rape at the cotyledon stage is not always correlated with adult plant resistance [28] and genes regulating these traits are different [29]. Lesions on cotyledons inoculated with IBCNC 2 were characterized by collapsed grey-green tissue, diffused margins, and numerous pycnidia at 14 dpi (Figure 4a); disease severity was >4.0 using the 0 to 6 scale. Plants inoculated with the non-aggressive IBCNC reference strains and the 'Thl' isolates showed a hypersensitive response, a limited dark lesion with no pycnidia or no symptoms (Figure 4b); disease severity ranged from 0 to 1.5. Isolate IBCNC 2 caused significant internal damage to stem tissue (Figure 4c); the other IBCNC strains and the 'Thl' isolates caused small, superficial stem lesions (Figure 4d). The disease severity of IBCNC 2 and 'Thl' isolates were expressed as VDT values and determined to be VDT >2.4 and 0.1 to 1.0, respectively ($p < 0.01$) (Figure 5). The pathogenicity tests confirmed that the 'Thl' isolates were not aggressive on susceptible *B. napus* cultivar 'Lirabon'.

Genetic diversity of *P. lingam* isolates

Preliminary genetic structure and variability of *P. lingam* isolates from different host plant species were studied using PCR-based ERIC and VNTR fingerprinting techniques. ERIC and VNTRs are located in non-coding regions and are distributed throughout many eukaryotic genomes with a high heterogeneity. These regions are useful to characterize microbial populations at the genus, species and strain level [21, 30, 31]. VNTR fingerprints exhibited considerable polymorphisms among the IBCNC reference isolates analyzed (Figure 4, lanes 2–10) (Figure 6). The band profiles clearly visualized diversity between the reference strains and the 'Thl' isolates, including the 'Thl' reference strain (IBCNC 64). The banding patterns of the 'Thl' isolates (Figure 6, lanes 12–29), showing 7 to 14 bands ranging from 350 bp to 3 kb, were visibly distinguishable from those of the IBCNC strains. The fingerprinting technique detected at least seven different

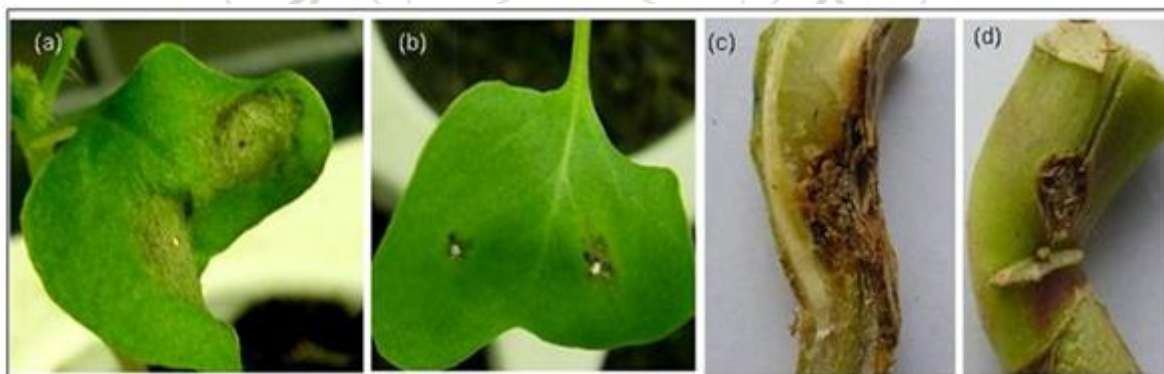


Figure 4: Response of cotyledons and stems of *B. napus* cultivar Lirabon to *Phoma lingam* isolates. (a) Cotyledon tissue collapse caused by the virulent reference strain IBCNC 2, and (b) hypersensitive-like symptoms caused by a 'Thl' isolate. (c) Strain IBCNC 2 shows canker on stem bases. (d) Small superficial canker lesion at stem bases caused by a 'Thl' isolate.

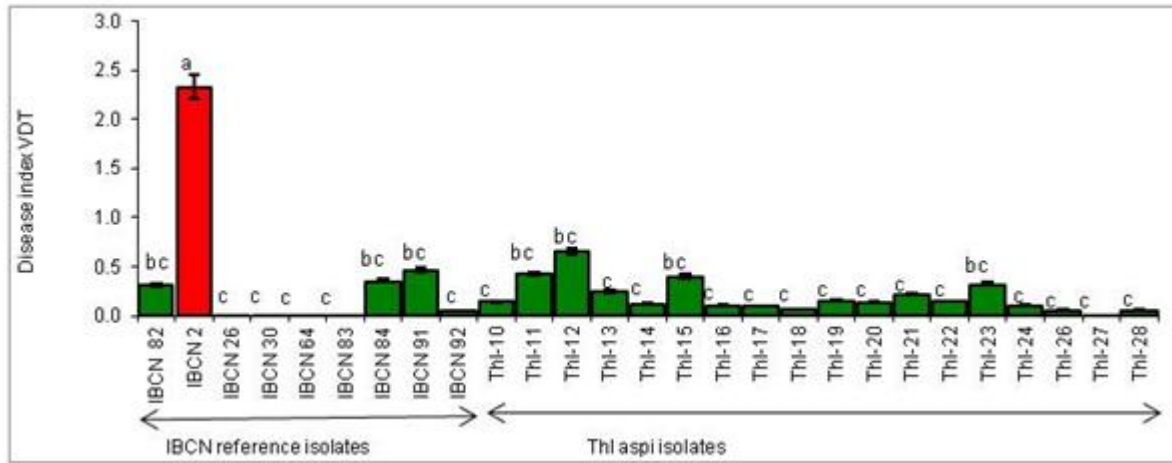


Figure 5. Disease severity on stem bases of *B. napus* cultivar ‘Lirabon’ 49 days post-inoculation with *Phoma lingam* isolates. Bars followed by different letters are statistically different from each other ($p < 0.05$). IBCN 2 and IBCN 26, aggressive and less/non-aggressive reference strains, respectively

band profiles among the ‘Thl’ isolates. Isolates Thl-10, -11, -12, -13 -18 and -24 generated separate band profiles comprised of 7, 8, 14, 14, 9 and 8 bands, respectively. The largest genotype, comprised of 13 isolates (Thl-14 to 17, 19 to 23 and 27 to 28), showed a profile of 14 bands. Based on ERIC band profile, isolate Thl-26 could be assigned to the last genotype. Similar to VNTR, the ERIC method revealed obvious variability in band profiles among ‘Thl’ isolates and between reference strains (data not shown). Analysis of ITS-rRNA gene sequences discriminated ‘Thl’ isolates from the IBCN reference strains, including a previously characterized ‘Thl’ strain (IBCN 65). The diversity could be visualized by different sizes of ITS-rRNA gene fragments of the isolates on an agarose gel (data not shown). The UPGMA dendrogram created from ITS-rRNA sequences revealed a highly heterogeneous ‘Thl’ population (Figure 7). The ‘Thl’

population evaluated in this study could be distributed into eight clusters, namely cluster - 1, 2, 3, 6, 7, 8, 9 and 10. Of the ‘Thl’ isolates, 10 showed relationships to eight non-aggressive reference strains belonging to *L. biglobosa*. This finding is supported by Balesdent and co-authors [32], who suggested the occurrence of highly heterogeneous *P. lingam* in western Canada. One isolate (Thl-12) showed a close relationship with a previously characterized ‘Thl’ isolate (IBCN 65) (cluster 3) that was collected from an area where the tested ‘Thl’ isolates were collected a few years later [33]. This suggests that significant temporal restructuring of *P. lingam* populations can occur on *T. arvense*. In regard to crop fields, the ‘Thl’ population from canola fields (Thl-10 to 13) and road allowance (Thl-24 to 27) appeared to be highly heterogeneous as compared to the population from barley fields.

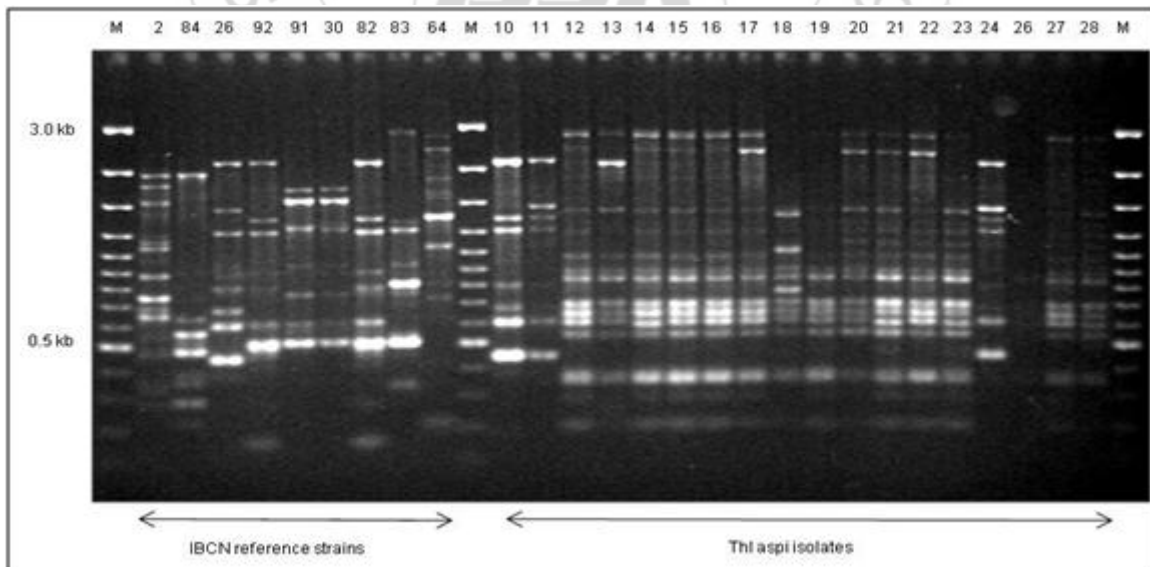


Figure 6: Band profiles of the Variable Number of Tandem Repeats (VNTR) locus generated from *Phoma lingam* isolates on 1.5% agarose gel. M, DNA marker.

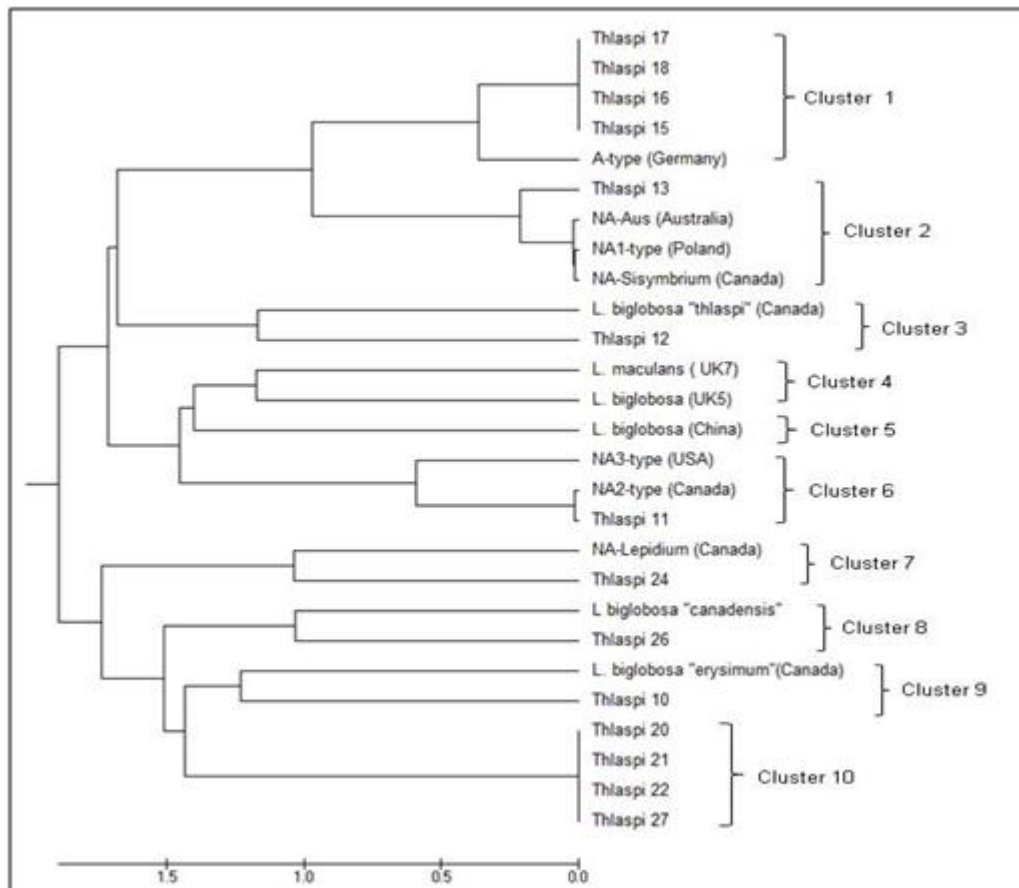


Figure 7: UPGMA distance tree shows genetic diversity of 'Thl' *Phoma lingam* isolates in comparison with reference strains. The dendrogram was created using ITS-rRNA gene sequences by MEGA6 software.

Populations from the two crop fields were found to be significantly diverse from each other. The 'Thl' isolates from barley fields could be divided into two distinct genotypes (cluster – 1 and 10). Four isolates (Thl-20, 21, 22 and 27) (cluster - 10) appeared to be distinct from all other 'Thl' and reference strains. None of the 'Thl' isolates were found to be related to a pathogenic *L. maculans* strain collected in UK, and two *L. biglobosa* strains from the UK and China [15]. Importantly, a set of four isolates from barley fields (Thl-15, 16, 17 and 18) showed a close relationship with an aggressive strain (IBCN 2) originating from *B. napus* in Germany (cluster – 1). This may be of concern if 'Thl' *P. lingam* population biology changes from non-pathogenic to pathogenic during their adaptation to global environmental changes [34]. The shift of non-aggressive *L. biglobosa* populations to an aggressive state, resulting in crop losses on different continents, has been reported [13, 35].

4. Conclusion

The morphological and biochemical characteristics, pathogenicity on *B. napus* cv. 'Lirabon' (susceptible to blackleg disease) and molecular analyses revealed that the *P. lingam* that infects *T. arvense* in Saskatchewan, Canada comprised of multiple genotypes and belonged to non/less pathogenic sexual state of *P. lingam*, namely *L. biglobosa*. Molecular fingerprinting and phylogenetic analysis of ITS sequences suggest the occurrence of significant genetic restructuring of the 'Thl' population, possibly due to the adverse effects of climate change and sexual recombination among 'Thl' and other strains co-existing in *T. arvense*

plants [36]. The dynamic nature of 'Thl' *P. lingam* populations implies further challenges in the development of disease management strategies, specifically, screening germplasm for introduction of varieties resistant to *L. biglobosa*. Although the life cycles of sexual states of the pathogen are similar and both may exist in a similar agroecological niche [35], isolates of *L. maculans* appear to be genetically stable and show gene-for-gene interactions with all their *Brassica* hosts (*B. napus*, *B. rapa*, *B. oleracea*, *B. juncea*, etc.). In contrast, such specialized interactions have not been found with isolates of *L. biglobosa* [13]. The pathogenicity-related genes of *P. lingam* vary significantly with host plant, and to new environmental conditions [34, 37]. Analysis of isolates at regular intervals on locally grown host plant cultivars is needed to understand their potential migration from a non-aggressive to an aggressive state.

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