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PHOMALIGIN A AND OTHER YELLOW PIGMENTS IN *PHOMA LINGAM* AND *P. WASABIAE*

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Key Word Index—*Leptosphaeria maculans*; *Phoma lingam*; *P. wasabiae*; fungi; blackleg disease; cyclohexadienones.

Abstract—The structure determination of a new yellow pigment obtained from weakly virulent isolates of *Phoma lingam*, the blackleg fungus, is described. Investigations of the secondary metabolite profile and the molecular genetic characteristics of weakly virulent isolates of *P. lingam* indicate it is closely related to *P. wasabiae*.

INTRODUCTION

The blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual state *Phoma lingam* (Tode ex Fr.) Desm.] causes widespread destruction among cruciferous crops and can be particularly devastating for rapeseed (*Brassica napus* L. and *B. campestris* L.) [1]. Crop losses caused by *P. lingam*, in Canada alone, exceed thirty million dollars annually. Three strains of the pathogen have been characterized on the basis of virulence, host range, and cultural tests: (i) a highly virulent strain causing leaf spots and severe stem cankers on rapeseed and cabbage (*B. oleracea* L.); (ii) a weakly virulent strain causing superficial leaf and stem lesions on rapeseed and cabbage; and (iii) a strain causing disease only on stinkweed (*Thlaspi arvense* L.) [2]. More recently, a general view has been expressed that virulent and weakly virulent isolates belong to different species [3–6]; however, no formal reclassification has been done. Varieties of spring rapeseed and canola (*B. napus* L. and *B. campestris* L.) commercially available for oilseed production in Canada are susceptible to both highly and weakly virulent strains of blackleg.

While virulent isolates of the fungus are readily recognized by their biosynthesis of epipolythiodioxopiperazines [7–10] and a depsipeptide [11], no characteristic metabolites appear to have been obtained from weakly virulent isolates. It is known, however, that weakly virulent isolates produce a water-soluble, reddish-brown pigment [12], whose chemical structure has not been reported. We have previously found that the broth extracts of weakly virulent isolates contained mainly lipids that are common to fungi and other living organisms; similar results were obtained for four different isolates of the *Thlaspi* strain [9]. Furthermore, in contrast with virulent isolates, the extracts of weakly virulent isolates were not toxic to *Brassica* species [9, 13].

In the course of screening mutants and transformants of *P. lingam* for the production of phytotoxins, an isolate producing a bright yellow broth was detected. Interestingly, we had observed that some weakly virulent isolates of *P. lingam* also produced yellow pigments sporadically. Because the chemical characterization of weakly virulent isolates might lead to the isolation of useful markers, it was of interest to examine the chemistry of those isolates producing yellow pigments. We have recently reported the chemical structures of phomaligols, phomaligadiones, and phomapyrones [14, 15] which were isolated from weakly virulent isolates producing yellow pigments. Now we describe the isolation and structure determination of three yellow metabolites (**1**, **5** and **6**) synthesized by weakly virulent isolates of *P. lingam*. To the best of our knowledge, **5**, which was named phomaligin A, has not been reported previously. Compounds **1** and **6** have been reported as metabolites of *P. wasabiae*, but the structure determination and spectroscopic data of **1** have not been described [16, 17]. Additionally, we present evidence that weakly virulent isolates of *P. lingam* are both chemically and genetically similar to *P. wasabiae*. Preliminary pathogenicity testing indicates that *P. wasabiae* and weakly virulent isolates of *P. lingam* cause similar disease symptoms on wasabi (*Eutrema wasabi* M.) plants. The implications of these results on grouping the weakly virulent isolates of *P. lingam* are discussed.

RESULTS AND DISCUSSION

Phoma lingam was grown in shake culture for five to seven days. Broth and mycelium were separated, freeze-dried, and extracted with ethyl acetate. Only the broth

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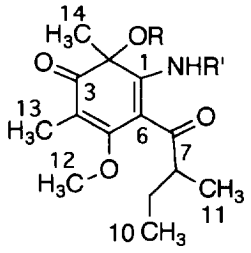
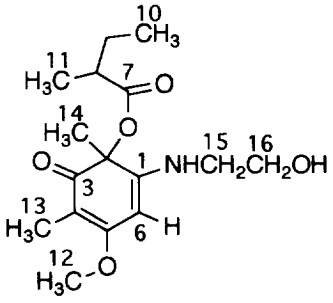


1 R = R '= H
2 R = CH_3; R' = H
3 R = R' = CH_3
4 R = Ac; R' = H
5 R = H; R' = CH_2CH_2OH



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extract showed yellow bands by TLC analysis. The weakly virulent isolates North Battleford-2 (NB-2) and Unity were initially investigated; however, the yield of yellow metabolites was rather low. Isolate FAN10B5, a derivative of the weakly virulent isolate Unity resistant to hygromycin, produced a bright yellow broth and whitish mycelium in Cove's medium [18]. As previously reported [15], the yields of the broth extracts of isolate FAN10B5 were higher than those of isolates NB-2 or Unity. Furthermore, TLC, HPLC, and ^{1}H NMR spectrometry of the fractionated extracts of isolate FAN10B5 indicated the presence of components similar to isolate NB-2 and Unity. For this reason, isolate FAN10B5 was the source of three metabolites: 1-amino-2,6-dimethyl-6-hydroxy-4-(2'-methyl-1-oxobutyl)-3-methoxy-2,4-cyclohexadien-1-one (**1**), 1-(amino-2'-hy-droxyethyl)-2,6-dimethyl-6-hydroxy-4-(2'-methyl-1-oxobutyl)-3-methoxy-2,4-cyclohexadien-1-one (**5**) and 1-(amino-2'-hydroxyethyl)-2,6-dimethyl-6-(2'-methyl-1-butyryloxy)-3-methoxy-2,4-cyclohexadien-1-one (**6**).

Fractionation of the broth extract by flash column chromatography (FCC) [19] followed by preparative TLC, led to the isolation of three yellow compounds, **1**, **5** and **6**. Compound **1** was the major yellow component of the extract and the least polar of the three metabolites. The CI-mass spectroscopy (NH_3 or *iso*-butane as carrier gas) of **1** revealed the base peak at *m/z* 268. Because the EI-mass spectrum showed a very weak peak for the likely molecular ion (*m/z* 267, < 1%) the HR-EI-mass spectroscopy, was carried out on the methyl (**2**) and acetyl (**4**) derivatives. From these data and the ^{13}C NMR spectrum, a molecular formula of $C_{14}H_{21}NO_4$ was deduced for **1**. The ^{1}H NMR spectrum of **1** (Table 1) showed only 19 hydrogens, which suggested the presence of at least two exchangeable protons. Furthermore, proton decoupling and D_2O exchange experiments indicated that **1** contained a *sec*-butyl group (9 hydrogens), in addition to two methyl groups (δ1.89 and 1.54), a methoxy group (δ3.76) and an exchangeable proton (δ4.03, *br s*). The proton decoupled ^{13}C NMR spectrum (Table 2) of **1** showed 14 resonances, four of which were attributable to carbonyl groups or equivalents (δ204.8, 200.1, 172.2 and 169.8) and one to an oxygenated quaternary carbon (δ76.2). Of

Table 1. ^{1}H NMR spectral data for **1–6** (in $CDCl_3$, 500 MHz)

H	1	2	3	4	5	6
6	—	—	—	—	—	5.06 *s*
8	3.19 *ddq* (7.0, 6.8, 6.8)	3.20 *ddq* (7.0, 6.8, 6.8)	3.17 *ddq* (7.0, 6.8, 6.8)	3.24 *ddq* (7.0, 6.8, 6.8)	3.18 *ddq* (7.0, 6.8, 6.8)	2.46 *ddq* (7.0, 6.8, 6.8)
9	1.59 *m* 1.30 *m*	1.60 *m* 1.37 *m*	1.61 *m* 1.36 *m*	1.62 *m* 1.34 *m*	1.65 *m* 1.33 *m*	1.71 *m* 1.44 *m*
10	0.79 *t* (7.5)	0.79 *t* (7.5)	0.81 *t* (7.5)	0.81 *t* (7.5)	0.83 *t* (7.5)	0.92 *t* (7.5)
11	1.09 *d* (6.8)	1.10 *d* (6.8)	1.07 *d* (6.8)	1.08 *d* (6.8)	1.07 *d* (6.8)	1.18 *d* (7.0)
12	3.76 *s*	3.72 *s*	3.69 *s*	3.71 *s*	3.73 *s*	3.85 *s*
13	1.89 *s*	1.86 *s*	1.84 *s*	1.86 *s*	1.86 *s*	1.74 *s*
14	1.54 *s*	1.50 *s*	1.54 *s*	1.55 *s*	1.56 *s*	1.51 *s*
15	—	—	—	—	4.25 *m* 3.70 *m*	3.23 *m*
16	—	—	—	—	3.84 *m* 3.78 *m*	3.79 *m*
Others	4.04 *br s*	3.14 *s* (OMe)	3.11 *s* (OMe) 3.30*d* (5.5) (NMe)	2.14 *s* (Me)	4.65 *br s* 2.25 *br s*	4.95 *br t*

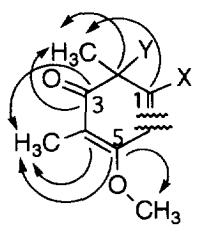
Coupling constants (*J* in Hz) in parentheses.

Table 2. ^{13}C NMR spectral data for **1–6** (in $CDCl_3$, 125.8 MHz)

C	1	2	3	4	5	6
1	169.8 *s*	166.3 *s*	167.2 *s*	165.7 *s*	168.6 *s*	160.8 *s*
2	76.2 *s*	81.9 *s*	82.0 *s*	79.1 *s*	76.2 *s*	78.6 *s*
3	200.1 *s*	196.9 *s*	196.2 *s*	194.2 *s*	198.4 *s*	192.8 *s*
4	107.1 *s*	110.8 *s*	109.6 *s*	110.3 *s*	105.3 *s*	101.9 *s*
5	172.2 *s*	170.4 *s*	172.1 *s*	170.1 *s*	174.1 *s*	171.2 *s*
6	99.3 *s*	101.8 *s*	101.0 *s*	100.8 *s*	99.0 *s*	79.6 *d*
7	204.8 *s*	205.2 *s*	204.7 *s*	205.8 *s*	205.0 *s*	175.0 *s*
8	45.2 *d*	45.3 *d*	45.5 *d*	45.2 *d*	45.6 *d*	40.4 *d*
9	27.9 *t*	28.2 *t*	28.1 *t*	27.9 *t*	27.6 *t*	26.4 *t*
10	11.8 *q*	11.7 *q*	11.9 *q*	11.6 *q*	12.1 *q*	11.7 *q*
11	16.9 *q*	16.9 *q*	17.6 *q*	16.8 *q*	17.9 *q*	16.5 *q*
12	60.3 *q*	60.2 *q*	60.4 *q*	60.2 *q*	60.6 *q*	55.6 *q*
13	8.8 *q*	8.6 *q*	8.6 *q*	8.6 *q*	8.9 *q*	7.2 *q*
14	34.9 *q*	31.9 *q*	26.3 *q*	27.6 *q*	31.6 *q*	27.9 *q*
15	—	—	—	—	47.6 *t*	44.5 *t*
16	—	—	—	—	62.0 *t*	59.8 *t*
Others	—	54.6 *q* (OMe)	54.0 *q* (OMe) 32.0 *q* (NMe)	168.8 *q* 20.5 *q* (Acetyl)	—	—

the remaining nine carbons, seven corroborated the presence of a methoxy, a *sec*-butyl, and two methyl groups, as indicated in the ^1H NMR spectrum. The two remaining carbons (δ107.1 and 99.3) could not be conclusively assigned. Finally, the structure of **1** was unambiguously assigned on the basis of its HMBC [20] and HMQC [21] spectral data, as well as spectroscopic data obtained for derivatives **2** and **3**, as described below.

Firstly, analysis of the HMBC spectrum of **1** indicated the two substructures **7** and **8**. Substructure **8** contained the lowest filed carbonyl carbon (δ204.8, C-7), which was connected with the *sec*-butyl group, indicated by its long-range correlations with the protons at δ3.19, 1.59 and 1.30, and 1.09 (H-8, H-9 and H-11, respectively). Substructure **7** contained the carbonyl carbon at δ200.0 (C-3) which showed long-range coupling with the methyl groups at δ1.89 (H_3-13) and 1.54 (H_3-14). The latter methyl group (δ1.54) showed further long-range coupling with the carbons at δ76.2 (C-2) and 169.8 (C-1). Additionally, the carbons at δ107.1 (C-4) and at 172.2 (C-5) displayed long-range coupling with the methyl group at δ1.89 (H_3-13). Since the carbon at δ172.2 (C-5) showed further long-range coupling with the methoxy group at δ3.76 (H_3-12) it could be assigned to C-5. In this way the partial structural units **7** and **8** were established. The last carbon remaining to be assigned (C-6, 99.3) had to join C-5 and C-1 (no correlations observed to any protons in the ring), completing a cyclohexadienone. Thus, the number of unsaturations predicted by the molecular formula (i.e. five) was fulfilled. It remained to determine the location of the 1'-oxo-2' methylbutyl, hydroxyl and amino groups. Chemical shift considerations suggested that the hydroxyl group should be attached to C-2 and the amino group to C-1 and, therefore, the 1'-oxo-2'-methylbutyl was attached to C-6. These assignments were unequivocally demonstrated by the HMBC spectra of the monomethyl (**2**) and dimethyl (**3**) derivatives of **1**. Methylation of **1** caused a downfield shift of C-2 and an upfield shift of C-3 in both **2** and **3**. More importantly, the correlations of the new methoxy groups (δ3.14 in **2** and 3.15 in **3**) with the carbons C-2 (δ81.9 in **2**, and 82.0 in **3**) proved the initial assignment. Furthermore, the HMBC spectrum of the dimethyl derivative **3** showed, as expected, an additional correlation of the *N*-methyl group with the carbon at δ167.2 (C-1), thus corroborating the attachment of the amino group to C-1. In this way the structure was established as the cyclohexadienedione **1**.



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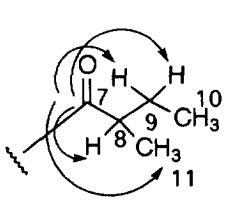

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Compound **5**, also a bright yellow compound, which we named phomaligin A, was produced in much smaller amounts than **1**. Both the ^1H and ^{13}C NMR spectra (Tables 1 and 2, respectively) clearly indicated that the structures of metabolites **1** and **5** were closely related. The HR-EI-mass spectrum of **5** indicated a molecular formula of $C_{16}H_{25}NO_5$, i.e. an additional C_2H_4O unit in relation to **1**. The spin system of the additional four hydrogens of **5** could be clearly assigned to a hydroxyethyl group, after D_2O exchange and ^1H NMR decoupling experiments. This assignment was confirmed by the ^{13}C NMR spectrum, which revealed two additional methylene carbons

at δ47.5 (C-15) and 62.0 (C-16). Analysis of the HMBC and HMQC spectra of phomaligin A (**5**) further confirmed these assignments and established that the hydroxyethyl group was located at the nitrogen. This assignment was deduced from the correlation observed between C-1 (δ168.6) and both H_2-15 (δ4.25, *m* and 3.70, *m*). Additionally, all of the carbon–proton correlations observed in the HMBC and HMQC spectra of **1** were present in those of **5**. The relevant correlations of the HMBC spectrum are summarized in Fig. 1. In this way the structure of phomaligin A was assigned as the cyclohexadienedione **5**, i.e. the N–CH_2CH_2OH derivatives of **1**.

Compound **6** was an orangish yellow compound, isomeric ($C_{16}H_{25}NO_5$) with phomaligin A (**5**). Comparison of the NMR spectral features of both compounds (Tables 1 and 2), indicated that **6** also contained a cyclohexadienone ring, a *sec*-butyl group (methine proton at higher field, δ2.46 in **6** vs 3.18 in **5**), two methyl groups (singlets), a methoxy group, and a N–CH_2CH_2OH group. An additional methine singlet (δ5.06) and a carbonyl group at much higher field (δ175.0 in **6** vs 205.0 in **5**) suggested that the *sec*-butyl group of **6** was attached to a different carbon. From the correlations revealed in the HMQC and HMBC spectra of **6**, the methine proton at δ5.06 could be assigned to H-6, i.e. one-bond coupling with C-6 (δ79.6) and additional coupling with C-1, C-2, C-4 and C-5. Similar to metabolite **5**, the *N*-hydroxyethyl group of **6** was attached to C-1, i.e. a correlation between C-1 and both H_2-15 was observed. Unlike **5**, the protons of the *sec*-butyl group (i.e. H-8, H-9 and H-11) were correlated with an ester carbonyl group at δ175.0 (C-7). The remaining carbon–proton correlations of the HMBC spectrum indicated the location of the methyl groups H_3-12 (OMe), H_3-13 and H_3-14, and carbonyl C-3. Finally, from these assignments it followed that the group O=C(O)CH(Me)CH_2Me was attached to C-2 (i.e. no other carbons available). The structure of this compound is, therefore, the cyclohexadienone **6**. The rather low chemical shift of C-6 may reflect the electron donating effect of the NH-group.

Fungal metabolites similar to both **1** and **6** had been isolated previously. The stereoisomer 2-(*R*), 8-(*S*) of **1**, aspersitin, was isolated from *Aspergillus parasiticus* [22]. The epimer 2-(*R*), 8-(*R*) of aspersitin, wasabidienone B, was later on isolated from *P. wasabiae* [16]. Both structures were determined by X-ray analysis. Additionally,

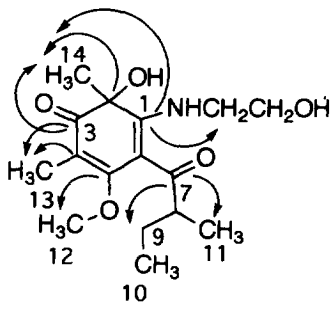

Fig. 1 Selected HMBC correlations for phomaligin A (**5**).

the structure **6** was assigned to wasabidienone E [17] and its stereochemistry was presumed to be identical to wasabidienone B [2-(*R*), 8-(*R*)]. No spectroscopic data has been published for wasabidienone B, and the spectroscopic data of aspersitin is similar, but not identical, to that obtained for **1**. However, the optical rotations that we obtained for **1** and **6** were considerably different (cf. Experimental) from those reported for either aspersitin ($[\alpha]_D$ 285°, MeOH), wasabidienone B ($[\alpha]_D$ 349.5°, $CHCl_3$), or wasabidienone E ($[\alpha]_D$ 50°, $CHCl_3$). Because we were unable to obtain samples of these compounds for direct comparison with **1** and **6**, we isolated them from an isolate of *P. wasabiae* (CCRC) 35135). The metabolites **1** and **6** obtained from *P. wasabiae* were identical in all respects, including optical rotations, to the metabolites isolated from *P. lingam*. Metabolite **5** was detected in *P. wasabiae*, but, owing to its small amount was not isolated. Based on these results, the configurations of C-2 and C-8 of **1** must be identical to those reported for wasabidienone B, that is 2-(*R*), 8-(*R*). However, the absolute configurations of these carbons in **5**, although presumably identical to **1**, remain undetermined.

Following these results, molecular genetic characteristics of isolate CCRC 35135 of *P. wasabiae* and weakly virulent isolates of *P. lingam* were compared to clarify the relationship of both species. Previously, we reported that the 5.8s rDNA sequences of several *P. lingam* isolates were identical; however, there were major differences in both internal transcribed spacers 1 and 2 (ITS1 and ITS2) that correlated with the pathogenicity grouping [5]. We have now compared the ITS1, ITS2 and 5.8s rDNA sequence of *P. wasabiae* and *P. lingam*.

Direct sequencing of the polymerase chain reaction (PCR) product amplified from *P. wasabiae* DNA with primers ITS1 and ITS4 confirmed its identity to the 5.8s rDNA gene surrounded by ITS1 and ITS2. The sequence was deposited in GenBank (accession number U 19274). It was aligned with the sequences previously reported for four *P. lingam* isolates representing different pathogenicity groups [5]. The isolates NB2 and Unity are weakly virulent rapeseed isolates, Thlaspi 5 is virulent on the cruciferous weed *Thlaspi arvense*, and PHW 1276 is a highly virulent rapeseed isolate. The close relationship of all the isolates was revealed by the absolute identity of their 5.8s coding region. This result places *P. wasabiae* in the Leptosphaerias, although no sexual stage has been reported for this organism.

The ITS1 of *P. wasabiae* was 98.3% homologous to this ITS in NB2 (four base pairs difference) and Unity (three base pairs difference), 89.5% homologous to the ITS1 of Thlaspi 5 (17 base pairs difference) and only 69% homologous to this spacer in PHW 1276 (56 base pairs difference). The similarity among isolates in the ITS2 sequence was much higher. The ITS2 of *P. Wasabiae* was 99.4% homologous to the sequence in NB2 and Unity (one base pair difference), 94.4% homologous to Thlaspi 5 (eight base pairs difference) and 81.4% homologous to PHW 1276 (30 base pairs difference). The presence of only four base differences in the ITS sequences between *P. wasabiae* and either of the two weakly virulent rapeseed

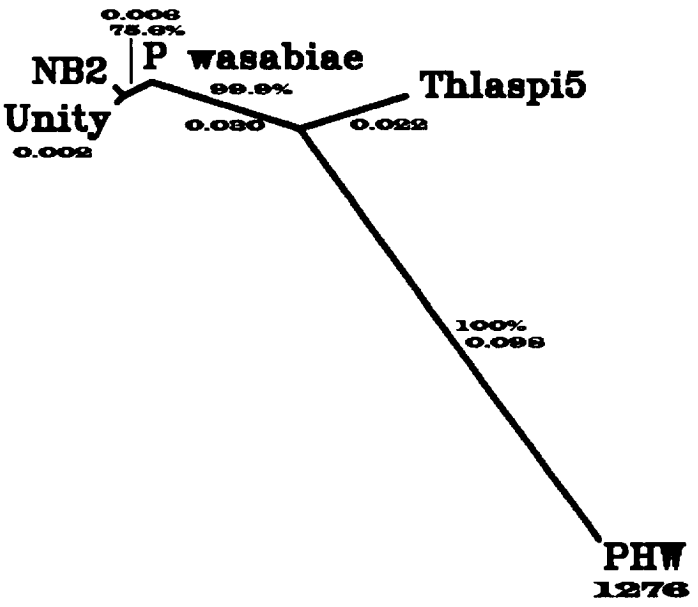

Fig. 2 Most parsimonious and best phylogenetic tree from maximum likelihood analysis obtained from the sequence alignments of *P. wasabiae* with the weakly virulent rapeseed isolates NB2 and Unity, stinkweed isolate Thlaspi 5 and highly virulent rapeseed isolate PHW 1276. The percentages represent the proportion of 1000 parsimony bootstrap replications in which the groups to the left were placed together. The 3-decimal numbers are branch lengths derived by the maximum-likelihood method and represent the expected number of base substitutions. This is an unrooted tree.

isolates indicates that they are the most closely related of the organisms examined.

The result of the phylogenetic analysis of the sequence alignments is illustrated in Fig. 2. The unrooted tree presented is the most parsimonious and also the best tree as determined by the maximum likelihood method [23]. The bootstrapped DNA parsimony analysis [24] placed *P. wasabiae* with NB2 and Unity in 99.9% of the replications. The maximum likelihood method places *P. wasabiae* at a very short genetic distance from NB2 and Unity. Two more base substitutions were present in the *P. wasabiae* sequence than were found in the sequences of the weakly virulent rapeseed isolates and this led to the separation of the isolates in the phylogenetic tree. There is little information on intraspecific variation in ITS sequence filamentous fungi. However, we have found four base differences among highly virulent rapeseed isolates [5]. It is, therefore, likely that *P. wasabiae* and weakly virulent isolates of *P. lingam* are members of the same species.

The above conclusions are also supported by the electrophoretic karyotype of *P. wasabiae* obtained by pulse-field gel electrophoresis (PFGE) (see Fig. 3). Weakly virulent rapeseed isolates have chromosome sizes ranging from 0.7 to 2.7 megabases (Mb) with the greatest concentration of chromosomes in the size range 0.7–2.0 Mb, Thlaspi isolates show a more even distribution of chromosome sizes ranging from 0.7 to 3.2 Mb, and the highly virulent rapeseed isolates have the largest chromosomes ranging from 0.7 to 3.7 Mb, concentrated in the upper molecular size range [6]. The karyotype of the examined *P. wasabiae* isolate is very similar to that of the adjacent weakly virulent isolates NB2, Outlook 2 and Unity. *Phoma wasabiae* has approximately 13 well separated chromosomes ranging in size from 0.7 to 2.5 Mb. In contrast, the karyotypes of WR5 and Leroy (virulent) and Thlaspi 5 isolates show heavy staining due to unresolved chromosomes in a higher molecular size range.

We have examined over 60 different isolates of *P. lingam*, either virulent (highly) or weakly virulent and established that the virulent isolates consistently synthesize characteristic epipolythiodioxopiperazines in liquid cultures (Pedras, M. S. C., unpublished results). We have now determined that weakly virulent isolates of *P. lingam* also synthesize characteristic metabolites. Perhaps the rather specific culture conditions necessary for their biosynthesis can explain the early failure in uncovering these metabolites.

Phoma wasabiae is the causative agent of blackleg disease in wasabi or Japanese horseradish (*Eutrema wasabiae* M.) [25], which is, like rapeseed and canola, part of the cruciferous family. Preliminary pathogenicity testing on Japanese horseradish employing weakly virulent isolate Unity (*P. lingam*), highly virulent isolate Leroy (*P. lingam*), and isolate CCRC 35135 (*P. wasabiae*) indicated that the foliar disease symptoms are similar in plants inoculated with either isolate CCRC 35135 or isolate Unity, whereas isolate Leroy caused a hypersensitive response. On the other hand, toxicity tests conducted on wasabi and canola leafs with **1** and crude culture extracts of *P. wasabiae* indicated that **1** is phytotoxic on both wasabi and canola leaves.

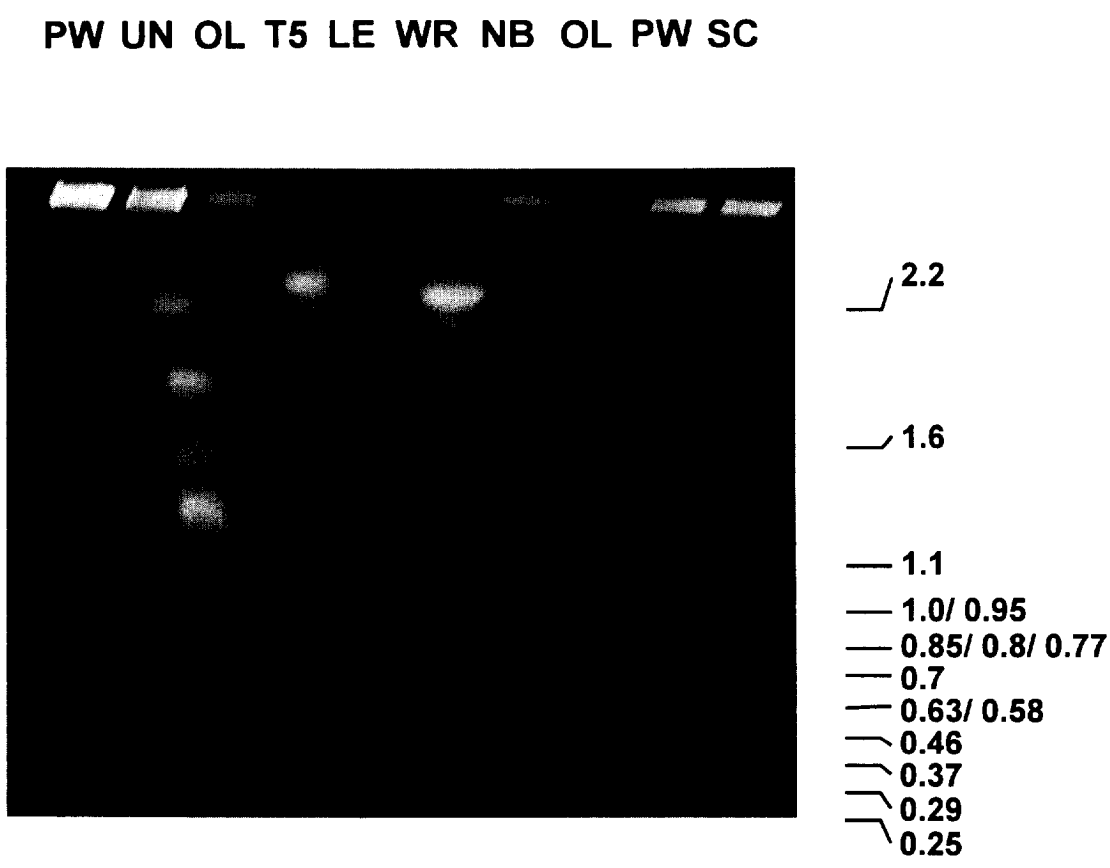

Fig. 3 Pulse-field gel electrophoresis of chromosomes from isolates of *P. wasabiae* and *P. lingam*. SC, *Saccharomyces cerevisiae*; PW, *P. wasabiae*; OL, Outlook 2; NB, North Battleford 2; WR, WR5; LE, Leroy; T5, Thlaspi 5; U, Unity. The sizes of the chromosomes are given on the right in megabases.

Our results indicate a very close relationship between the weakly virulent isolates of *P. lingam* and *P. wasabiae*. It should now be possible to take further steps to formally reclassify the weakly virulent isolates of *P. lingam* as a different species.

EXPERIMENTAL

General. All solvents were HPLC grade and used as such. Prep. TLC: (Kieselgel 60 F_{254}), 20 × 20 cm × 0.25 mm; analytical TLC (Kieselgel 60, F_{254}, aluminum sheets) 5 × 2 cm × 0.2 mm; compounds were visualized by exposure to UV and by dipping the plates in a 5% aq. (w/v) phosphomolybdic acid soln containing a trace of ceric sulphate and 4% (v/v) H_2SO_4, followed by heating at 150°. Flash CC: silica gel, grade 60, mesh size 230–400, 60 Å. NMR spectra: ^1H (500 MHz), δ values were referenced to $CHCl_3$ (7.24 ppm) and for ^{13}C (125.8 MHz) referenced to $CDCl_3$ (77.0 ppm). Mass spectra were obtained with a solids probe.

Fungal isolates and culture conditions. Fungal isolates of *Phoma lingam* were obtained from G. A. Petrie and R. K. Gugel, Agriculture Canada Research Station, Saskatoon, Saskatchewan. An isolate of *P. wasabiae* (CCRC 35135), isolated from *Eutrema wasabi* was obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Taiwan. Fungai isolates were maintained in Petri dish cultures in V8 medium [26], or, for long storage in autoclaved barley seed cultures at 4°. The isolate FAN10B5 was obtained as previously reported [15]. Cultures were inoculated and grown in the usual way [15].

Pathogenicity testing. The pathogenicity of the highly virulent rapeseed isolate Leroy, the weakly virulent isolate Unity and the isolate CCRC 35135 of *P. wasabiae* was tested on 4 fully expanded leaves of *E. wasabi* plants following published protocol [27].

Phytotoxicity bioassay. Wasabi (susceptible to *P. wasabiae*) and canola (susceptible to *P. lingam*) plants were grown under controlled environmental conditions [9]. The phytotoxicity of broth extracts of cultures of *P. wasabiae* (2 mg ml^{-1}, in 50% aq. MeOH) and **1** (1 and 2 mg ml^{-1}, in 50% aq. MeOH) was tested on young leaves of 3–5-month-old wasabi plants and on 3-week-old rapeseed plants as previously described [9]. Both the extract and **1** (2 mg ml^{-1}) caused lesions on both plants of variable size and irregular shape.

Sequence analysis of the 5.8s rDNA and transcribed spacers 1 & 2. The fungal culture conditions, DNA prepn and polymerase chain reaction (PCR) amplification reactions were as described [5]. The PCR pro-

ducts from the amplification of *P. wasabiae* DNA were directly sequenced with the primers ITS1, ITS2, ITS3 and ITS4 using the Taq Dye-deoxy terminator system [28]. The DNA sequences were aligned by the PILEUP program of the GCG package [29]. Phylogenetic analysis of DNA sequences was performed with programs in the PHYLIP package, version 3.5 [30]. The global rearrangement option was invoked and PHW1276 was used as the outgroup. Trees were constructed using the programs DNAML (maximum likelihood method) [23] and DNABOOT (bootstrapped DNA parsimony) [24].

Pulse-field gel electrophoresis (PFGE) of fungal chromosomes. The culture conditions, PFGE sample prepn, and electrophoresis conditions were essentially as described [6] with the following modifications. For sample prepn of *P. wasabiae*, spores were incubated in 0.8 M NaCl containing lysing enzymes (25 mg ml^{-1}), Driselase (5 mg ml^{-1}), β-glucoronidase (16 mg ml^{-1}), chitinase (0.25 mg ml^{-1}, and BSA (10 mg ml^{-1}). The PFGE was performed in a CHEF mapper employing 4 blocks with 2 states each in the multi-state mode. The blocks consisted of the following electrophoretic conditions: Block 1, run time (RT) = 100 hr; voltage (V) = 1.8 V cm^{-1} angle (A) = state 1 + 60°, state 2 − 60°; initial pulse time (IT) = 300 sec; final pulse time (FT) = 825 sec, linear ramp (LR); Block 2, RT = 41 hr; V = 1.4 V cm^{-1}; A = + 60°, − 60°; IT = 825 sec; FT = 1180 sec, LR; Block 3, RT = 37 hr; V = 1.1 V cm^{-1}; A = + 60°, − 60°; IT = 1180 sec; FT = 1500 sec, LR; Block 4, RT = 18 hr; V = 1.1 V cm^{-1}; A = + 60°, − 60°; IT = 1500 sec; FT = 2700 sec, LR.

Extraction and isolation of metabolites. The culture broth (4 l) was filtered (Whatman no. 2), freeze-dried, diluted with H_2O (200 ml) and extracted with EtOAc (4 × 200 ml). The dried (Na_2SO_4) EtOAc extract was evapd to dryness (450 mg), dissolved in CH_2Cl_2–MeOH (9:1), and adsorbed into silica gel (*ca* 10 ml). After evapn of the solvent, the extract in silica gel was applied to the prepd column (silica gel *ca* 200 ml) and subjected to flash CC (CH_2Cl_2–MeOH), 93:7). Ten frs of 50 ml were collected. Compound **1** was eluted in frs 2–3 (74 mg), phomaligin A (**5**) in fr. 4 (20 mg) and **6** in frs 5–8 (60 mg). Each one of the frs was further purified by prep. TLC using the solvent systems indicated for each compound: **1**, hexane–EtOAc (2:3, multiple elution), **5**, CH_2Cl_2–MeOH (19:1, double elution); **6**, CH_2Cl_2–MeOH (19:1, multiple elution), followed by further prep. TLC, hexane–EtOAc (1:4, multiple elution).

Compound **1**. Yellow oil (*ca* 15 mg l^{-1} of broth); R_f 0.60, hexane–EtOAc (1:1); $[\alpha]_D$ 416° ($CHCl_3$; *c* 1.04); $[\alpha]_D$ + 237° (MeOH; *c* 0.90); ^{1}H and ^{13}C NMR: Tables 1 and 2; UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 400 (3.60), 310 (3.95), 245 (3.90); FTIR ν_{max} cm^{-1}: 3405, 2964, 1537, 1462, 1275, 1222, 984; CI-MS: *m/z* (rel. intensity) 268 ([M + 1]$^+$, 100).

Phomaligin A (**5**). Yellow oil (*ca* 1 mg l^{-1} of broth) R_f 0.43, CH_2Cl_2–MeOH (19:1); $[\alpha]_D$ + 139° ($CHCl_3$; *c* 0.28); ^{1}H and ^{13}C NMR: Tables 1 and 2; UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 415 (3.65), 330 (3.83), 245 (3.93); FTIR ν_{max} cm^{-1}: 3341, 2927, 1741, 1628, 1500, 1229, 1151, 1070; HR-EI-MS: *m/z* obtained 311.1735, calcd for $C_{16}H_{25}NO_5$ 311.1733; CI-MS: *m/z* (rel. intensity) 312 ([M + 1]$^+$, 100).

Compound **6**. Yellow oil (*ca* 2 mg l^{-1} of broth) R_f 0.27, CH_2Cl_2–MeOH (19:1); $[\alpha]_D$ + 90.2° ($CHCl_3$; *c* 0.57); ^{1}H and ^{13}C NMR: Tables 1 and 2; UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 406 (3.60), 275 (3.69), 245 (3.93); FTIR ν_{max} cm^{-1}: 3372, 2962, 1591, 1536, 1453, 1339, 1224, 1142, 1074; HR-EI-MS: *m/z* obtained 311.1737, calcd for $C_{16}H_{25}NO_5$ 311.1733; CI-MS: *m/z* (rel. intensity) 312 ([M + 1]$^+$, 100).

Methylation of **1**. A solution of phomaligin A (9 mg) in MeI (2 ml) was treated with NaH (washed with hexane, *ca* 10 mg), at 24°. After 22 hr the reaction mixt. was diluted with EtOAc, filtered (cotton plug), and the solvent evapd. Prep. TLC (CH_2Cl_2–MeOH, 24:1, double elution) yielded **2** (3.2 mg) and **3** (2.2 mg).

Compound **2**. R_f 0.72, CH_2Cl_2–MeOH (24:1, double elution), R_f 0.20, hexane–EtOAc (1:1); ^{1}H and ^{13}C NMR: Tables 1 and 2; HR-EI-MS: *m/z* obtained 281.1650, calcd for $C_{15}H_{23}NO_4$ 281.1627; CI-MS: *m/z* (rel. intensity) 282 ([M + 1]$^+$, 100).

Compound **3**. R_f 0.80, CH_2Cl_2–MeOH (24:1, double elution), R_f 0.20, hexane–EtOAc (1:1); ^{1}H and ^{13}C NMR: Tables 1 and 2; EI-MS: *m/z* (relative intensity) 295 ([M]$^+$, 54), 280 ([M − 15]$^+$, 35), 262 (29), 252 (36), 236 (25); CI-MS: *m/z* (rel. intensity) 296 ([M + 1]$^+$, 100).

Acetylation of **1**. Compound **1** (7 mg) was acetylated with Ac_2O/DMAP, at room temp. for 20 hr, to yield, after prep. TLC (hexane–EtOAc, 1:1, double elution), **4** (6 mg).

Compound **4**. Obtained as small needle-like yellow crystals (CH_2Cl_2–hexane), mp 148–150°; R_f 0.30, hexane–EtOAc (1:1); $[\alpha]_D$ 183° ($CHCl_3$; *c* 0.67); ^{1}H and ^{13}C NMR: Tables 1 and 2; HR-EI-MS: *m/z* obtained 309.1571, calcd for $C_{16}H_{23}NO_5$ 309.1576; CI-MS: *m/z* (rel. intensity) 310 ([M + 1]$^+$, 100.

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