Health Testing and Evaluation of *Leptosphaeria maculans* (*Phoma lingam*) Contamination in *Brassica napus* Seed Samples from Poland and England

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Abstract: Mycological analysis and evaluation of *Leptosphaeria maculans* (*Phoma lingam*) contamination in *Brassica napus* seed samples from England and Poland were conducted. The results showed that 26% ~ 40% of the seeds were contaminated by fungi and bacteria with *Alternaria*, *Fusarium* and *Penicillium* as the most commonly recovered genera. *P. lingam* contamination in Polish and English seed samples were 0.5% and 0.6% respectively, of which 7.1% of Polish isolates and 12.3% of English ones were classified as Tox+ pathotype. Seed surface sterilization prior to seed health testing resulted in an increase of the detection *of P. lingam*.

Key words: *Leptosphaeria maculans* / *Phoma lingam*; seed health testing; pathotype
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Oilseed rape (*Brassica napus*) is an important economic crop in the world. Many pathogens can attack this crop and very often fungi are observed to be the most damaging group. More than ten fungal species are seed-borne in rape, of which extensive attentions are attached to blackleg or stem canker caused by the fungus *Phoma lingam* (Tode ex Fr.) Desm, sexual stage *Letosphaeria maculans* (Desm.) Ces. Et de Not.

This fungus is parasitic to wild and cultivated genera of Cruciferae, such as *Brassica* spp. (rutabaga, rape, turnip, cabbage etc.), *Sinapis alba*, *Raphanus* spp., *Thlaspi arvense* and *Camelina sativa*, and is present throughout the *Brassica*-growing areas of the world, but causing damage as a pathogen only in temperate regions or at high altitudes in the tropics with yield losses from 10% to 56%[1,2].

The fungus can be differentiated into two pathotypes, Tox0 and Tox+. Generally, Tox+ pathotype is strongly aggressive or virulent, whereas Tox0 pathotype is less aggressive or virulent[3]. In China, only Tox0 pathotype has been reported until now[4].

Seed-borne pathogen is found to be one of inoculum sources for an epidemic and is thought to be the major cause of the introduction of the disease into previously uninfected areas, or new strains into existing infected areas[5]. The growing seed importation and the active agency for international seed production due to its cheap labor will probably bring China a risk of aggressive strains. Seed health testing should be taken into consideration for this reason. The current work is the first try in China to evaluate the contamination of *Brassica napus* seeds from abroad (England and Poland), and classify the obtained *P. lingam* isolates with morphological and biochemical methods.

1 Materials and methods

1.1 Materials

Brassica napus seeds of two cultivars, Lipton (susceptible to *P. lingam*) and Capitol (resistant), were comprised in the experiments, which were kindly provided by Dr. Jonathan West from Department of Plant Pathology in Institute of Arable Crops Research, Rothamsted, England, and collected from the seed production field belonging to Institute of Plant Genetics at Poznań, Poland.

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1.2 Methods

1.2.1 Mycological analysis of seeds For mycological analysis, 10 000 seeds per cultivar were used for English seed sample and 6 000 for Polish seeds. The deep-freezing blotter method was used following the ISTA rules[6], with a minor modification. For each 9 cm Petri dish, 25 seeds were plated on two pieces of well-soaked paper (Whatman, 3 mm). After one day at 20 ℃ in the darkness for imbibition, the seeds were exposed to −20 ℃ for one day to inhibit seed germination and then were incubated at 20 ℃ for 10 days with alternating cycle of 12 hours of near ultraviolet light (NUV) and 12 hours darkness. The seeds were examined for presence of fungi based on their colony morphology and spore shapes by a stereomicroscope and a light microscope.

In another experiment, pretreated English seeds were compared with untreated ones to determine if other fungi hinder or obscure the growth of *Phoma* spp. For pretreatment, 5 000 seeds were surface-sterilized with a 1% (*w*) sodium hypochlorite (NaOCl) solution for 10 minutes before plating, then rinsed thoroughly with sterile distilled water.

1.2.2 Identification of *P. lingam* isolates One of pycnidia of *Phoma* spp. developed on seed surface or on the paper near the seed was picked out and the isolates were purified on PDA medium with 100 mg/L streptomycine sulfate. The final identification of *P. lingam* was made according to colony characters and the fruit body morphology[1]. The cleaned isolates were maintained on PDA medium at 25 ℃ in the darkness.

1.2.3 Classification of *P. lingam* isolates Classification of obtained isolates was made based on the growth properties on PDA medium and the pigmentation in liquid medium by comparison with two accompanying control strains, PL10 (Tox0) and PL56 (Tox+). An agar disc (5 mm diameter) overgrown with mycelium was placed in the center of a 9 cm Petri dish with 15 mL medium. Dishes were sealed with Parafilm and incubated at 25 ℃ in 12 hours dark/12 hours NUV light. The diameters of fungal colonies were measured every 2 days until the mycelium covered the whole dish. Meanwhile, the pycnidia production was recorded. Three agar discs were put into a 100 mL conical flask containing 50 mL of Czapek-Dox liquid medium supplemented with yeast extract (2 g/L). The pigment production was observed after 20 days at 25 ℃ in the darkness.

2 Results

2.1 Mycological analysis

Fifteen fungal genera were recovered from the studied seed samples, as listed in Table 1. Seed samples from England and Poland had similar contamination percentages in respect with every fungus species and bacteria. *Alternaria* spp. was the most common genus recovered, followed by *Fusarium* spp. and *Penicillium* spp. Species which did not sporulate within 14 days of incubation were not further studied. Unidentified fungal species were those that produced few spores only, so the identification was impossible at this developmental stage of the fungal colony.

Tab. 1 Contamination percentage of *Brassica napus* seed samples from England and Poland %

fungal species	seed sample from England		seed sample from Poland	
	cv. Lipton	cv. Capitol	cv. Lipton	cv. Capitol
Alternaria spp.	19.55	11.72	18.77	14.82
Fusarium spp.	5.53	4.84	6.43	4.55
Penicillium spp.	1.82	3.05	4.87	3.37
Phoma spp.	0.55	0.85	0.53	0.47
Cladosporium spp.	0.24	1.43	0.62	0.68
Aspergillus spp.	0.01	0.13	0.07	0.00
Botrytis cinerea	0.03	0.06	0.06	0.33
Mucor spp.	0.47	0.17	0.08	0.25
Epicocum spp.	0.31	0.25	0.04	0.30
Rhizopus spp.	0.04	0.02	0.01	0.00
Gonatobotrys simplex	0.00	0.23	0.00	0.00
Drechslera spp.	0.00	0.00	0.08	0.17
Sclerotinia sclerotionum	0.08	0.06	0.12	0.00
Stracoderma spp.	0.00	0.05	0.00	0.00
Verticillium spp.	0.00	0.00	0.00	0.00
unidentified fungal species	0.13	0.13	0.08	0.18
non-sporulating species	0.24	1.42	0.67	1.39
bacteria	10.21	1.15	1.23	1.72
total	39.21	25.56	33.66	28.23

Great attention was paid to *Phoma* spp., which was identified on the basis of the pycnidia characters. *Phoma* spp. occurred on seeds of the susceptible cultivar (Lipton) and the resistant one (Capitol) and no considerable difference between two cultivars could be deduced from the obtained data.

Additional tests were employed to separate *P. lingam* from other *Phoma* spp. by its colony characters

and the fruit body morphology on PDA medium[1]. As a result, 119 among 140 *Phoma* spp. isolates from English seed sample, and 56 among 70 from Polish seed sample were identified as *P. lingam*.

2.2 The detection of *Phoma* spp. after surface sterilisation

After surface sterilisation with NaOCl solution, fungal species were considerably reduced and only *Phoma* spp. and *Alternaria* spp. were observed on seeds.

In comparison with untreated seeds, an increase in percentage of *Phoma* spp. was found after pretreatment. The incidence of *Phoma* spp. was nearly two fold in the case of cv. Capitol and three fold for cv. Lipton, respectively 1.21% and 1.98%. This indicated that detection of *Phoma* spp. was partly masked by other fast developing fungi, resulting in an underestimation of infection percentage.

2.3 Classification of *P. lingam* isolates

Two distinct types of *P. lingam* isolates were observed on PDA medium, differing in colony morphology, sporulation and pigmentation.

For one type, the colonies developed rapidly (4.9～6.8 mm/d) and usually reached the dish edge within 14 days. The colonies were round with smooth edges. The mycelium was either white or yellow to brown coloured, loose aerial mycelium was produced and the sporulation ranged from negligible to abundant. Very often, some aerial mycelium became gradually orange or brown and visible droplets of brown ooze were produced from them. Yellow or brown pigment could be observed in the media from the bottom of the Petri dish. When the cultures were kept at 25 ℃ with an alternating NUV light, the production of ooze and pigment was absent, but abundant pycnidia were developed even in 5 days. The pigment production could be seen again when Petri dishes kept in NUV light were transferred into darkness for 4—6 days. By comparing with the Tox0 control and on the basis of the criteria in *P. lingam* research[1], all these isolates could be classified as Tox0 pathotype.

In contrast, the other type showed a slower growth (1.7—3.9 mm/day) and did not reach the edge even within 20 days. The colonies had an irregular margin with white or grey compact aerial mycelium. There was no ooze from mycelium and no pigment in media, but much more pycnidia than Tox0 colonies. The isolates had similar cultural properties in dark and in NUV light except that pycnidia developed a little earlier in NUV light. According to the growth characteristics, this type of isolates was attributed to Tox+ pathotype.

The pigmentation of *P. lingam* Tox+ and Tox0 cultures growing in liquid Czapek-Dox medium could be clearly differentiated. Tox0 isolates produced different amount of pigments varying in colour from yellow, red to dark brown, whereas Tox+ isolates remained colourless.

Combining the observation of growth properties and pigmentation, only 14 in 119 English *P. lingam* isolates and 4 in 56 Polish isolates were classified as Tox+ strains, accounting for 12.3% and 7.1%, respectively.

3 Discussion

Without a great deal of experience, it is difficult to differentiate *P. lingam* from other saprophytic pycnidia-forming fungi such as *Phoma glomerata* and *Phoma herbarum*. The variation in pycnidia shape, size, colour and ooze complicated the identification of isolates by the characters of pycnida. In our experiments, colony morphology and pigmentation were employed as assistance for further identification. This two-step method was time-consuming and could not be used to analyse large number of isolates. A simple, rapid and reliable method is needed and molecular technique probably provides a promising future.

Maguire et al[7] found that NaOCl pretreatment resulted in the highest *P. lingam* counts, which was confirmed once again in the experiment described herein. Pretreatment gets rid of fungal inocula carried on the seed surface and thus facilitates the development of latent pathogens in seed or under seed coat. Taking into consideration this factor, the risk of plant infection in the field is higher than that estimated by seed testing.

Although seed contamination with *P. lingam* was relatively low (less than 1%), one contaminated seed can become the source for epidemic due to fast spread of ascospores and conidia through air and water. In order to keep our country away from this destructive disease, plant quarantine and seed treatment should be strongly emphasised. As a fact, in some countries phytosanitary certificates showing the seed to be free from *P. lingam* have been obligatory for seed importation and the production of certified seed[8].

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波兰、英国油菜种子样本的健康测试及黑胫病病原菌感染分析

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摘要: 对来自波兰及英国的 2 个相同品种的油菜种子样品进行了健康测试, 并重点观察了油菜黑胫病病原菌(*Letosphaeria maculans*/ *Phoma lingam*)感染的程度及菌系类型. 结果表明来自 2 个国家的种子都一定程度受微生物感染, 品种间差异不大, 所检测到的较多的菌属为 *Alternaria*, *Fusarium* 及 *Penicillium*. 黑胫病病原菌感染的百分率波兰种子样品平均为 0.5%, 英国种子样品平均为 0.6%, 菌系鉴定表明, 7.1%的波兰株系及 12.3%的英国株系为强致病的 Tox+ 类型. 此外, 还发现健康测试前种子表面消毒可提高黑胫病病原菌的检出率.

关键词: 油菜黑胫病; 种子健康测试; 致病型

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