Black root rot caused by *Diaporthe sclerotioides* threatens cucurbit cultivation in Japan

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Abstract: In Japan, since black root rot of cucurbitaceous crops was found more than 30 years ago, the disease has caused severe economic losses to the cucurbit crop industry. Subsequent to the pathogen being correctly identified as *Diaporthe sclerotioides* based on morphology and DNA sequence, knowledge about DNA sequences has developed making technical tools to detect and quantify the pathogen in natural samples of plants and soils available. In addition to chemical soil disinfectants, solarization and biological soil disinfestation have been developed as environment-friendly methods to effectively control this disease. Although it is difficult to apply such temperature-dependent methods in open-fields especially under cool climate conditions, an alternative approach, which changes soil pH to weak alkaline with amending steel converter slag, has also proved effective against the disease. In this mini-review, the process of *D. sclerotioides* identification, detection and quantification methods developed for this fungus, host specificity, and disease control measures available and practiced in Japan are discussed concisely.

1. Introduction

Black root rot of cucurbitaceous crops causes severe root destruction leading to growth depression, non-vascular wilt and premature death of plants. The disease was originally described in gherkin (*Cucumis sativus*) roots in the Netherlands by Van Kesteren, who classified its causal agent as *Phomopsis sclerotioides* Kesteren (Van Kesteren, 1966). It has since spread to other European regions, including U.K., Germany, Denmark, Norway, France (Ebben and Last, 1973), and Italy (Cappelli *et al.*, 2004). In Japan, the disease was first reported in 1985 in squash root which was the rootstock of a cucumber (*Cucumis sativa*) (Hashimoto and Yoshino, 1985). Since then, the disease has also been found in melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), pumpkin (*Cucurbita maxima*), and other cucurbits, threatening the production of these major cucurbit crops with severe economic damage. Shishido *et al.* (2006) identified the causal agent of this disease as *P. sclerotioides* Kesteren through both morphological and phylogenetical analyses. Because of recent development in DNA sequence comparisons, asexual state of genus, *Phomopsis*, has connected to sexual state, *Diaporthe*, which has priority over *Phomopsis*, and should be the generic name adopted for these taxa (Udayanga *et al.,* 2012; Gomes *et al.*, 2013), and thus I use *Diaporthe sclerotioides* as the name of the pathogen of this disease in this article.

To control black root rot of cucurbits, solarization with a combination of soil fumigants such as chloropicrin has been widely applied in greenhouses, especially in warmer climate regions (Kobayashi *et al.,* 1997). However, the disease has been consistently spreading in cucumber production areas in northern parts of Japan, where climate conditions are not warm enough to apply such temperature-dependent measures. In addition, the disease has not yet been contained in the southern parts of Japan even though the incidence is sporadic and inconsistent (Shishido, 2006). The present paper offers a concise review of recent developments in knowledge about black root of cucurbit crops as well as control measures against this disease in Japan.

2. Taxonomy of *Diaporthe sclerotioides*

Morphology

The telemorphic stage of *D. sclerotioides* has not yet been discovered, while anomorphic stages of *Diaporthe* species are often characterized with specific pycinidia. *D. sclerotioides* forms pycinidium which are mostly subglobose or spherical under the epidermis (Van Kesteren, 1966). In our experiment, pycnidia were found submersed in sterilized beanpods after three weeks of incubation, mostly globose and varying in size (200-500 μm in diameter) (Fig. 1A). The internal cavity of a pycnidium was often divided by protrusions of the proliferous layer (Fig.

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1B). Numerous pycnospores (Fig. 1C), mostly ellipsoidal to ovoid, hyaline, 7-12 × 3-6 µm in size, usually with two guttules, were observed in the pycnidium (Fig. 1D) and apparently were produced on conidiogenous cells (Fig. 1E). Conidiophores filiform, hyaline, septate at the base, rarely branched, up to 25 µm, were formed from the inner cells of the locular walls. These only α-type conidiospores were unlike other *Diaporthe* species and no β-type were observed. Although we found that an isolate produced pycnidia and conidiospores, the event was very rare and no such organs have been reported in the natural environment. Therefore, we suspect that conidiospores are not the primary source of inoculum of this pathogen.

On the other hand, *D. sclerotioides* easily forms dull, grayish-brown mycelial mats on common agar media including potato dextrose agar (Shishido *et al.*, 2006). The mycelium consisted of thin hyaline hyphae (2-5 µm in diameter) and thick hyaline to dark brown hyphae (10-20 µm in diameter) (Fig. 1F). A layer of the thick hyphae and dark brown, thick-walled cells formed a small sclerotium (Fig. 1G), and later became superficial or submersed pseudo-sclerotia, mostly flattened, of various sizes (Fig. 1H). Because these pseudo-sclerotia are commonly observed on diseased roots as well, they are likely the primary inocula of black root rot of cucurbit crops.

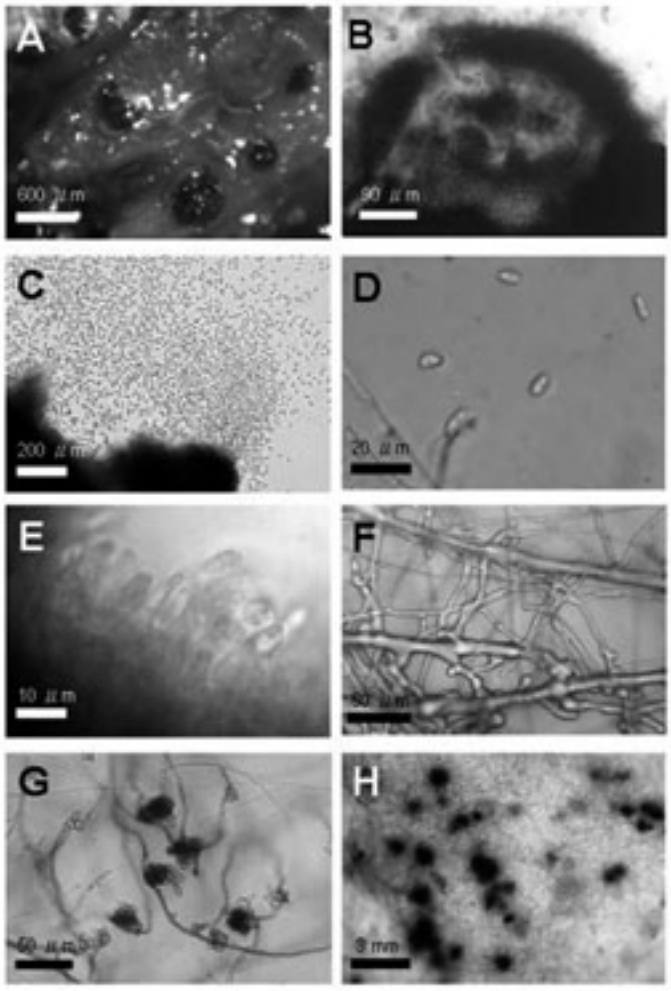

Fig. 1 - Typical features of *Diaporthe sclerotioides* grown under gnotobiotic conditions. A, Pycnidia on a sterilized beanpod. B, Vertical section of pycnidium. C, Numerous pycnospores mechanically released from pycnidium. D, Pycnospores. E, Pycnophores on the internal wall of pycnidium. F, Thick-walled, crenate aerial mycelium commonly found in various agar cultures. G, Masses of dark-colored mycelium, later become pseudomicrosclerotia. H, Small, brownish-black pseudomicrosclerotia on a boiled cucumber leaf. Reprinted from reference Shishido *et al.* (2006) with permission from the publisher.

Molecular phylogeny

Phylogenic analyses of the genus *Diaporthe* have been conducted in a number of studies (Rehner and Uechker, 1994; Zhang *et al.*, 1998; Kanematsu *et al.,* 2000; Farr *et al.*, 2002; Murali *et al.,* 2006; Shishido *et al.*, 2006; Santos *et al.,* 2010; Udayanga *et al.*, 2012; Gomes *et al.,* 2013). DNA sequences of the ITS regions of *D. sclerotioides* isolated from various parts of Japan formed a single distinct clade without differing from the ex-type *D. sclerotioides* (CBS 296.67) by even a single nucleotide (Shishido *et al.,* 2006). The close kin species of *D. sclerotioides* were *D. columnaris* (Farr *et al.,* 2002) and *D. strumella* var. *longispora* (Udayanga *et al.,* 2012). These species differ 11 and 26 bases of the DNA sequences in their ITS regions out of 329 bases in total (ITS 1 and 2). The relationships detected with the DNA sequences of the ITS region were also found in other loci including elongation factor 1-α (Udayanga *et al.,* 2012) and a mating type gene, *MAT1-1-1* (Santos *et al.*, 2010).

Interestingly, we found no host-specific DNA sequences within the ITS regions among the Japanese isolates of *D. sclerotioides* that originated from four different host species: melon, watermelon grafted on bottlegourd, pumpkin, and cucumber. Rehner and Uecker (1994) argued that the host-based species concept was not reliable for *Diaporthe* because of the genetic diversity among isolates of this genus from various hosts. Kanematsu *et al.* (2000) supported this hypothesis by demonstrating that the phenotypic divergence of *Phomopsis* species, i.e. W type (mainly white colonies, weakly virulent, bearing both α- and β-type conidia) and G type (mainly gray colonies, highly virulent, bearing only α-type conidia) was dependent on their ITS sequences rather than on the host species. Since the Japanese *D. sclerotioides* isolates of black root rot are all G type, it may not be surprising that these isolates create a single clade in the phylogenetic tree.

3. Methods for detection and quantification of *Diaporthe sclerotioides*

Plant pathogenic fungi are usually detected by their growth on selective media or by biochemical, chemical, and immunological analyses. However, none of these conventional techniques are available for *D. sclerotioides*. Moreover, morphological identification of these fungi on nonselective media is time-consuming and requires expert knowledge of classical taxonomy. In recent years, PCR-

based molecular techniques have contributed to the detection and identification of various types of plant pathogens. Real-time PCR techniques combine the sensitivity of conventional PCR with the generation of a specific fluorescent signal. This signal can be measured throughout the procedure, providing real-time analysis of the reaction kinetics, and thereby allowing for quantification of specific DNA targets (Schena *et al.*, 2004).

Shishido *et al.* (2010) constructed conventional and real-time PCR primers to detect *D. sclerotioides*, utilizing a DNA sequence in the ITS region specific to this fungus. The designed primers, CPs-1 (forward) and CPs-2 (reverse), successfully detected the fungus in soil and plant samples collected from fields naturally infested with the disease. Furthermore, the CPs-1/CPs-2 primer pair was applied to a real-time PCR assay with SYBR Green I, and the protocol achieved a linear response with a high correlation coefficient between input DNA and cycle threshold. However, because SYBR Green I is a DNA-intercalating dye without sequence specificity (Wittwer *et al.,* 1997), and because DNA in natural samples usually contains unknown sequences, the amount of DNA will only be accurate if no presence of other DNA similar to this fungus is confirmed in the sample. This problem was solved using a TaqMan probe-based real-time PCR assay, which is highly specific, sensitive and quantitative (Shishido *et al.,* 2013). In addition, the TaqMan probe-based protocol allows multiplex real-time PCR, and thus using internal standard DNA such as GFP for soil samples (Klerks *et al.,* 2004) and COX for plant samples (Weller *et al.,* 2000), quantification of the fungal DNA should be more accurate than the mono-plex counterparts. Table 1 summarizes primer sequences that can be used for detecting and quantifying *D. sclerotioides* DNA in natural samples.

4. Host range and specificity of *Diaporthe sclerotioides*

In general, it is important in breeding programs to know if there are specific interactions between pathogenic microorganisms and host species. Although *D. sclerotioides* can cause black root rot only in cucurbit species, until recently little has been known about the degrees of either the host susceptibility to this disease or host specificity of the pathogen. Shishido *et al.* (2014) hypothesized that *D. sclerotioides* isolates were more infective and virulent to the cucurbit species from which the pathogens were originally isolated than to other host species. They conducted cross-inoculation experiments using cucumbers, melons, pumpkins, watermelons, and bottlegourd (*Lagenaria siceraria* var. *gourda*), by inoculating 12 *D. sclerotioides* isolates from these cucurbit species. The virulence of the isolates was evaluated as the area under the disease progress curve (AUDPC). All cucurbit species were susceptible to each isolate, but AUDPCs were significantly different among the hosts as melon > cucumber ≥ watermelon ≥ bottlegourd ≥ pumpkin.

The infectiveness of isolates, on the other hand, was assessed as the quantity of *D. sclerotioides* DNA detected in the hypocotyls of seedlings two weeks after inoculation using the TaqMan-based real-time PCR protocol described above. The fungal DNA quantities varied among the species in the same order as the AUDPCs. Orthogonal contrasts indicated no specificity in either the fungal virulence or infectiveness between *D. sclerotioides* isolates and the cucurbit hosts from which these isolates originated (Fig. 2). Based on these results, the original hypothesis was refuted and they concluded that though host susceptibility to black root rot varies among cucurbit species, *D. sclerotioides* isolates are unlikely to have specificity to the host

Table 1 - PCR protocols for detecting and quantifying *Diaporthe sclerotioides* in natural samples

Detection of *D. sclerotioides* (Shishido *et al*., 2010)	
CPs-1 (forward) CPs-2 (reverse)	5´-GCCTCGGCGCAGGCCGGCCTCACC-3´ 5´-GGGGCCTTCCAGAACGAAATATAATTT-3´
Note: Not recommended for real-time PCR, Expected amplicon size: 392 bp	
Detection and quantification of *D. sclerotioides* (Shishido *et al*., 2013)	
CPs2f (forward) CPs2r1 (reverse) CPs2t (probe)	5´-ACTGCTTGGTGTTGGGGCACC-3´ 5´-TCCAGAACGAAATATAATTTACTACGCT-3´ 5´- [FAM]-AAAGGGCGGGCCCTGAAATCTAGTGGCGA-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe, Expected amplicon size: 101 bp	
Internal standard of soil samples (Klerks *et al*., 2004)	
FPGFP (forward) RPGFP (reverse) PYYGFP (probe)	5´-TGGCCCTGTCCTTTTACCAG-3´ 5´-TTTTCGTTGGGATCTTTCGAA-3´ 5´- [VIC]-AACCATTACCTGTCCACACAATCTGCCC-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe as an external standard.	
Internal standard of plant samples (Weller *et al*., 2000)	
COX-F (forward) COX-R (reverse) COX-P (probe)	5´-CGTCGCATTCCAGATTATCCA-3´ 5´-CAACTACGGATATATAAGAGCCAAAACTG-3´ 5´- [VIC]-AGGGCATTCCATCCAGCGTAAGCA-[TAMRA] -3´
Note: Applicable with SYBR Green I instead of TaqMan probe as an external standard.	

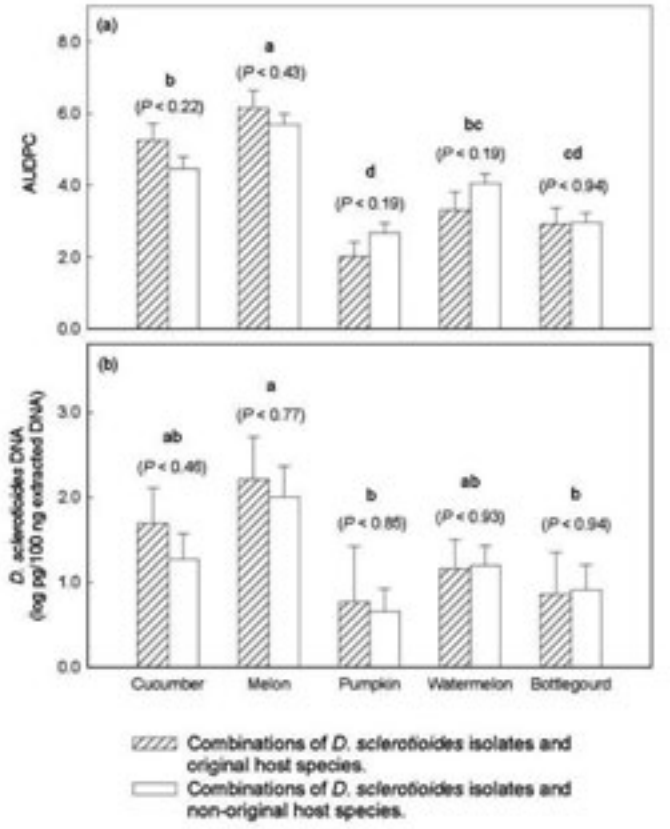

Fig. 2 - The area under the disease progress curve (AUDPC) for black root rot in cucumber, melon, pumpkin, watermelon, and bottlegourd plants (a), and the quantity of *Diaporthe sclerotioides* DNA detected in the hypocotyls of these plants (b) after root inoculation with 12 isolates of *D. sclerotioides*. In both charts, orthogonal contrasts were tested between original host-fungal isolate combinations and non-original host-fungal isolate combinations with *P* values showing type I error probabilities of null hypotheses. The same letters indicate no significant difference between plant species as determined by Tukey's HSD test ($P < 0.05$). Error bars denote the standard error of the mean. Reprinted from reference Shishido *et al.* (2014) with permission from the publisher.

species in terms of either virulence or infectiveness. This research implies that in practice seedlings and soils from infested areas should be handled carefully because the pathogen may spread to various cucurbit species irrespective of the original host.

Interestingly, the relationship between the AUDPC and *D. sclerotioides* DNA quantity in hypocotyls provided evidence that the virulence of this pathogen was not highly correlated with its infectiveness, although some degree of fungal invasion was obviously required for disease development (Fig. 3). The correlation analyses also showed that, although for most of the cucurbit species there were statistically significant correlations between AUDPCs and *D. sclerotioides* DNA quantities in the hypocotyls, their relatively low coefficients of determination indicated limited associations for these variables, therefore suggesting that the virulence of *D. sclerotioides* may be due to factors in addition to infectiveness.

5. Control measures against black root rot of cucurbit crops

Van Kesteren (1966) indicated that *Cucurbita ficifolia* was tolerant to the disease despite apparent root infection by *D. sclerotioides*. However, no true resistant variety or *R*-genes as such has been reported to black root rot. It is possible to use *C. ficifolia* for rootstocks of cucumber, and in fact it used to be a common practice in cucumber production in Japan. Unfortunately, the rootstock of *C. ficifolia* causes a fine white powder on the surface of the cucumber fruits, called "bloom", primarily composed of silica (Mitani *et al.*, 2011). In the late 1980s, cucumber without any bloom (bloomless cucumber) became popular in Japan because of its more attractive and distinctly shiny appearance; and thus *C. ficifolia* has no longer be used as rootstock of cucumber in the commercial production.

Because *D. sclerotioides* is a soil-borne pathogen, soil disinfestation is a common control measure against black root rot of cucurbits. Although chloropicrin appears to be the most effective among disinfectant chemicals, its high toxicity to humans and unpleasant odor limits its popularity in practical applications (Shishido and Takeuchi,

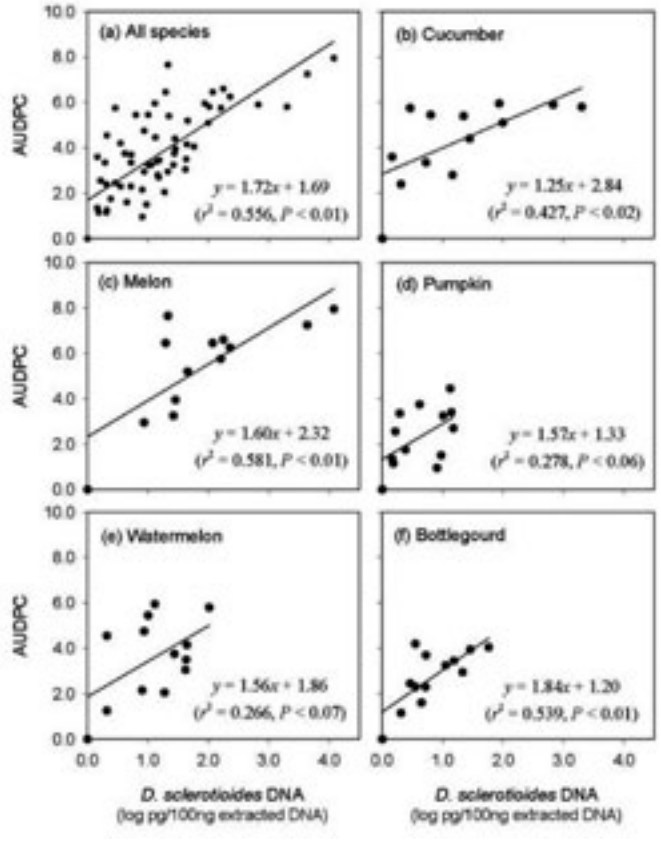

Fig. 3 - Correlations between the area under the disease progress curve (AUDPC) and the quantity of *Diaporthe sclerotioides* DNA detected in the hypocotyls of all the cucurbit species combined (a), cucumber (b), melon (c), pumpkin (d), watermelon (e), and bottlegourd (f) after root inoculation with 12 isolates of *D. sclerotioides*. Reprinted from reference Shishido *et al.* (2014) with permission from the publisher.

2005). Soil solarization is an alternative and environmentally-healthy measure against the disease since the pathogen is known to be heat-sensitive (Kobayashi *et al.,* 1997). One of the problems in soil solarization is to maintain high temperatures (37.5°C for 2 days or 35.0°C for 6 days) throughout the root range of soil depth, *ca.* 30 cm. In recent years, biological soil disinfestation (BSD), or anaerobic soil disinfestation (ASD) has been successfully applied to control some soil-borne pathogens and pests including *Fusarium oxysporum*, *Ralstonia solanacearum*, and parasitic nematodes (Momma *et al.,* 2006; Lamers *et al.,* 2010). BSD increases the effect of solarization by amending organic substances such as wheat bran or green manure crops under anaerobic conditions. Yokoyama *et al.* (2012) demonstrated that BSD with a low concentration of ethanol (0.5%-1.0%) was sufficiently effective to control black root rot of cucumber. They recommend a low concentration of ethanol instead of wheat bran for the soil amendment because the former produces almost the same level of disease control efficacy to the latter with little unpleasant odor during the anaerobic process.

Although BSD may be a promising control measure against black root rot, the practice may only be applicable in protected facilities such as greenhouses and walk-in tunnels in relatively warm climate regions. Therefore, other control measures are needed that are applicable in vast, unprotected fields especially in cool climate regions. Iwadate (2012) demonstrated that changing soil pH to weak alkaline, i.e. pH=7.5, with amending steel converter slag, significantly reduced the disease severity of cucumber in unprotected fields. All of these cucumber fields were in Iwate Prefecture, located in the north-eastern part of Japan, an area known for its cool climate. Although the mechanisms of disease suppression of this method have not yet been elucidated, it is certainly interesting since steel converter slag is easily available at a low cost as a byproduct of steel manufacturing.

To date, only a few studies of biological control have been conducted regarding black root rot, e.g. *Gliocladium roseum* by Moody and Gindrat (1977) and *Pseudomonas* sp. by Fuchs and Defago (1991). Nonetheless, because *D. sclerotioides* is a slow growing fungus compared with other major soil-borne fungal pathogens such as *Fusarium*, *Rhizoctonia*, and *Pythium*, the fungus may be less competitive in searching for nutrients and habitats in soil. Interestingly, hypovirulent elements such as double-stranded RNA are also known to infect *Diaporthe* species (Ghabrial, 2013). Although the detailed mechanism has not been elucidated, some isolates of *D. sclerotioides* significantly reduce in virulence (Shishido *et al.,* 2014). Therefore, biological control including hypoviruses may have potential for controlling black root rot of cucurbit crops in future.

6. Conclusions

In Japan, more than 30 years have passed since black root rot was first reported in cucumber. Since then, the fungal isolates were correctly identified as *Diaporthe sclerotioides* based on their morphology and DNA sequence. In addition, the knowledge of DNA sequence has developed technical tools for detecting and quantifying the pathogen in natural samples of plants and soils. On the other hand, aside from chemical soil disinfestation, environment-friendly control measures have also been developed by applying solarization as well as biological soil disinfestation. Although such temperature-dependent methods may not properly be applicable to out-fields especially in cool climate regions, an alternative measure by changing soil pH to weak alkaline with amending steel converter slag has proved effective to control this disease. Nonetheless, the area of its infestation is still expanding to northern parts of Japan. To prevent further damage to cucurbit production from black root rot and reducing the infestation of *D. sclerotioides*, studies are needed especially in the area of breeding of resistant varieties and understanding the ecology of the pathogen to develop more promising and effective measures to control of this disease.

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