

MOLECULAR DETECTION AND COMPARISON OF *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* ISOLATES ORIGINATING FROM WHEAT AND RYE

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Abstract: *Gaeumannomyces graminis* is an etiologic agent of take-all, economically important disease of cereals worldwide. A polymerase chain reaction with variety-specific primers was successfully used for detection of *G. graminis* var. *tritici* in plant tissue. Obtained results showed that this diagnostic method is a very sensitive and useful tool for detection of the pathogen even before disease symptoms arise. DNA polymorphism revealed by RAPD-PCR with three arbitrary primers was suitable for assessing genetic variation among *Ggt* isolates originating from wheat and rye.

Key words: *Gaeumannomyces graminis*, molecular analysis, RAPD, take-all, variety-specific PCR

INTRODUCTION

Gaeumannomyces graminis (Sacc.) Arx & Olivier var. *tritici* Walker is the etiologic agent of take-all disease, one of the most economically important root diseases of wheat and barley worldwide. Rye is considerably less susceptible than wheat to var. *tritici* (Hornby 1998). Take-all starts as a root rot, causing stunting and nutrient-deficiency symptoms in shoots, and progresses upward into the bases of the stems where it can then disrupt the flow of water to shoots and cause premature death of the plant. The pathogen then survives saprophytically in dead roots and stem bases invaded through parasitism, and uses these substrata as a food base to infect the next wheat crop (Cook 2003). Although *G. graminis* var. *tritici* (*Ggt*) is one of the most frequently studied root disease of cereals, yet it still remains the most important root disease of wheat world wide.

Diagnosis of take-all disease is generally based on visual symptoms, host identification, predisposing environmental conditions, and the presence of darkly pig-

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mented, ectotrophic runner hyphae on plant roots and/or crowns (Fouly and Wilkinson 2000). Moreover, visual diagnosis of stem base disease, where several fungi may be present in the same plant is difficult, particularly during the early growth stages when the symptoms can be confused.

Classical identification of *G. graminis* is based on colony morphology and teleomorphic state observations. However, the formation of sexual stage in field infected plants is rare, can take weeks to complete in the laboratory and may not be produced by some isolates limiting the number of available morphological features (Henson *et al.* 1993).

Because of some difficulties in quick diagnosing of take-all, molecular techniques have been applied to identify *G. graminis*. The polymerase chain reaction (PCR) and its applications in plant disease diagnosis are highly sensitive and reproducible. Herdina *et al.* (1996) used DNA probes to identify and quantify *Ggt* in soil for the prediction of take-all in a wide range of field soils. ITS region polymorphism was often used to identify *Gaeumannomyces* species and its varieties (Goodwin *et al.* 1995; Fouly *et al.* 1997; Fouly and Wilkinson 2000; Ward and Akrofi 1994). Rachdawong *et al.* (2002) to detect *G. graminis* varieties used sequences of avenacinase and avenacinase-like genes. Although many diverse identification methods are available, few of these can reliably identify all varieties of *G. graminis*, and there are many isolates that give atypical results (Freeman and Ward 2004).

There has been relatively little published work concerning genetic variability of *Ggt* isolates originating from different hosts. Randomly amplified polymorphic DNA (RAPD) has provided a useful tool for detecting intraspecies and even intravarietal variations (Fouly *et al.* 1996; Bryan *et al.* 1999).

The presented studies were undertaken to determine whether the variety-specific primers defined by Fouly and Wilkinson (2000) for *Ggt* isolates originated from the USA could be useful for identification of Polish isolates and detection the pathogen in naturally infected plants. Additionally, genetic variability of isolates originated from wheat and rye were assessed.

MATERIALS AND METHODS

Fungal isolates and culture maintenance

Wheat and rye plants, at the end of tillering stage, displaying take-all symptoms were collected in 2006 from western Poland. Forty-three isolates of *G. graminis* var. *tritici* derived from roots of wheat and rye plants and used in laboratory experiment are listed in Table 1. Cultures were maintained in Petri plates on potato dextrose agar (PDA; Merck, Darmstadt, Germany).

DNA preparations

Mycelia from two-weeks-old cultures grown on a liquid medium (5 g/l of glucose, 1 g/l of yeast extract) were collected by vacuum filtration using Büchner funnel. Fungal DNA was extracted and purified using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations with slight modification. Additionally, total DNA was extracted from wheat tissue by grinding 100 mg roots (with and without disease symptoms) with carborundum using DNeasy Mini Kit.

Table 1. Isolates of *G. graminis* var. *tritici* derived in Poznań region in 2006 from roots of wheat and rye

Isolates	Host plants
M-1d, M-1e, M-1g, M-1h, M-2f, M-4a, M-5f, H-10aI	spring wheat
A-15aI, A-15bI, A-15cIB, A-15dIB, B-13aIB, B-14aI, B-14bI, B-14c, B-14d, B-15aI, B-15eIB, Z-6a, Z-6b, Z-7e, Z-8a, Z-8c, Z-8d	winter wheat
C-13bI, C-13dI, C-13f I, C-13gI, C-14aI, C-14cI, C-14dI, C-15aI, C-15b I, C-15cI, C-15d I, C-15eI, C-15gI, D-13aI, D-13b, D-14bI, D-14dIB, C-15b	winter rye

Species-specific PCR

Primers NS5 (White *et al.* 1990) and GGT-RP (Fouly and Wilkinson 2000) (Sigma-Genosys, Pampisford, UK) were used for specific detection and identification of *G. graminis* var. *tritici*. The amplification reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Hilden, Germany) in a small total volume (5 µl). The reaction mixture contained 5 ng of fungal DNA, 1x reaction buffer, 2mM magnesium chloride, 200 µM of each dNTP, 0.4 µM each of the primers and 0.5 U of *Taq* DNA polymerase. Amplification was carried out in a Biometra Tpersonal 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following programme: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 53°C for 1 min and extension at 72°C for 1 min. The amplification was ended with an additional extension at 72°C for 5 minutes. Additionally the efficiency of variety-specific reaction was checked. Amplification of specific fragment was performed by adding different amount of fungal DNA to reaction mixture. The total DNA extracted from field-infected plants with visible and latent disease symptoms was also used in PCR assay as a potential source of *Ggt*. Additionally, DNA extracted from *Fusarium culmorum* (*Fc*) was used in PCR. The reactions were carried out as described above. Parts of roots used in PCR assay were also placed in Petri plates on PDA medium.

RAPD assays

The RAPD-PCR reactions were carried out using a *Taq* PCR Core Kit (QIAGEN Inc., Hilden, Germany) in a total volume of 4.5 µl. The reaction mixture and PCR profile were described earlier (Irzykowska *et al.* 2005). Three random 10-mer primers: OPJ-04, OPJ-05, OPJ-14 (Qiagen Operon, Cologne, Germany) were used to screen the isolates for polymorphism. Amplification was carried out in a Biometra Tpersonal 48 thermocycler (Whatman Biometra, Goettingen, Germany). PCR was repeated twice to check reaction reproducibility.

The electrophoresis conditions

The PCR products were separated by electrophoresis (4 V/cm) in 1.5% agarose gels with 1x TBE buffer (89mM Tris-borate and 2mM EDTA, pH 8.0) and visualised under UV light following ethidium bromide staining. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas GMBH, St. Leon-Rot, Germany) was used as a molecular size standard for PCR products.

Statistical analysis

Polymorphic bands were scored and analyzed by Treecon for Windows version 1.3b software (Van de Peer and de Wachter 1994). The coefficients of genetic similarity (GS) of the investigated isolates were calculated according to the formula given by Nei and Li (1979)

$$GS_{ij} = 2N_{ij}/(N_i + N_j)$$

where: N_{ij} – the number of alleles present in i -th and j -th isolates,

N_i – the number of alleles present in the i -th isolate,

N_j – the number of alleles present in the j -th isolate, $i, j = 1, 2, \dots, 43$.

The coefficients were used to group the isolates hierarchically using the Unweighted Pair Group Method of Arithmetic Means (UPGMA).

RESULTS

Variety-specific amplification of genomic DNA from *G. graminis* var. *tritici* cultures and estimation of reaction sensitivity

The NS5 and GGT-RP primers were designed based on sequence differences among American *Ggt* isolates. To examine the suitability of these primers for identification of Polish isolates, *Ggt* cultures derived from Poznań region were analyzed. Temperature of primer annealing was enhanced to 53°C to increase reaction specificity and reaction mixture volume was decreased to 5 µl. A 410 bp DNA fragment was amplified in PCR from all *Ggt* isolates examined (Fig. 1).

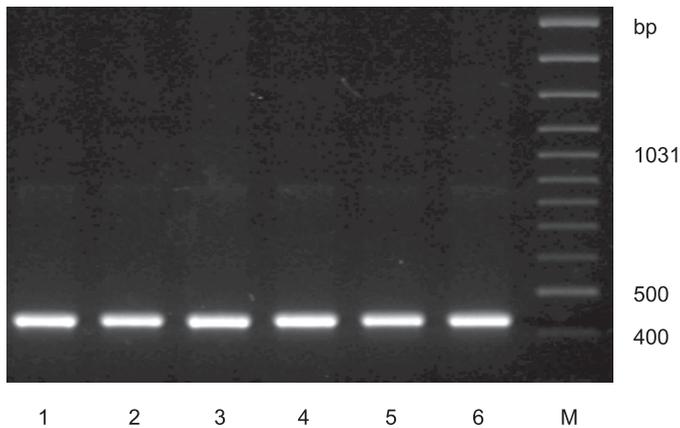


Fig. 1. Variety-specific PCR of genomic DNA from *G. graminis* var. *tritici* cultures

Lane M – Gene Ruler™ 100 bp DNA Ladder Plus; Lane 1–3 isolates from wheat; lane 4–6 isolates from rye

To determine the minimum amount of fungal DNA sufficient for detection by specific PCR, reactions were set up with variable quantities of DNA ranging from 100 ng to 0.1 pg (Fig. 2). As little as 1 pg of *Ggt* genomic DNA was sufficient for reliable amplification of the 410 bp fragment typical for *Ggt*. By subjecting total sample volumes (5 µl) of the PCR product to an agarose gel, the specific fragment was visualized.

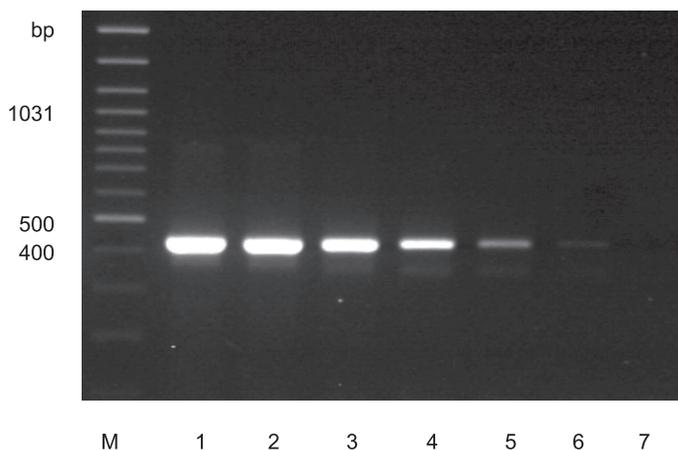


Fig. 2. Sensitivity of the *G. graminis* var. *tritici*-specific PCR assay

PCR product amplified from decreasing amounts of *Ggt* genomic DNA: lane 1 – 100 ng, lane 2 – 10 ng, lane 3 – 1 ng, lane 4 – 100 pg, lane 5 – 10 pg, lane 6 – 1 pg, lane 7 – 0.1 pg, line M – Gene Ruler™ 100 bp DNA Ladder Plus

Detection of *G. graminis* var. *tritici* in naturally infected wheat roots

To investigate usefulness of a variety-specific assay for diagnostic applications, a total DNA from wheat roots was extracted. Wheat roots with darkened fragments and without visible disease symptoms were taken from field-cultivated plants. DNA from darkened roots was amplified specifically, revealed the presence of 410 bp fragment typical for *Ggt* (Fig. 3, lane 5). To make obtained results completely reliable, PCR product was sequenced and obtained sequence was analysed and compared with data base (unpublished data).

Additionally, a variety-specific product was amplified in sample with DNA from apparently healthy roots (Fig. 3, lane 3 and 4). Fragments of the same roots were placed on PDA medium and after seven days mycelium with curling back of the hyphae on the colony edge, typical for *Ggt*, was grown confirming molecular identification. Considering that mixed infections of *Fc* and *Ggt* occur frequently in field condition also DNA extracted from *Fc* mycelium was used in PCR assay. No band was amplified in samples with *Fc* DNA confirming reaction specificity (Fig. 3, lane 2).

Genetic variability

Genetic variability among 43 isolates was determined using the data generated by three 10-mer primers. Only reproducible bands of sufficient intensity were scored. The number of polymorphic fragments varied from 6 to 10 per primer (Fig. 4). Twenty-four polymorphic RAPD markers were obtained. The PCR products size ranged from 0.1 to 3.4 kb. Because RAPD-PCR is particularly prone to contamination, a negative control was included (Fig. 3, lane 1). The comparison of each profile for each primer was done on the basis of the presence [1] versus absence [0] of RAPD products of the same length. Each band was assumed to represent a single genetic locus. These binary data from RAPD analyses were used for grouping isolates by the UPGMA method. The relationship among isolates is presented in the form of a dendrogram (Fig. 5). Some of isolates were identical in screened genome regions (similarity equal 1).

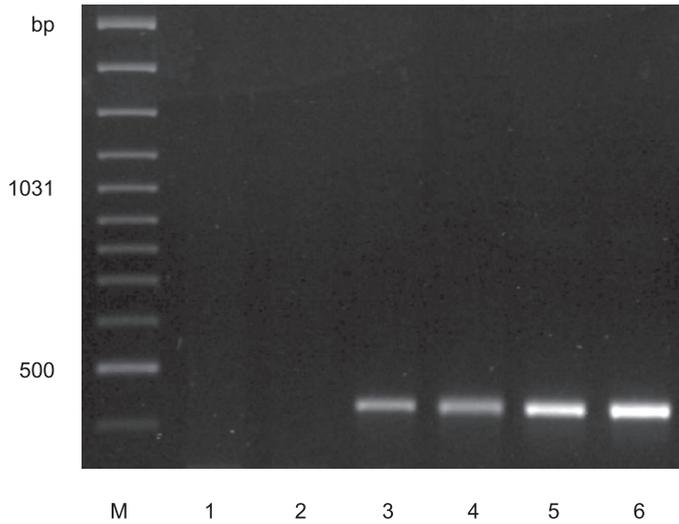


Fig. 3. Variety-specific PCR of DNA from wheat tissue

Lane M – Gene Ruler™ 100 bp DNA Ladder Plus; lane 1 – negative control; lane 2 – reaction with DNA from *F. culmorum*; lanes 3 and 4 – amplicon from total DNA of wheat roots without take-all symptoms; lane 5 – amplicon from total DNA of wheat roots with take-all symptoms; lane 6 – amplicon from DNA extracted from *Ggt* culture as a positive control

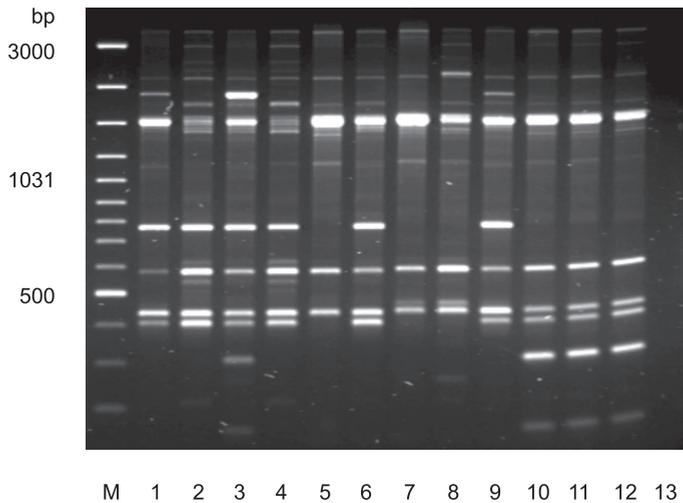


Fig. 4. RAPD-PCR patterns of *G. graminis* var. *tritici* isolates

Lanes 1-8 isolates from wheat, lanes 9-12 isolates from rye amplified with primer OPJ-04; lane 13 – negative control; lane M – Gene Ruler™ 100bp DNA Ladder Plus

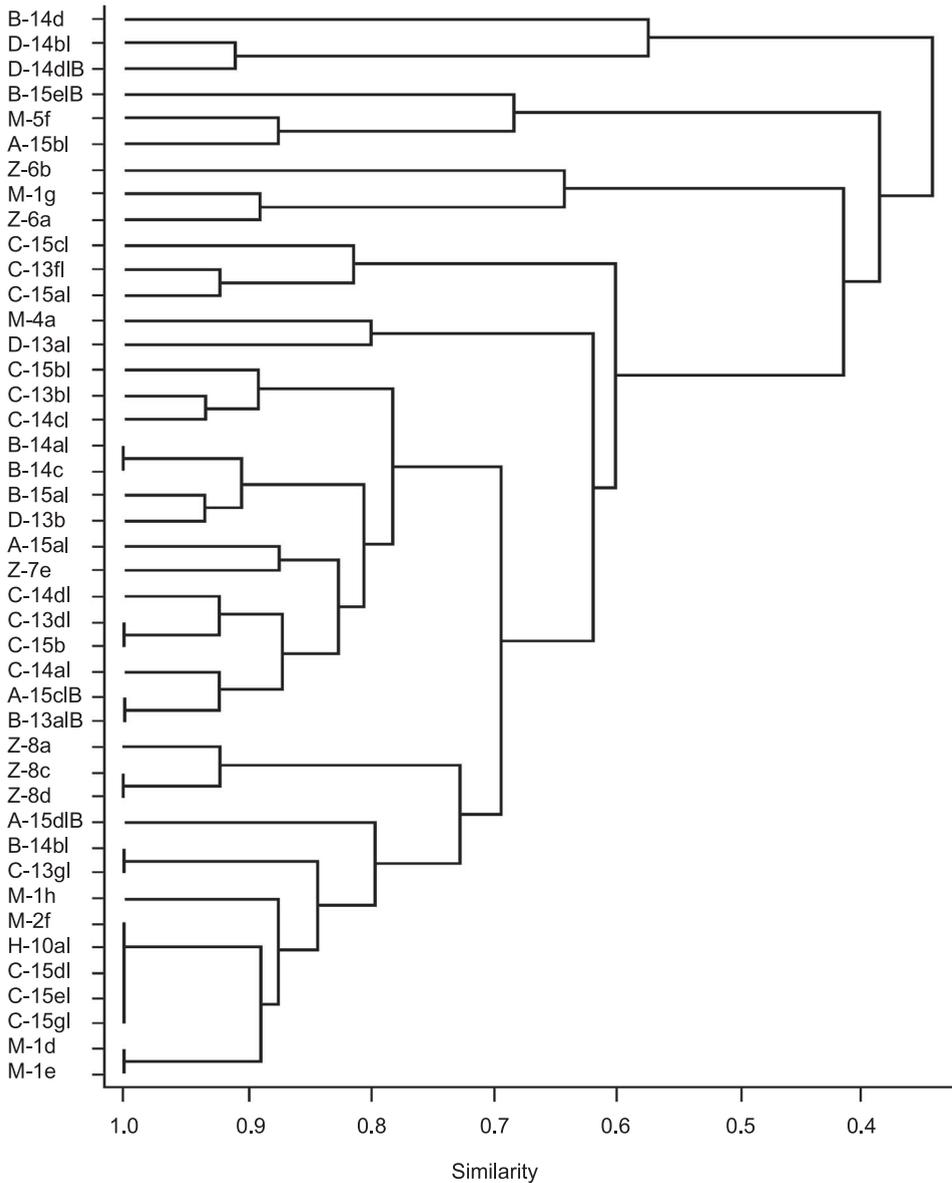


Fig. 5. Dendrogram of *G. graminis* var. *tritici* isolates based on random amplified polymorphic DNA data analysis. The isolates were grouped hierarchically using the unweighted pair group method of arithmetic means (UPGMA)

DISCUSSION

PCR technology offers a number of advantages over conventional methods for detection and differentiation of take-all fungus (Irzykowska 2006). Molecular analyses are fast, universal and precise. Several PCR primer pairs for specific detection of a particular *Gaeumannomyces* species and also *G. graminis* varieties have been devel-

oped (Bryan *et al.* 1995; Goodwin *et al.* 1995; Schesser *et al.* 1991; Ward and Bateman 1999). However, in some cases diagnostic methods developed in one part of the world may not be applicable universally (Freeman and Ward 2004).

Specificity of primers used in this work for identification of *Ggt* is based on nucleotide sequence differences in the middle region of the small subunit of nuclear rDNA (18S rDNA) and was tested earlier on American isolates (Fouly and Wilkinson 2000). Using variety-specific primers it was possible to identify Polish *Ggt* isolates, originated from completely different gene pool. Small volume of reaction mixture (5 µl) decreases analysis costs. Obtained results showed that this molecular method is a useful tool for detection of the pathogen even before symptoms arising. Moreover, very high reaction efficiency was observed, so as little as 1 pg of *Ggt* DNA can be detected. The copy number of the rDNA repeat is more than 50 per genome, explaining a high sensitivity for detection if used as a target gene for PCR (Fouly and Wilkinson 2000).

A few different methods have been used to assess genetic variability of various *Gaeumannomyces* species including time consuming RFLP and isozyme analysis (Harvey *et al.* 2001). However RAPD method is a useful tool for genetic variability analysis. RAPD markers generated with three arbitrary primers revealed DNA polymorphism suitable for assessing differences among 43 isolates examined, this being in accordance with results obtained by other researchers (Augustin *et al.* 1999; Weber *et al.* 2005). Dendrogram produced from analysis of RAPD data divided isolates into small subgroups. However, isolates of *Ggt* originated from rye did not form a distinct subgroup at dendrogram as it was described earlier (Bryan *et al.* 1999). Bateman *et al.* (1997) distinguished groups of the pathogen corresponding with different host species using a mitochondrial rDNA probe. In agreement with Hornsby's (1998) opinion, some isolates of *Ggt* (R-type isolates) can cause more root blackening on rye than do others (N-type isolates). Perhaps in this experiment only R-type isolates were analysed or *Ggt* genome should be screened more extensively to find regions responsible for host-pathogen interaction.

Presented results demonstrate that detection method based on NS5 and GGT-RP primers provides rapid, sensitive and accurate diagnosis not only of American *Ggt* isolates (Fouly and Wilkinson 2000) but also Polish isolates. Further work is needed to find an association between host preference and molecular markers grouping and to determine the importance of such association.

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POLISH SUMMARY

MOLEKULARNA IDENTYFIKACJA ORAZ PORÓWNANIE IZOLATÓW *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* POCHODZĄCYCH Z PSZENICY I ŻYTA

Gaeumannomyces graminis jest czynnikiem etiologicznym zgorzeli podstawy źdźbła i korzeni, ekonomicznie ważnej choroby zbóż o zasięgu światowym. W badaniach zastosowano łańcuchową reakcję polimerazy z odmianowo-specyficznymi starterami do wykrywania *Gaeumannomyces graminis* var. *tritici* (Ggt) zarówno w czystych kulturach jak i tkance roślinnej. Na podstawie uzyskanych wyników stwierdzono, że zastosowana metoda diagnostyczna jest bardzo czułym i użytecznym narzędziem do wykrywania patogena jeszcze przed wystąpieniem objawów choroby na korzeniach. Polimorfizm DNA ujawniony na drodze analizy RAPD-PCR z trzema losowymi starterami był wystarczający do określenia zróżnicowania genetycznego izolatów Ggt pochodzących z pszenicy i żyta.