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Genetic diversity and effect of temperature and pH on the growth of *Macrophomina phaseolina* isolates from sunflower fields in Hungary

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Abstract The effects of temperature and pH on the growth of 45 Hungarian *Macrophomina phaseolina* isolates from different locations and hosts were compared on the basis of their genetic diversity. One Spanish and two Serbian isolates were also included in the experiment. The most favourable temperature regimes for the development of the isolates ranged between 25 and 35°C. The optimal pH for the pathogen varied between 4.0 and 6.0, but growth was observed on potato dextrose agar even at pH values of 3.0, 7.0 and 8.0. RAPD analysis with 13 different primer pairs generated 148 unambiguous bands. RFLP analysis involving 8 different restriction endonucleases was performed on a 1550 bp fragment of the rDNA region containing internal transcribed spacers (ITS1, ITS2), the 5.8S rDNA and part of the 25S rDNA. The greatest genetic distance values were obtained for three isolates, two from Hungary and one from Spain, which had similar values, but were quite distinct from all the others. A strong positive correlation was observed between the genetic distances and the growth parameters measured at various temperatures, and between the geographical data and the growth data sets at different pH values, but the correlation was less strong in the latter case. While Hungarian *M. phaseolina* populations are thought to reproduce clonally, the present results indicate the coexistence of different haplotypes in this area, and besides the geographical dominance of a given haplotype it was found that a closer genetic relationship might exist between spatially distinct haplotypes.

Keywords Charcoal rot disease · Population structure · Nuclear ribosomal (rDNA) regions · Genetic differentiation · Internal transcribed spacer (ITS)

Introduction

The economic importance of charcoal rot disease caused by *Macrophomina phaseolina* (Tassi) Goidanich [*Rhizoctonia bataticola* (Taubenhaus) E.J. Butler] is still considerable. This polyphagous pathogen infects more than 700 plant species [1]. The disease can be diagnosed on the basis of the symptoms: ash grey spots on the stems and small, black microsclerotia developing in the pith and root tissues. Microsclerotia survive in plant residues in the soil, and serve as the primary inoculation source [2]. The infectivity of the pathogen is highly influenced by environmental factors. The effect of temperature on the growth of *M. phaseolina* has been investigated by several authors, some of whom found 30°C to be the optimal temperature for both mycelium growth and the development of microsclerotia [3–5]. Up to now no satisfactory answer has been given on the optimal

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epidemiological factors of this polyphagous pathogen. Similarly, the effect of different pH values on the growth of *M. phaseolina* has been examined. The pathogen was able to develop over a pH range of 2.0–9.0. As the pH increased the growth intensity gradually increased, but it decreased again above pH 7.0. The in vitro pH optimum for mycelium development was between 5.0 and 6.0 [3, 6].

Over the last 10 years several authors have investigated the structure of *M. phaseolina* populations with molecular genetic methods. Contradictory results were obtained in these studies. While some authors found a significant correlation between the geographical data, host plants and genetic diversity of the isolates, others were unable to confirm these findings. Su et al. [7] and Aboshosha et al. [8] detected host-specific RAPD markers, while Das et al. [9] found a correlation between RAPD markers and the site of origin of different isolates. Mayek-Perez et al. [10] and Reyes-Franco et al. [11] found a tendency of AFLP genotypes to cluster with groups of isolates of different geographical origin and also from different hosts. On the other hand, Purkayastha et al. [1], Rajkumar and Kuruvinashetti [12] and Omar et al. [13] detected no correlation between genetic (RAPD) and geographical data. In the latter two studies however, only a limited number of isolates (10) were used. Almeida et al. [14] studied *M. phaseolina* isolates from Brazil using RAPD markers and were able to divide all the 55 samples into three groups, but these were not host-specific. These authors also used ten endonuclease enzymes to digest a 620 bp fragment amplified with the ITS1 and ITS4 primers from nine *M. phaseolina* samples isolated from various host plants (soybean, maize, sorghum, sunflower, wheat), but polymorphism was not detected. When analyzing the ITS region Chase et al. [15] and Su et al. [7] found no polymorphism using two (*Bst*U I and *Taq* I) and five (*Msp* I, *Hae* III, *Mbo* I, *Rsa* I and *Taq* I) restriction enzymes, respectively. Purkayastha et al. [1], on the other hand, reported a high degree of polymorphism in the restriction patterns of the ITS region containing part of the 25S rDNA when using *Eco*R I and *Taq* I enzymes.

The objectives of the present study were (i) to analyze the diversity and genetic relationships of *M. phaseolina* isolates collected from sunflower fields in Hungary and (ii) to investigate whether the genetic diversity of different isolates is correlated with factors such as geographical origin and/or environmental conditions (temperature, pH). For this purpose the growing patterns of 48 *M. phaseolina* isolates collected from Hungarian, Spanish and Serbian localities were measured under different conditions and compared with molecular data on the RAPD and PCR-RFLP patterns of the ITS region containing 5.8S rDNA and part of the 25S rDNA using various statistical methods.

Materials and methods

Collection and maintenance of isolates, analyses of temperature and pH

Systematic disease surveys were undertaken in commercial sunflower growing regions, as well as on experimental farms in Hungary. *M. phaseolina* isolates were collected

Fig. 1 Collection localities of *Macrophomina phaseolina* isolates in Hungary. Further details are given in Electronic Supplementary Material 1

from 39 localities in Hungary (Fig. 1). Diseased plants were collected from sunflower in or near crop fields and from non-sunflower hosts (soybean, corn, sugar beet). These samples were also included in the analysis as outgroup representatives, together with one Spanish and two Serbian isolates. The collection dates and sites, together with host plant information for the isolates, are given as Electronic Supplementary Material 1. Scrapings from infected plant debris were placed on potato dextrose agar medium (PDA, Merck KGaA, Germany) and incubated at 25°C. Pure cultures were made by repeated passage.

One-week-old pure cultures growing on PDA medium were used as inocula. Discs (five mm in diameter) containing mycelia and microsclerotia from the margins of the colonies of *M. phaseolina* were transferred to the centre of 90 mm Petri-dishes filled with 10.0 ml sterile PDA medium (original pH 5.5). After inoculation the Petri-dishes were placed in dark thermostats adjusted to temperatures of 10, 15, 20, 25, 30, 35 and 40°C. To check the most favourable pH, PDA with eight different pH values (pH 2.0–9.0) was used in 90 mm Petri-dishes. The cultures were grown at 25°C. The pH of the medium was adjusted using 1 N HCl or 1 N NaOH solutions and was checked with a 206-pH2 pH-meter, after autoclave sterilization. After this procedure the pH of the medium was not measured. The effect of temperatures and pH on the growth patterns of *M. phaseolina* was tested in four replications. Colony diameters were measured 3, 5 and 6 days after inoculation. Statistical analysis was carried out using the Microsoft Excel software program package (Microsoft Office XP). Significance was estimated from SD at the 5% probability level.

DNA extraction, RAPD and data analysis

Each isolate from stock cultures of *M. phaseolina* was grown for 7 days at 25°C on 5 ml PDA in 50 mm Petri-dishes in dark thermostats. Five mg samples from 1-week-old pure fungal cultures were ground in liquid nitrogen for DNA extraction. Total genomic DNA was extracted using the Gentra Genomic DNA Purification Kit (Gentra Systems, USA). DNA from 48 *M. phaseolina* isolates was amplified by the RAPD method [16]. Twenty RAPD primer pairs were selected for further study, after a screening and optimization process with 50 primer pairs. The primers were paired arbitrarily, but palindromes and complementarities within and among primers were avoided (see Electronic Supplementary Material 2). The sequence of each primer was generated randomly, comprising 12 base pairs and 50-70% GC content. The RAPD primers were modified to contain the ATG start codon or stop codons (e.g. TAA, ACT) based on consensus sequences for the flanking regions reported by Joshi et al. [17] and Sawant et al. [18]. Non-conserved nucleotide positions were also exploited by designing primers where these non-conserved nucleotides typically occurred within the last three or four nucleotides at the 3′end [19]. Replicate experiments, containing one negative and positive control were performed to verify reproducibility and to check the reliability of the primers and the patterns produced. The selected primers yielded stable, reproducible banding patterns. The PCR reaction mixture was prepared in a final volume of 20 µl containing: 2 µl 10× PCR buffer, 2 µl 2 mM dNTP, 20 pmol of each primer, 20 ng DNA, and 0.8 unit DynaZyme DNA polymerase (Finnzymes, Finland). Amplification was carried out in a Robocycler (Stratagene, USA) with the following profile: 94°C for 3 min; 35 cycles at 94°C for 30 s, 37°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Amplified products were separated on 1.5% agarose gels and post-stained with ethidium-bromide, then photographed with a GeneGenius gel documentation system (Syngene, UK). To estimate molecular weight, 50 bp, 100 bp DNA ladders (Fermentas, Lithuania) were used. Only distinct, well-resolved and clear bands were scored, where reliable bands were considered as amplicons found in replicate reactions. The amplified fragments were coded in an absence/presence (0/1) data matrix. It was presumed that fragments with equal length had been amplified from corresponding loci and represented a single, dominant locus with two possible alleles. From this binary matrix, a distance matrix was computed according to Nei and Li [20] based on Dice's similarity coefficient [21]. A dendrogram was constructed using the unweighted pair-group method with an arithmetic average (UPGMA) algorithm. This method was used to identify unique and clonal haplotypes within and among the various populations of *M. phaseolina*. The original matrix was bootstrapped 1,000 times, in order to measure the reliability of the branching patterns, and the quality of the resulting groups. The TREECON program [22] was used for all calculations. Distance matrices were computed from the geographical data set for the collection sites (in km), and from the growth patterns at different pH and temperature values (in mm), using Jaccard's coefficient [23]. The matrices were tested with the XLstat program (XLSTAT 2008 software, Addinsoft SARL, Paris, France). The correlation between the data sets containing estimates of the geographical distances, between the ranges of the collection sites of the isolates, and between the genetic distance matrixes, obtained with molecular genetic methods, was calculated with the Mantel test [24]. This procedure was repeated for the correlation between the genetic distances and the growth parameters at different pH and temperature values.

ITS amplification and PCR-RFLP

The PCR primers LR5 (5′-TCCTGAGGGAAACTTCG-3′), designed by Rehner and Samuels [25], and ITS5 (5′-G

GAAGTAAAAGTCGTAACAAGG-3′), described by White et al. [26], were used to amplify the ITS region including the 5.8S gene and part of the 25S rDNA according to Purkayastha et al. [1]. The PCR reaction mixture was the same as that used for the RAPD analysis. The restriction enzymes used for initial screening were *Bst*U I, *Eco*R I, *Hae* III, *Hinf* I, *Mbo* I, *Msp* I, *Rsa* I and *Taq* I. For digestion 10 µl samples of the PCR products were used. Each digestion contained 10 units of restriction enzyme and was carried out according to the manufacturer's instructions. Electrophoresis and gel documentation were done as described for RAPD analysis.

Results

Effect of temperature on the growth of mycelia and microsclerotia colonies

It is typical of *M. phaseolina* that mycelia start to grow first, followed by the formation of microsclerotia, with which the fungus reproduces asexually. In Hungary pycnidia of *M. phaseolina* were only detected once on the stem of bean plants [Vajna and Békési, personal communication]. Unfortunately, the way in which this pathogen reproduces in Hungary is not well documented. The mean (in mm) mycelia and microsclerotia colony diameters of *M. phaseolina* isolates as a function of temperature and incubation time together with SD (%) values are available as Electronic Supplementary Material 3. The colony growth parameters for all 48 isolates are presented in Fig. 2. The heat demand and tolerance of the isolates varied over a wide range. The spread of the pathogen was very slow at 10°C. Even on the 6th day only nine isolates showed slight mycelium growth. The highest daily mycelium growth rate was only 3.13 mm/day (Mp 45). On the 6th day microsclerotium growth was only observed for one isolate (Mp 45). This measured 13.50 mm, averaged over the replications. At 15°C only 25 isolates started mycelium growth even on the 6th day. The Mp 45 isolate exhibited the highest mean daily mycelium growth rate (10.29 mm). Only two isolates showed microsclerotium growth, with rates of 19.00 mm (Mp 42) and 59.00 mm (Mp 45). At 20°C most of the isolates completely covered the 90 mm Petri-dishes on the 6th day (Fig. 2), while the diameter of the mycelium colony was still only 33.25 mm for Mp 34, 55.25 mm also for Mp 38 and 72.00 mm for Mp 45. At the same time the microsclerotium diameter of some isolates almost reached the edge of the Petri-dishes, while it was only 23.00 mm for Mp 34, 42.75 mm for Mp 38 and 66.25 mm for Mp 45. At 25, 30 and 35°C the mycelia and microsclerotia of the isolates reached the maximum diameter of 90 mm on the 6th day (Fig. 2), except for Mp 34, Mp 38 and Mp 45 (see Electronic Supplementary Material 4). These isolates differed significantly from the others. At 40°C great differences in the extent of mycelium growth could be observed on the 6th day, ranging from 68.75 mm for Mp 17 to 4.25 mm for Mp 20, while isolates Mp 25, Mp 32, Mp 33, Mp 34, Mp 38 and Mp 45 developed no mycelia at 40°C. At this temperature none of the isolates produced microsclerotia even on the 6th day (Fig. 2). It was observed that isolate Mp 45 produced the largest mycelia at 10 and 15°C, while at 20, 25, 30 and 35°C, it did not reach the margin of the Petri-dishes. The same tendency was observed in the case of Mp 34 and Mp 38 at all temperatures. The mycelia of isolate Mp 45 did not exceed a diameter of 72.00 mm at any temperature, while the production rate of microsclerotia was only 73%

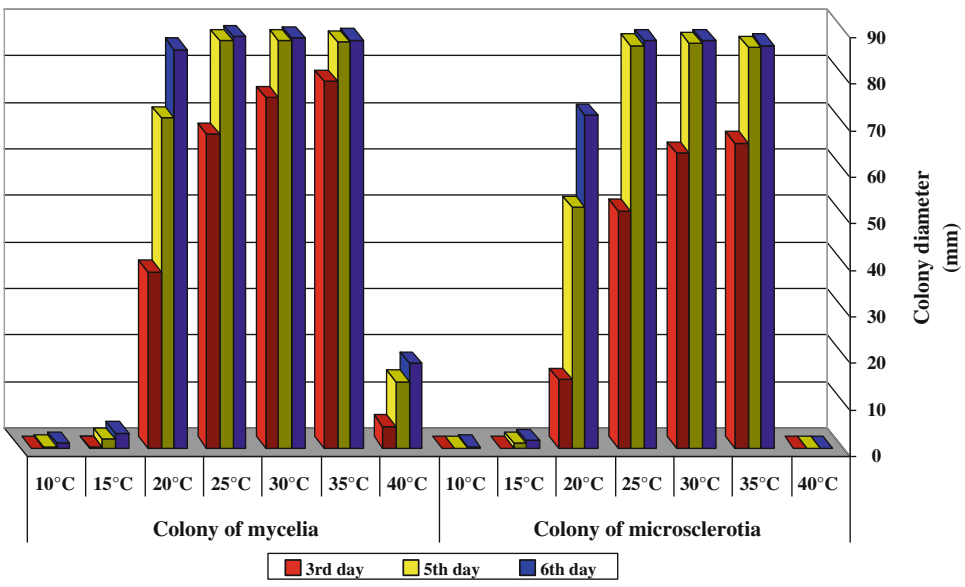

Fig. 2 Mean diameters of mycelia and microsclerotia colonies depending on temperature and incubation time. This figure demonstrates the tendency of colony growth measured at different temperatures on the 3rd, 5th and 6th days

of the average for the isolates even on the 6th day. These results are surprising, because *M. phaseolina* is known as a mesophile fungus and Mp 45 was collected in Spain, where the climate is much warmer than in Hungary. In summary it can be stated that the most favourable temperature interval for the development of the analysed isolates was between 25 and 35°C, though the isolates still grew relatively well at 20°C.

Effect of pH on the growth of mycelia and microsclerotia colonies

The mean colony diameter growth of mycelia and microsclerotia measured for each pH and day, together with SD (%) values, are available as Electronic Supplementary Material 5, while the colony growth measured at different pH values on the 3rd, 5th and 6th days for all 48 isolates is shown in Fig. 3. The optimal pH for the development of *M. phaseolina* was between 4.0 and 6.0. On the 3rd day maximum growth was observed at pH 4.0 (Fig. 3). On the 5th day, averaged over the isolates, the largest mycelium colony diameter, 87.68 mm, was observed at pH 6.0 and the largest microsclerotium diameter, 86.95 mm, at pH 5.0. On the 6th day the mean diameters of mycelium and microsclerotium colonies were above 80.00 mm over a pH range of 3.0-7.0 (Fig. 3). A pH of 2.0 was the least favourable for the pathogen, though with the exception of Mp 34 and Mp 38 the isolates started to form mycelia and microsclerotia even at this pH. Averaged over all the isolates the mycelium colonies were not greater than 24.36 mm even on the 6th day and the mean diameter of microsclerotium colonies was 22.63 mm. At pH 3.0, 4.0, 5.0 and 6.0 the mycelia and the microsclerotium colonies reached a diameter of 90 mm on the 6th day, except for isolates Mp 34, Mp 38 and Mp 45, which differed significantly from the other isolates on the 6th day at these pH values (see Electronic Supplementary Material 6). At pH 7.0, 8.0 and 9.0 all the isolates showed mycelium growth. The mean diameter of the mycelium colonies of all the isolates was 87.85 mm at pH 7.0, 78.30 mm at pH 8.0 and 70.49 mm at pH 9.0 on the 6th day. At pH 7.0, 8.0 and 9.0 all the isolates formed microsclerotia, with a mean diameter of 83.42 mm at pH 7.0, 70.06 mm at pH 8.0 and 61.68 mm at pH 9.0 on the 6th day. These results contradicted the findings of Singh and Chohan [3], who observed no microsclerotium formation at pH 9.0. At pH 7.0, 8.0 and 9.0 the mycelium and microsclerotium colonies of Mp 34, Mp 38 and Mp 45 were smaller than average, in many cases being less than half the average of the other isolates (see Electronic Supplementary Material 6).

RAPD analysis

The RAPD analysis was carried out with 20 primer pairs, 13 of which gave reproducible amplification patterns. With the use of these primers 148 unambiguous bands were amplified from the 48 *M. phaseolina* isolates. One primer pair, 5 + 66, gave a specific banding pattern for the three isolates that proved to be different from the other isolates in the temperature and pH tests (Fig. 4). These isolates were Mp 34 (Iregszemcse, Hungary), Mp 38 (Kaposvár-Toponár, Hungary) and Mp 45 (Cordova, Spain). Moreover, with this primer pair a 200 bp fragment was obtained in 13 of the 34 Trans-Danubian (West Hungary) isolates, which was also present in one of the Serbian samples. In East Hungarian

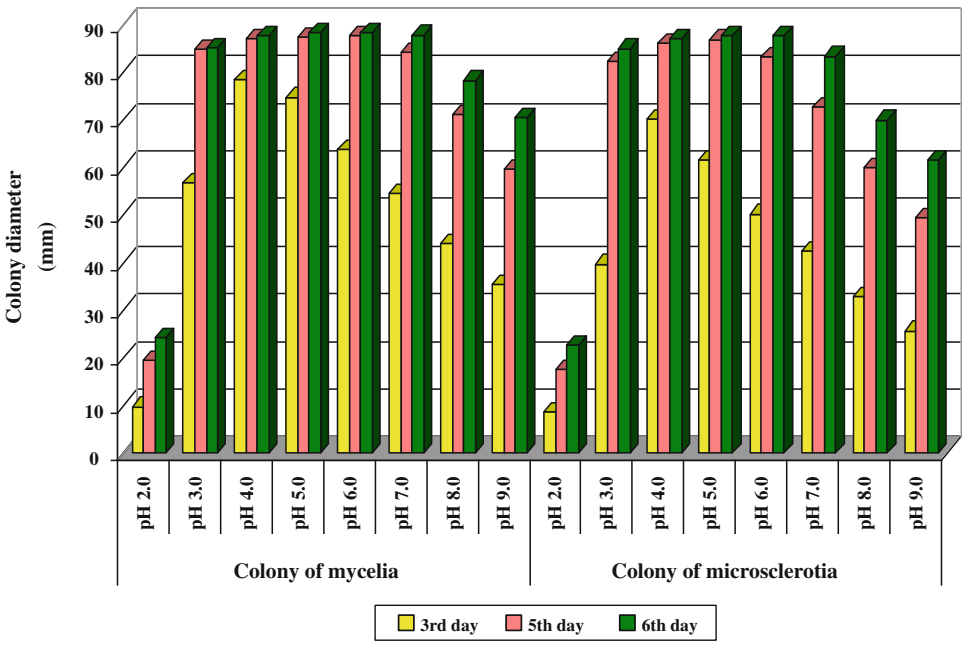

Fig. 3 Mean colony diameters of mycelia and microsclerotia of *M. phaseolina* isolates measured on different pH media and days

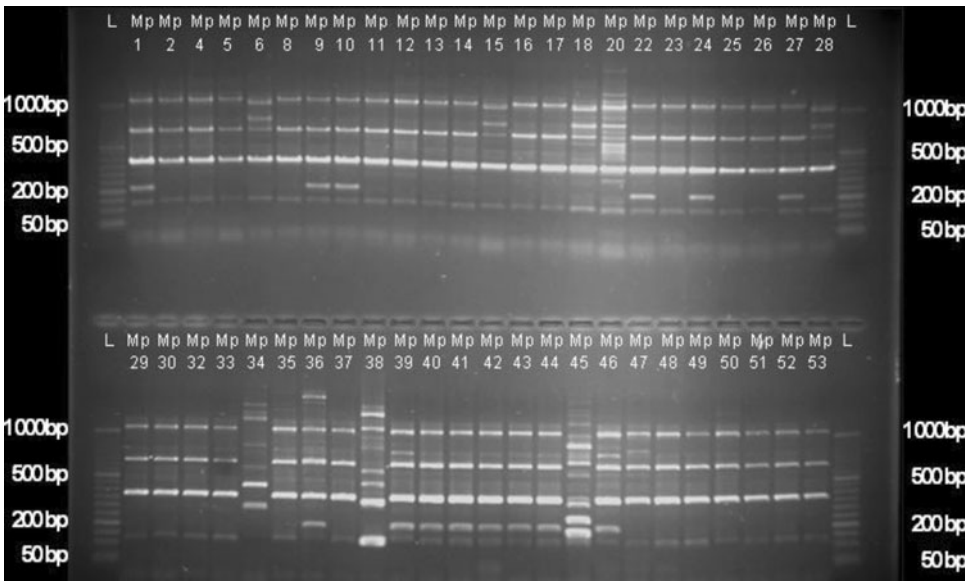

Fig. 4 Agarose gel electrophoresis pattern of the RAPD profiles of *M. phaseolina* isolates showing a specific 200 bp fragment obtained with random primer pair 5 + 66. *Note L* 50 bp DNA ladder, *Mp* indicates the codes of isolates given in Electronic Supplementary Material 1

isolates and in those of other origin this fragment could not be detected. This indicated a loose geographical linkage, which is why genetic diversity analysis was performed to detect possible spatial and genetic correlations.

The dendrogram (Fig. 5) generated by the UPGMA method clearly illustrates that most of the isolates were grouped together in a larger cluster (Group I) with a genetic distance of less than 0.2. In contrast to the narrow genetic distances, the topology of the dendrogram reveals small subgroups, based on collection localities. Isolates from small subgroups, such as Mp 49-53, collected near Bicsérd, formed the South Hungary cluster. The Western Hungarian region split into two fairly mixed clusters together with other samples from Central Hungary or Serbia (West Hungary I and II). Within the West Hungary clusters small subgroups appeared composed of Mp 16, 17, 26 and 35,

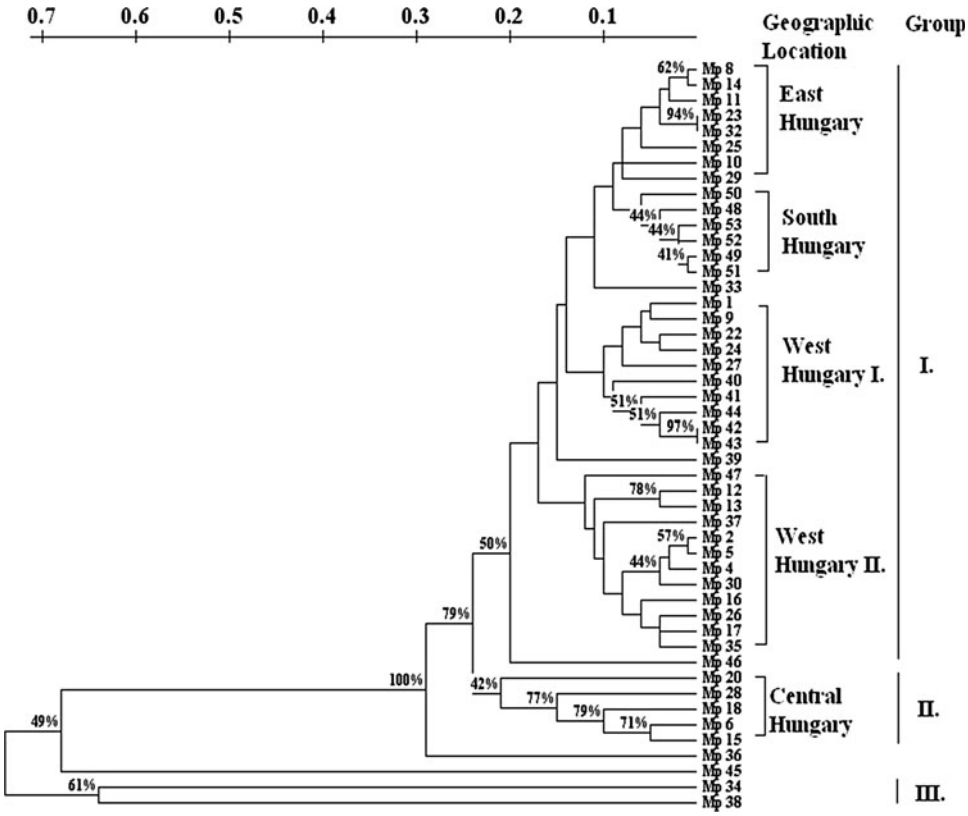

Fig. 5 Dendrogram constructed with the UPGMA clustering method for 48 isolates of *M. phaseolina* based on 148 RAPD loci. Numbers at nodes represent bootstrap values generated by 1000 replications

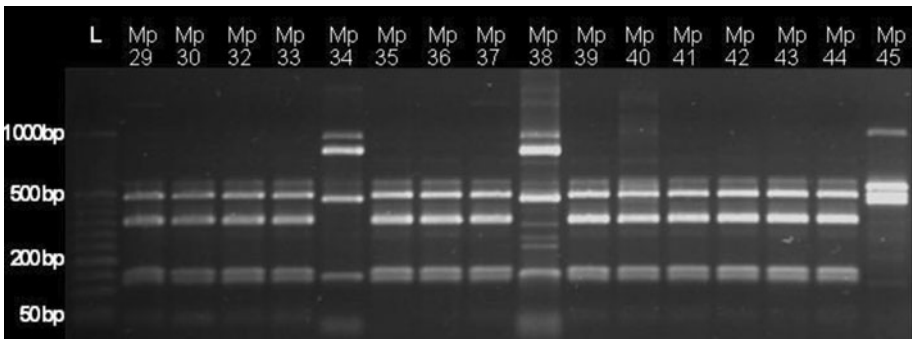

Fig. 6 Agarose gel electrophoresis pattern of representative restriction digestion of the ITS region with the *Msp* I restriction enzyme. Patterns were the same for all isolates (partially shown) except for Mp 34, Mp 38 and Mp 45. Further details are discussed in the text. *Note L* 50 bp DNA ladder, *Mp* indicates the codes of isolates given in Electronic Supplementary Material 1

collected in the Keszthely region (part of West Hungary). Eastern Hungarian samples formed a group together. This cluster also appeared to be a somewhat mixed formation, as isolates from Northern and Central Hungary were nested within this group. One isolate collected in Serbia, Mp 47, was grouped with the Hungarian isolates in Group I. The other Serbian isolate (Mp 46) was at the largest genetic distance (0.2) from the first group. Group II was composed of isolates from Central Hungary. Group III represented a distinct cluster in the dendrogram. In the genetic sense, these were distant from the rest of the isolates analyzed. This group consisted of Mp 34 and Mp 38, both collected in Hungary. Isolate Mp 45 from Cordova (Spain), and Mp 36 collected in Hungary occupied a basal position to most of the other isolates. The topology of these samples cannot be explained on a geographic basis. According to the topology of the dendrogram, Mp 34 and Mp 38 had the greatest genetic distance (0.75). During the mycological analysis the results obtained for Mp 34, Mp 38 and Mp 45 were similar, and this difference from the other isolates could also be detected at the DNA level. From the topology of the dendrogram it is clear that the geographical origin of the isolates had a significant impact on the clustering patterns. The Mantel test between the geographical data set and the genetic distance matrix indicated a weak but significant correlation ($r = 0.402$, $P = 0.001$) between the two data sets. The greatest difference between the data measured (microsclerotium and mycelium growth) at various temperature and pH values was highly significant on the 3rd day at 25°C on standard PDA medium at pH 5.5, and for the same medium and temperature at pH 7.0. The genetic distance matrix and the mycelium growth measured at 25°C (pH 5.5 on the 3rd day) showed a strong correlation ($r = 0.734$, $P = 0001$), as did the genetic data and the microsclerotia colony size ($r = 0.703$, $P = 0.001$). A weak but significant correlation ($r = 0.483$, $P = 0.001$) could be observed between the genetic distance and the mycelium growth measured at pH 7.0 on the same day and temperature. A similar correlation was found between the genetic distance and the microsclerotium formation at 25°C on the 3rd day at pH 7.0 ($r = 0.567$, $P = 0.001$).

PCR-RFLP on the rDNA of the ITS region

An approximately 1550 bp fragment was amplified from all 48 isolates with the ITS5 and LR5 primers. This fragment was digested with eight restriction enzymes (see Electronic Supplementary Material 7). The restriction patterns obtained with *Eco*R I and *Taq* I were monomorphic, while the enzymes *Bst*U I, *Hae* III, *Hinf* I, *Mbo* I, and *Msp* I (Fig. 6) produced the same restriction pattern for all the isolates except Mp 34, Mp 38 and Mp 45. Digestion with *Rsa* I not only distinguished these three isolates but also divided the other isolates into two groups. In the case of *Bst*U I, *Hinf* I and *Rsa* I, Mp 45 could be distinguished from Mp 34 and Mp 38, while *Hae* III and *Msp* I also gave different restriction patterns for Mp 34 and Mp 38.

Discussion

A critical trait of pathosystems established in agricultural plant populations is the degree to which the same pathogen populations are present in dispersed crop fields. The analysis of the population genetic structure of generalist pathogens such as *M. phaseolina* is an approach to understand the dynamics of such pathosystems. Molecular markers are necessary to partition the genetic variance into within- and among-population components, which is the prerequisite for characterizing the genetic structure of pathogens [27–29]. In the present work the effect of different temperatures and pH on the growth of 48 *M. phaseolina* isolates and the genetic variation between the isolates was studied using RAPD assay and PCR-RFLP on the rDNA region.

The most favourable temperature interval for all the isolates was between 25 and 35°C. At these favourable mesophile temperatures, all the isolates in the experiments exhibited good growth by the 6th day, except Mp 34, Mp 38 and Mp 45. In general this is in line with other findings reporting an optimal growth rate between 30 and 35°C [30], but this may vary depending on the geographical region the fungus was isolated from. Manici et al. [31] concluded that the isolates from various climatic regions in Italy grew best at temperatures close to those in the soils from which they were isolated. They also suggest that high soil temperatures may lead to the selection of hyphae, microsclerotia and spores that are adapted to better growth at higher or lower temperatures [31]. The results suggest that higher temperature regimes are favourable for *M. phaseolina* in Hungary, explaining the increased incidence of the disease in warmer seasons [32]. Dry summers provide the best conditions for the development of mass infection. The present results confirm that drought and high temperature may increase crop losses in fields infected with *M. phaseolina* [33]. It was also reported that *M. phaseolina* may become more problematic in agricultural areas where climate change results in higher temperature accompanied by long periods of drought [34], in contrast to many pathogens which favour a change to moister conditions [35]. However, it is unclear whether this is because of increased infection rates under drought or because of increased impacts per infection event [36]. In regions of Hungary subject to greater heat and drought stress the importance of *M. phaseolina* is likely to increase. This is in line with other reports stating that in warmer countries where sunflower is cultivated, charcoal rot is the most important disease [37], while in colder, wet areas, *Sclerotinia sclerotiorum* (Lib.) de Bary is the dominant pathogen affecting sunflower [38]. It was reported by Saleh et al. [39] that an increase in the *M. phaseolina* infections occurring in natural and agricultural ecosystems may alter the relative frequency of plant species in the community. Furthermore, in agricultural relevance such larger fungus biomass should also enhance the diversity of this pathogen increasing the likelihood of developing more aggressive lines [39].

Edaphic factors such as soil moisture, temperature, salinity and pH critically affect the survival of *M. phaseolina* and influence charcoal rot incidences in other crops [40, 41]. Several studies on the growth rates of this fungus have been reported in the literature [30, 31]. According to the present results, the optimal pH for the pathogen is between 4.0 and 6.0. Other studies showed that the optimal pH ranged from 3.6 to 5.0 [42]. However, PDA medium with pH 3.0, 7.0 and 8.0 also provided satisfactory growing condition. This is in line with reports which found good growth between pH 5.0 and 8.0 [30], while others concluded that the best value was between pH 4.0 and 6.0 [43]. It can thus be concluded that the growth rate of *M. phaseolina* at different pH values depends mostly on the isolate and the conditions of origin. All the isolates were able to produce mycelia and microsclerotia under all the pH conditions examined, except for Mp 34 and Mp 38, which only grew between pH 4.0 and 9.0. This may be explained by the fact that some isolates prefer an acidic or alkaline environment [30]. In the course of the mycological analysis the Mp 34, Mp 38 and Mp 45 isolates gave similar results, but differed from the other samples. Certain differences were also recorded between the other isolates, but these were less typical.

The dendrogram clearly illustrates that the comparison of isolates revealed three groups and that many of the isolates analysed had a genetic distance of less than 0.2 (Group I), as also reported by Monga et al. [44]. Isolates Mp 34, Mp 38 and Mp 45 showed the highest genetic dissimilarity values of 0.75 or more. The correlation between mycelium and microsclerotium growth and genetic distances was variable. Statistical analysis of the spatial and genetic data of the isolates revealed a significant but weak correlation ($r = 0.402$, $P = 0.001$), indicating that some groups were geographically mixed populations. This means that the RAPD analysis did not detect a pronounced divergence between the isolates according to geographical origin, as also reported by Purkayastha et al. [1] and Rajkumar and Kuruvinashetti [12]. The two Serbian isolates (Mp 46, Mp 47) could not be separated from the Hungarian isolates according to geographical origin. This might indicate a spread from Hungary to Serbia or vice versa. It is assumed that the same or very similar haplotypes may spread over long distances, probably due to the transportation of seeds or crops contaminated with microsclerotia. Another explanation for the resulting tree topology could be that Serbian and Hungarian populations overlap or the same haplogroups may occur in both countries. These assumptions on introduction routes between the two countries can be interpreted as preliminary hypotheses, since the results presented here could be challenged by larger analysis, both in terms of molecular characters and samples included.

The restriction digestion of the ITS-25S fragment of the rDNA region was performed with eight different enzymes. The restriction patterns obtained with *Eco*R I and *Taq* I were monomorphic, confirming the findings of Purkayastha et al. [1]. Digestion with *Bst*U I, *Hae* III, *Hinf* I, *Mbo* I and *Msp* I resulted in similar restriction patterns for all the isolates except Mp 34, Mp 38 and Mp 45. These results contradict the findings of Almeida et al. [14], who detected no polymorphism with restriction endonuclease enzymes *Hae* III and *Mbo* I. Additionally, Su et al. [7] were unable to identify polymorphic fragments in an analysis involving

Hae III, *Mbo* I, *Msp* I and *Rsa* I. The restriction analysis of the ITS region (PCR-RFLP) did not prove to be a suitable method for detecting variability among *M. phaseolina* isolates, as also concluded by other authors [7]. In the present analysis it produced fairly monomorphic patterns within/among populations. These results suggest that RFLP analysis on the ITS region only effectively reveals polymorphism between isolates where high genetic distances are expected, based either on the results of preliminary mycological analysis or on the geographical distance between the collection localities. This can be explained by the results of recent studies showing that only the ITS1 region accumulates numerous SNPs in its sequence, compared to the other spacer region ITS2, while the 5.8S rDNA gene includes no polymorphism in *Macrophomina* [39, 45–47]. This is not surprising because ITS1 has a higher mutation rate than ITS2, since this region plays a role in the ribosomal maturation process and proved to be more conserved in its sequence [48]. Although the suitability of ITS as a molecular marker for population genetic studies has long been debated [49–54], it should be noted that it separated the Mp 34, 38 and 45 isolates from all the others, which was also confirmed by phenotypic and RAPD analysis.

While *M. phaseolina* is a generalist pathogen with clonal reproduction affinity [39, 55], the results indicate the coexistence of different haplotypes in Hungary. Besides the geographical dominance of a given haplotype, the possibility of a closer genetic relationship between spatially distinct haplogroups is suggested. Despite the asexual nature of this pathogen [30, 56] genetic diversity levels were responsible for cluster formation. This may indicate that genetic variability between isolates of *M. phaseolina* is due to the fusion of vegetative cells, favouring heterokarions or parasexual recombination between nuclear genes, as suggested by previous genetic studies [39, 57]. Although no teleomorph for *M. phaseolina* is known, Baird et al. [58] detected heterogeneity in solely asexually reproducing populations from the USA using SSR markers. Contrary to the parasexual recombination theory, there was never more than one allele detected per isolate in their analysis, implying that parasexuality is absent [59]. Presumably genetically admixed isolates, together with possible clonal isolates, were recovered from multiple locations found in close geographic proximity (e.g. Mp 42, Mp 43) or from more distantly related crop fields (e.g. Mp 23, Mp 32). It was also shown previously that asexual isolates originating from the same host taxa from different locations in different years appeared to be clonal [60]. These findings might indicate that both geographical and genetic mixing occurs in the Hungarian populations of this phytopathogenic fungus. Considering the contradiction between the findings of other labs and the results presented here, the comprehensive genetic analysis of *M. phaseolina* at infraspecific level would be desirable. The taxonomic identity of this fungus has challenged researchers, as has been the case for many generalist pathogens. Although only one taxon is included in the *Macrophomina* genus, this species has many synonyms [56]. It has also been reported that isolates from different hosts and soils differ in morphology [1, 8], suggesting the subdivision of *M. phaseolina* into more than one species [61]. It is also reported that three isolates (Mp 34, Mp 38, Mp 45) in the analyzed samples were genetically and morphologically distinct from the other isolates. These patterns remain unresolved due to the small sample size and support the need for further sequence information. However, it can be noted that this differentiation may be attributable not to geographical separation but to the taxonomic complexity of *Macrophomina*. Despite the many studies, there is still not sufficient evidence to warrant splitting the species [7, 11, 61]. The most effective approach resolving this problem would be the sequence analysis of multiple genomic regions, e.g. rDNA internal transcribed spacers (ITS), translation elongation factor 1-alpha (EF1-α) or the β-tubulin gene. This phylogenetic treatment should include isolates from different parts of the world, but so far no such complete taxonomic analysis has been undertaken. Although the latest study by Saleh et al. [39] utilized ITS sequences together with AFLP data, also it still proved impossible to split *M. phaseolina* into distinct minor species, and the authors concluded that all the isolates belonged to a single complex taxon. Multi-locus methods, such as RAPD or AFLP, which generate a large number of data, are mainly informative in the classification of clonally reproducing populations. The patterns produced could be used to develop, locus-specific markers characteristic of a given genotype. To clarify genetic and geographic, as well as genetic and host specific relations the above mentioned classification would be required. It is hoped that finding answers to these questions will head to a better understanding of the biology of *M. phaseolina*.

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