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# Anastomosis groups and molecular variation in *Pseudocercospora griseola*

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**ABSTRACT.** The fungus *Pseudocercospora griseola* is the causal agent of angular leaf spot, a common bean (*Phaseolus vulgaris* L.) disease. The successful development of angular leaf spot-resistant cultivars depends on understanding the levels of variability in pathogen populations. *P. griseola* shows great pathogenic and genetic variation using inoculation on differential bean cultivars and molecular markers. Nevertheless, how this variability develops is not clearly understood. Parasexuality has been presented as a mechanism used by asexual fungi to increase variation. Hyphal fusion is the first step for the parasexual cycle, and it can be considered an additional trait for population studies. The aim of this study was to identify hyphal fusion (anastomosis) among *P. grisola* isolates and to evaluate the variability of isolates using analyses of anastomosis groups and RAPD markers. Hyphal anastomosis was observed in all isolates. Three isolates showed 85.0% compatibility and were compatible with 17 isolates. This is the first report of the occurrence of anastomosis between *P. griseola* isolates.

 

Sixteen anastomosis groups were observed and only one group was formed by five isolates (Pg-01, Pg-02, Pg-07, Pg-08, and Pg-12). There was a large number of anastomosis groups and absence of clustering among isolates for hyphal fusion, demonstrating the existence of genetic variability for loci involved in the control of this trait. High genotypic variability by RAPD markers was detected, but there was no relationship between genotype and anastomosis group. More studies are necessary to elucidate further the process of asexual recombination in this phytopathogen.

**Key words:** *Phaeoisariopsis griseola*; Angular leaf spot; Common bean; RAPD markers; Parasexual cycle

## INTRODUCTION

Angular leaf spot of common bean (*Phaseolus vulgaris* L.) is caused by *Pseudocercospora griseola* (Sacc.) (Crous et al., 2006). It is one of the most important diseases in tropical and subtropical regions, where the production losses caused by this disease may reach 80%, depending on environmental conditions, cultivar susceptibility and development stage of the crop (Mahuku et al., 2002).

Several control strategies have been proposed for this pathogen. However, the most commonly used strategy is the use of genetic resistance. The successful development of cultivars resistant to angular leaf spot depends on understanding the levels of variability in pathogen populations. Usually, variability has been evaluated by determining the reaction pattern of isolates on a set of differential cultivars (Pastor-Corrales and Jara, 1995). The adoption of a standard nomenclature system allowed the comparison of data obtained by different investigators. In Brazil, over 50 pathotypes have been identified (Nietsche et al., 2002; Sartorato and Alzate-Marin, 2004; Damasceno e Silva et al., 2008), which reflects the high level of variability that has been observed for this pathogen.

Genetic variability using molecular markers has shown to be greater than pathogenic variability between isolates (Busogoro et al., 1999; Nietsche et al., 2001; Mahuku et al., 2002; Sartorato, 2004; Stenglein and Balatti, 2006). Twenty-seven isolates of *P. griseola* were studied using ISSR markers, which indicated that this type of marker is highly sensitive in genetic diversity studies (Abadio et al., 2012).

The sexual cycle of *P. griseola* is unknown, and the mechanism responsible for its high level of variability has not been studied. The occurrence of migration of isolates from one region to another (Busogoro et al., 1999) and of mutation followed by selection in a specific locus (Mahuku et al., 2002) has been suggested. The parasexual cycle is a potential mechanism for genetic recombination in fungi, especially for those with asexual reproduction, which can lead to increased genetic variability (Hastie, 1981). Anastomosis formation between hyphae of different strains is the first step for heterokaryosis and consequently parasexual recombination to occur. Furthermore, anastomosis is important for intra-hyphal communication in the colony and homeostasis during growth and reproduction (Saupe, 2000), allowing exchange of cytoplasmic and genetic material (Roca et al., 2004, 2010; Ruiz-Roldan et al., 2010; Ishikawa et al., 2010, 2012), and it could be related to pathogenicity as shown in *Alternaria brassicicola* (Craven et al., 2008). Anastomosis between hyphae also allows the horizontal transfer of my-

 

coviruses (McCabe et al., 1999), and the occurrence of mycoviruses has been demonstrated in *P. griseola* (Lima et al., 2010).

Anastomosis formation between hyphae is a character that must be considered in studies of population structure of this pathogen, to gain knowledge of its variability, providing information about how to handle the host population.

There is no report of the occurrence of anastomosis groups in *P. griseola*. Some studies have been done for the common bean fungus *Colletotrichum lindemuthianum*, from which several anastomosis groups have been identified in natural populations (Rodríguez-Guerra et al., 2003; Ishikawa et al., 2008). According to the authors, the occurrence of anastomosis could be an important mechanism that can lead to the formation of different lineages of *C. lindemuthianum*. Genetically different isolates of *C. lindemuthianum* showed the capacity for hyphal anastomosis between one another, which could contribute to increased genetic variability (Ishikawa et al., 2008). However, studies using nit mutants showed a predominance of vegetative incompatibility in *C. lindemuthianum* (Castro-Prado et al., 2007; Barcelos et al., 2011), and despite anastomosis formation, vegetative incompatibility reaction occurs afterwards, leading to cell death (Ishikawa et al., 2012).

The objective of this study was to identify the occurrence of anastomosis between isolates of *P. griseola* and to evaluate variation between isolates of the fungus collected in Minas Gerais State, Brazil, through anastomosis groups and RAPD markers.

## MATERIAL AND METHODS

### Isolates of *P. griseola*

The 20 isolates of *P. griseola* used in this study (Table 1) were collected in Minas Gerais State, Brazil, and belonged to the Laboratory of Plant Resistance to Disease, Department of Biology, Federal University of Lavras (UFLA).

**Table 1.** Isolates of *Pseudocercospora griseola* used in this study, host cultivar, origin counties/state and pathotype.

| Isolates identification | Cultivar | counties/state | Pathotype |
|---|---|---|---|
| Pg-01 | CV-13 | Ijaci-MG | 63-47 |
| Pg-02 | CV-78 | Ijaci-MG | 63-15 |
| Pg-03 | ESAL 507 | Ijaci-MG | 63-47 |
| Pg-07 | LH-10 | Ijaci-MG | 63-55 |
| Pg-08 | CI - 257 | Ijací-MG | 63-15 |
| Pg-12 | RC-I-3 | Ijaci-MG | 63-23 |
| Pg-16 | - | Ijaci-MG | 63-31 |
| Pg-19 | - | Lavras-MG | 63-47 |
| Pg-24 | RC[1]/ | Lavras-MG | 63-63 |
| Pg-35 | RC | Lavras-MG | 63-63 |
| Pg-41 | Mulatinho vagem roxa | Lavras-MG | 63-31 |
| Pg-45 | Talismã | Lavras-MG | 63-63 |
| Pg-46 | Talismã | Lavras-MG | 63-63 |
| Pg-48 | Talismã | Ijaci-MG | 63-63 |
| Pg-52 | CNFC 10453 | Lambari-MG | 63-47 |
| Pg-53 | Roxo 90 | Lambari-MG | 63-63 |
| Pg-54 | MN 3420 | Lambari-MG | - |
| Pg-55 | A-95 | Lavras-MG | - |
| Pg-63 | Carioca MG | Lambari-MG | - |
| Pg-65 | RC | Lavras-MG | - |

[1]/RC: Lineages from recurrent selection.

 

## Analysis of hyphal anastomosis

Each isolate (Table 1) was allowed to confront itself and all other isolates (Rodríguez-Guerra et al., 2003). Briefly, a sterilized microscope slide was placed on a Petri dish containing water-agar (2%) and covered with a thin layer of $M_3$ medium (Junqueira et al., 1984). Fragments of isolates under confrontation were placed on the slide 0.5 cm apart and incubated for 15 days at 22° ± 2°C. All confrontations were carried out at least in duplicate. Following incubation, the slide was lifted from the Petri dish and the original fungal fragments were carefully removed to leave only the newly formed hyphae. Hyphae were stained with a 0.05% solution of trypan blue-lactophenol, the slide was covered with a cover slip and examined under a light microscope. Anastomosis was classified as positive following observation of fusion of hyphae between paired isolates.

## DNA extraction and RAPD analysis

Mycelia from each of the 20 isolates were seeded on separate Petri dishes containing PDA medium (200 g/L potato, 10 glucose and 20 g/L agar) and incubated at 24°C for 15 days. Five agar plugs were taken from the actively growing margins of each of the colonies and transferred to Erlenmeyer flasks containing 50 mL liquid PDA medium. Flasks were incubated at 24°C for 15 days in the dark under constant agitation (110-120 rpm). The mycelial masses were filtered through cheesecloth, washed with 0.05 M EDTA, and kept at -20°C until required for DNA extraction. DNA was extracted according to the method developed by Raeder and Broda (1985), with slight modifications. After mycelium maceration, the material was kept at 65°C for 30 min, with gentle inversions every 10 min; the samples were cooled for 5 min. Thereafter, 10 mL chloroform:isoamyl alcohol (24:1) were added, followed by homogenization and centrifugation for 10 min at 4000 rpm. The supernatant was mixed with 30 mL 95% ethyl alcohol:7.5 M ammonium acetate (6:1) and placed in a freezer (-20°C) for 6 h at least. DNA was collected and dissolved in 300 µL TE (1 mM Tris, 0.1 mM EDTA, pH 7.7). DNA solution was subjected to a second extraction with chloroform:isoamyl alcohol, and the supernatant was mixed with three volumes of 95% ethyl alcohol:3 M ammonium acetate (20:1) and kept in a freezer (-20°C) for 6 h. DNA was recovered and dissolved in 50-100 µL TE.

DNA was quantified by fluorometry with a TKO100 fluorometer (Hoeffer Scientific Instruments, San Francisco, CA, USA). DNA solution (2 µL) was added to 2 mL buffer (10 mM Tris, 1.0 mM EDTA, 0.1 M NaCl, pH 7.4), with 0.1 µL/mL H32258 stain. The samples were diluted in TE to 10 ng/mL for RAPD reaction.

The RAPD reactions were carried out using the primers OP AN11, OP AP18, OP AQ01, OP AQ02, OP AQ03, OP AQ04, OP AQ08, OP AS03, OP AS04, OP AS05, OP AS06, OP AS07, OP AS08, OP AS11, OP AS15, OP AS19, OP AT19, OP BB06, and OP BB08 (Operon Technologies, Alameda, CA, USA), the sequences of which are shown in Table 2. PCR amplification was performed in a MasterCycler Gradient 5331 thermal cycler (Eppendorf, Hamburg, Germany). The reaction mixture contained 35 ng genomic DNA, 50 µM of each dNTP, 0.4 µM oligonucleotide primer, 50 mM Tris-HCl, pH 8.0, 2.0 mM $MgCl_2$, 20 mM KCl, 0.6 U Taq polymerase and 4 µL water in a final volume of 12 µL. The PCR amplification program involved: 1) two initial cycles of 2 min at 94°C (denaturation), 15 s at 37°C (annealing) and 60 s at 72°C (elongation); and 2) 38 cycles of 15 s at 94°C (denaturation), 15 s at 37°C (annealing) and 60 s at 72°C (elongation), followed by a final

extension step of 2 min at 72°C. Amplification products were separated by 2.0% (w/v) agarose gel electrophoresis with 1X TBE buffer (0.45 M Tris-borate, 0.01 M EDTA, pH 8.0) for 4.5 h at 70 V, stained with ethidium bromide (0.5 µg/mL), visualized under UV light (Fotodyne Ultraviolet Trans-Illuminator) and photographed using a Kodak EDA-290 camera.

**Table 2.** Sequences of primers used in RAPD reactions and numbers of associated polymorphic bands.

| Primers | Sequences | Numbers of associated polymorphic bands |
|---|---|---|
| OP AN11 | 5'-GTCCATGCAG-3' | 5 |
| OP AP18 | 5'-GTCGTCGACA-3' | 2 |
| OP AQ01 | 5'-GGCAGGTGGA-3' | 3 |
| OP AQ02 | 5'-ACCCTCGGAC-3' | 4 |
| OP AQ03 | 5'-GAGGTGTCTG-3' | 4 |
| OP AQ04 | 5'-GACGGCTATC-3' | 4 |
| OP AQ08 | 5'-TCGGTAGACC-3' | 4 |
| OP AS03 | 5'-ACGGTTCCAC-3' | 4 |
| OP AS04 | 5'-GTCTTGGGCA-3' | 3 |
| OP AS05 | 5'-GTCACCTGCT-3' | 2 |
| OP AS06 | 5'-GGCGCGTTAG-3' | 3 |
| OP AS07 | 5'-CACGAGCAGG-3' | 4 |
| OP AS08 | 5'-GGCTGCCAGT-3' | 3 |
| OP AS11 | 5'-ACCGTGCCGT-3' | 6 |
| OP AS15 | 5'-CTGCAATGGG-3' | 5 |
| OP AS19 | 5'-TGACAGCCCC-3' | 5 |
| OP AT19 | 5'-ACCAAGGCAC-3' | 7 |
| OP BB06 | 5'-CTGAAGCTGG-3' | 4 |
| OP BB08 | 5'-TCGTCGAAGG-3' | 4 |
| Total | | 76 |

## Statistical analysis

The DNA bands obtained for each isolate were scored on a presence (1) or absence (0) basis. Only polymorphic bands were considered in the analysis, and such bands were confirmed in at least two replicates. The genetic similarity between isolates *i* and *j* ($sg_{ij}$) was estimated from the Sorensen-Dice coefficient given by the expression $sg_{ij} = 2a / (2a + b + c)$, where *a* represents the presence of a particular band in *i* and *j*, *b* represents the presence of the band in i and absence in *j*, and *c* represents the absence of the band in *i* and presence in *j*. The estimated standard error ($s_{sg}$) associated with each similarity was determined by $s_{sg} = \{sgij [(1 - sgij) / (n - 1)]\}^{0.5}$ and *n* is the sum of *a*, *b* and *c* for each isolate pair (Skroch et al., 1992). The maximum significant similarity value ($sg_m$) was estimated from the expression $sg_m = 1 - (t.\bar{s}_{sg})$, where *t* is the tabulated value of the Student-*t* distribution at the 1% level of probability with (N - 2) degrees of freedom and $\bar{s}_{sg}$ is the mean of the estimated standard errors of the comparisons considered. Cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) using the NTSYS-PC 2.1 program (Rohlf, 2000).

Analysis of anastomosis was carried out as described above except that genetic similarities between individuals were estimated using the similarity coefficient of Russel and Rao (1940) given by the expression $sg_{ij} = a / a + b + c + d$, where *a* represents compatibility for two isolates *i* and *j*, *b* represents compatibility only for isolate *i*, *c* represents compatibility only for isolate *j*, and *d* represents incompatibility for both isolates *i* and *j*.

## RESULTS

### Anastomosis occurs in *P. griseola* and there are different anastomosis groups

All strains showed the capacity of anastomosis. Anastomosis observed was in the H form (Figure 1). The strains Pg-02, Pg-07 and Pg-08 were collected in the city of Ijaci-MG, in the same season, but under different bean lines and showed the higher percentage of compatible reactions (85%) in relation to the other strains (Table 3). The reactions were different when these strains were compared, which indicated different loci involved in this trait. The Pg-48 collected in the same county, Ijaci-MG, showed a lower percentage of anastomosis formation (45%) with the other strains.

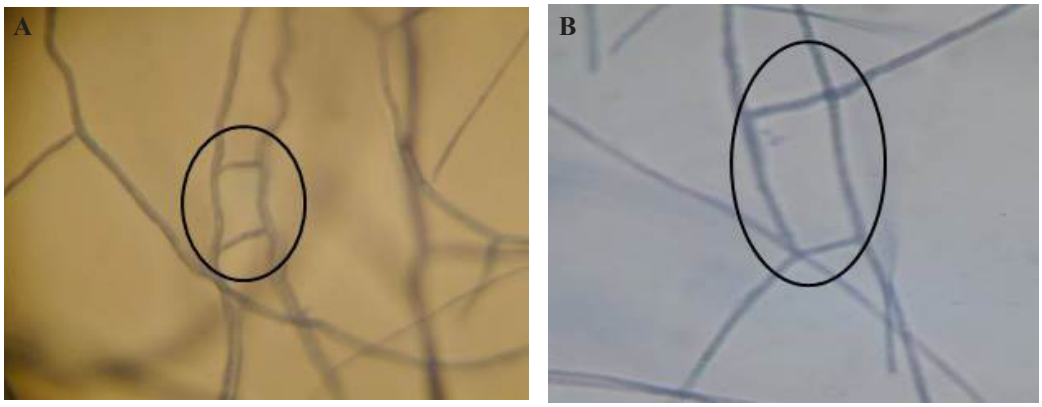


**Figure 1.** Hyphal anastomosis in H between the isolates Pg-01 and Pg-08 (**A**), and Pg-16 and Pg-52 (**B**).

**Table 3.** Compatibility reactions (determined by anastomosis fusion) between isolates of *Pseudocercospora griseola*, and the proportion of compatible reactions for each isolate.

| Isolates | Pg-01 | Pg-02 | Pg-03 | Pg-07 | Pg-08 | Pg-12 | Pg-16 | Pg-19 | Pg-24 | Pg-35 | Pg-41 | Pg-45 | Pg-46 | Pg-48 | Pg-52 | Pg-53 | Pg-54 | Pg-55 | Pg-63 | Pg-65 | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pg-01 | + | + | - | + | + | + | + | - | + | + | + | + | - | + | - | + | - | + | + | + | 75.0 |
| Pg-02 | | + | - | + | + | + | + | + | + | + | + | + | - | + | - | + | + | + | + | + | 85.0 |
| Pg-03 | | | + | + | + | - | - | + | + | + | + | + | + | - | + | + | + | + | + | + | 75.0 |
| Pg-07 | | | | + | + | + | + | - | + | + | + | + | + | + | - | + | + | + | - | + | 85.0 |
| Pg-08 | | | | | + | + | + | - | + | + | + | + | - | + | + | + | + | - | + | + | 85.0 |
| Pg-12 | | | | | | + | + | - | + | - | + | + | + | + | - | + | + | + | + | + | 80.0 |
| Pg-16 | | | | | | | + | + | - | + | - | - | + | - | + | - | - | + | + | - | 60.0 |
| Pg-19 | | | | | | | | + | + | + | + | + | + | - | - | - | + | + | - | + | 60.0 |
| Pg-24 | | | | | | | | | + | - | + | + | + | - | + | - | + | + | + | - | 75.0 |
| Pg-35 | | | | | | | | | | + | + | - | + | + | + | - | + | + | + | + | 80.0 |
| Pg-41 | | | | | | | | | | | + | - | + | - | + | + | + | + | + | - | 80.0 |
| Pg-45 | | | | | | | | | | | | + | + | - | + | - | + | - | + | + | 70.0 |
| Pg-46 | | | | | | | | | | | | | + | + | + | + | - | + | - | + | 75.0 |
| Pg-48 | | | | | | | | | | | | | | + | - | - | - | + | - | - | 45.0 |
| Pg-52 | | | | | | | | | | | | | | | + | + | + | - | + | + | 65.0 |
| Pg-53 | | | | | | | | | | | | | | | | + | - | - | + | - | 55.0 |
| Pg-54 | | | | | | | | | | | | | | | | | + | - | + | - | 65.0 |
| Pg-55 | | | | | | | | | | | | | | | | | | + | + | + | 75.0 |
| Pg-63 | | | | | | | | | | | | | | | | | | | + | + | 80.0 |
| Pg-65 | | | | | | | | | | | | | | | | | | | | + | 70.0 |

+ compatible; - incompatible.

Similarity estimates for anastomosis formation between strains varied from 0.15 to 0.85. Figure 2 shows a dendrogram and maximum similarity value ($sg_m$) at 1% probability was 0.69. Sixteen anastomosis groups were formed, and only five isolates were in the same group (Pg-01, Pg-02, Pg-07, Pg-08, and Pg-12). Each one of the remaining groups was formed by only one strain. Isolate Pg-48 showed the lowest similarity coefficient as well, according to results obtained for the percentage of compatibility.

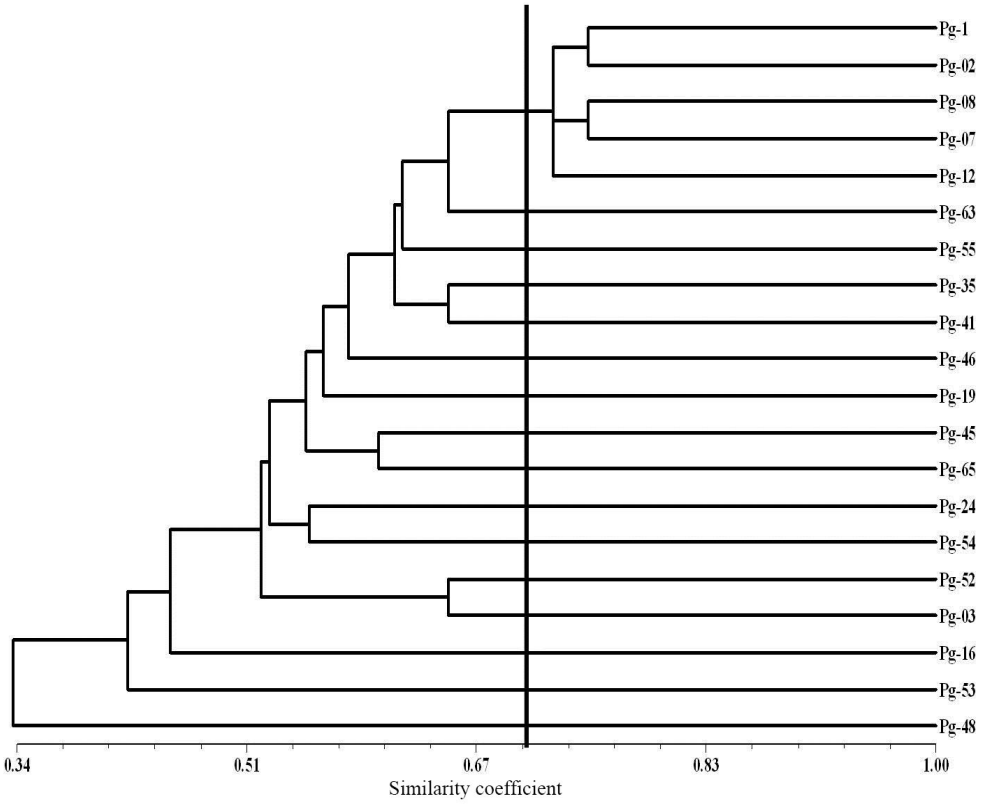


**Figure 2.** Dendrogram of genetic similarities for anastomosis formation among 20 isolates of *Pseudocercospora griseola* studied.

## Molecular diversity

Genetic analysis of diversity was performed using RAPD markers. Seventy-six polymorphic bands using 19 primers were found. The Sorensen-Dice coefficient was estimated and the matrix was constructed. Genetic similarities varied from 0.26 to 0.95. These data revealed a great genetic variability for the isolates studied. Molecular markers have already been used to study genetic variation of *P. griseola* (Mahuku et al., 2002; Sartorato, 2004; Stenglein and Balatti, 2006; Abadio et al., 2012). All these studies showed great genetic variation in the fungus.

Dendrograms were constructed using genetic similarities (Figure 3) and grouped by UPGMA. The maximum genetic similarity value was represented by the line ($sg_m$ = 0.86) at 1% probability. Only six isolates were in the same group (Pg-01, Pg-03, Pg-02, Pg-16, Pg-12,

and Pg-24). All these isolates were collected in Ijaci county-MG, except the isolate Pg-24, which was collected in Lavras-MG. The grouping of isolates according to counties only occurred for the isolates from Ijaci-MG.

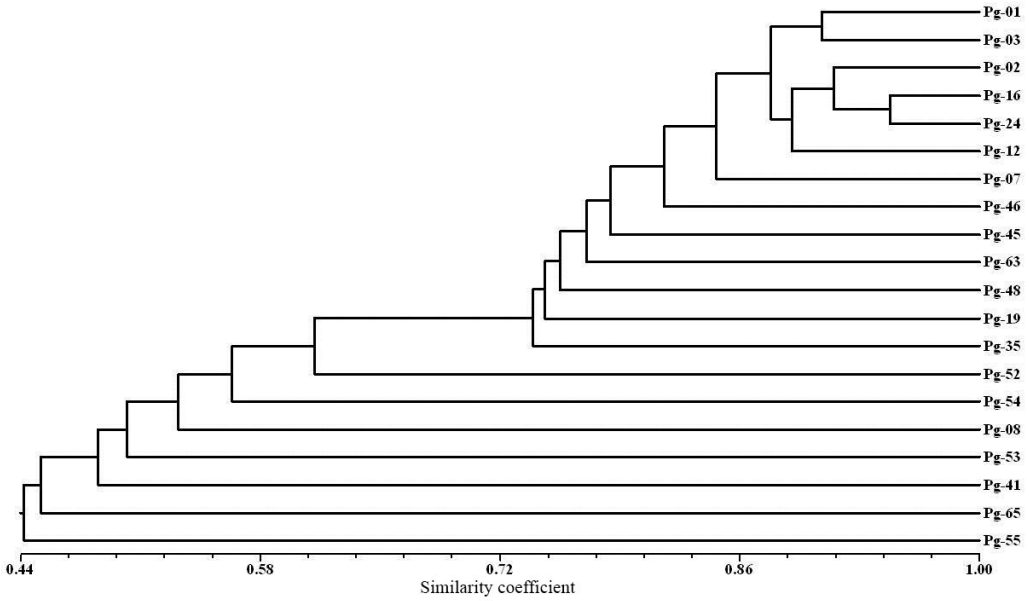


**Figure 3.** Dendrogram of genetic similarities based on RAPD markers for the 20 isolates of *Pseudocercospora griseola* studied.

## DISCUSSION

This is the first report of hyphal anastomosis in *P. griseola* fungus. Although this fungus has shown pathogenic and molecular variability (Busogoro et al., 1999; Mahuku et al., 2002; Nietsche et al., 2002; Sartorato, 2004; Stenglein and Balatti, 2006; Damasceno e Silva et al., 2008; Abadio et al., 2012), little is known about the mechanism underlying this variation in *P. griseola*.

Hyphal fusion can lead to heterokaryon formation and could cause variation within the fungus population. Anastomosis is the first step for the parasexual cycle. However, incompatible reactions were found to be frequent between isolates in the same or different populations (Saupe, 2000). Incompatibility could be a mechanism of self-defense (Glass et al., 2000). The large number of anastomosis groups was in agreement with the results obtained by Rodriguez-Guerra et al. (2003) and Ishikawa et al. (2008), who showed variability for the isolates of *C. lindemuthianum* studied and a great variation in the number of anastomosis groups.

It is important to mention that although most of the isolates were not grouped, this is not a limitation for anastomosis bridges between isolates from one group and another. For example, isolate Pg-48, showed the lowest compatibility similarity but was compatible with eight isolates (Table 3), which showed the capacity for information exchange. Rodriguez-Guerra et al. (2003) found that one strain with the capacity to fuse with isolates of another group is considered a “bridge”, allowing information exchange between different groups. The

number of compatible reactions between isolates (Table 2) could allow the horizontal transfer of mycoviruses by fused hyphae in this species. In the evaluation of 61 isolates of *P. griseola*, dsRNA fragments of mycovirus were detected in 42 isolates, showing that mycoviruses were common in this fungus (Lima et al., 2010).

As with pathogenicity, there was a high variability in the capacity of hyphal fusion in this species, which indicated that several genes must be involved in the control of this trait. Vegetative incompatibility genes were better studied in the ascomycete *Neurospora crassa*. The initial pre-fusion step is under the control of genes associated with heterokaryon self-incompatibility (*hsi*). The actual fusion step is controlled by self/non-self recognition genes termed heterokaryon incompatibility (het) genes. After fusion, the cascade of reactions leading from non-self recognition to cell death is influenced by various genes, including a number of suppressor genes that modify the signaling pathway. Finally, cell death occurs under the control of apoptotic genes (Leslie and Zeller, 1996). The fact that anastomosis occurs does not necessarily imply the formation of heterokaryon and a compatibility group.

Comparison of molecular and anastomosis grouping indicated that only three isolates (Pg-01, Pg-02 and Pg-12) were in the same group for both. These isolates were collected in the same location, but from different common bean lines. Although these isolates were similar, they were classified as different races (Damasceno e Silva et al., 2008). Isolates Pg 07 and Pg 08 were in the same compatibility group, but they were genetically different (Figure 3). Anastomosis formation depends on some genes, and genetic similarities using RAPD markers are based on a wide sample of genomic DNA, and thus, it is not expected that the traits are associated (Ishikawa et al., 2008).

Ishikawa et al. (2008) showed that genetically different isolates have the capacity to fuse. Anastomosis between these isolates that were in the same compatibility group or from different groups are important for the parasexual cycle and can lead to increased genetic variation. In this study, a relationship between anastomosis group and genotype was not observed.

It was not possible to prove the parasexual cycle in this study, which requires auxotrophic mutants as described for other species (Stromnaes and Garber, 1963; Castro-Prado et al., 2007; Milgroom et al., 2009; Barcelos et al., 2011). Other tools have recently been used such as nucleus-targeting fluorescent proteins and insertion of hygromycin- and phleomycin-resistance genes. This technique provides a better understanding of the mechanism of hyphal fusion and heterokaryon formation using fluorescence or confocal microscopy. This method was used for other plant pathogens such as *C. lindemuthianum* and *Fusarium oxysporum* (Ruiz-Roldan et al., 2010; Ishikawa et al., 2012). Additional studies are needed to elucidate further the role of hyphal fusion in *P. griseola*, as well in the population dynamics of the pathogen.

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