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Diversity among Isolates of the Tangerine Pathotype of *Alternaria alternata*

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Abstract

The tangerine pathotype of *Alternaria alternata* is the aetiological agent of Alternaria brown spot on tangerines. In the state of Paraíba, Brazil, its occurrence on "Dancy" tangerine trees is associated with genetic aspects as well as the influence of environmental conditions on reproduction and dissemination within and between populations. The aim of the present study was to evaluate the diversity of isolates of this pathogen using morphophysiological and molecular markers. For the analysis of mycelial growth and sporulation, 30 isolates from different locations were examined at 24-hour intervals until the seventh day, when the spores were quantified. The 30 isolates were characterised based on molecular markers (ISSR) and genetic similarity (Jaccard index). A factor arrangement was used: 30 isolates, four media (ODA, PDA, LEA and V8), three light regimes (continuous dark, alternating light and continuous light) and three temperatures (15˚C, 25˚C and 35˚C), with 12 repetitions. Groups 1, 2 and 3 presented low genetic variability. Group 4 showed high genetic variability of the isolates obtained from the Massaranduba (state of Paraíba-Brazil) producing region and higher mycelial growth and sporulation of *A. alternata*. The continuous light regime and the temperature 25˚C in PDA and V8 media were the ideal conditions for the mycelial growth and sporulation, respectively, of the isolates of *A. alternata*.

Keywords

Citrus tangerine, Alternaria Brown Spot, Fungal Variability, Environment Factors, ISSR Markers

1. Introduction

Alternaria diseases of citrus caused by *Alternaria* spp. (mainly *Alternaria alternata*) are important diseases with worldwide distributions. They have been documented in the USA [1], Turkey [2], Spain [3], Argentina, Brazil [4], China [5] and Iran [6]. Different diseases caused by *Alternaria* spp. on citrus species include brown spot of tangerines (*Citrus reticulata* Blanco) and their hybrids (namely the tangerine pathotype), leaf spot of rough lemon (*Citrus jambhiri* Lush) (namely the rough lemon pathotype), black rot of fruits and leaf spot on Mexican lime [7].

Alternaria brown spot, caused by the fungus *Alternaria alternata* (Fr.:Fr.) Keissler is the principal fungal disease of tangerines (*Citrus reticulate*) and their hybrids. This disease is considered a biggest problem on the fruit of many citrus cultivars grown for fresh markets. It causes lesions on leaves, stems, and fruit and reduces yield and fruit quality [5]. The symptoms of Alternaria brown spot are small black or brown spots on young leaves and small lesions with a corky appearance, with or without a chlorotic halo, which occur on the twigs and fruits [8].

This fungus produces a toxin that is responsible for most if not all of the disease symptoms. Sometimes the toxin is translocated up the veins and produces a necrosis of the veinlets that is characteristic of the disease. On highly susceptible cultivars, entire areas of the leaf may be killed and the leaves may abscise [9]. Most spores of *Alternaria* are produced by lesions on mature leaves on the tree, or recently fallen infected leaves on the grove floor. Even though spores are airborne, plantings of healthy trees will remain disease-free for long periods. If present from the outset, it builds to high populations during the period of vegetative growth on young trees and subsequently is on fruits [10].

There are more than eleven new species of *Alternaria* described as causal agents of the brown spot in different parts of the world, however, due to the conflicting morphological data, molecular studies have been expanded through polymorphism sequencing and mitochondrial restriction fragment length DNA and regions internally transcribed in the ribosome, where it has favored the identification of the close phylogenetic relationship between the isolates producing toxins and ensuring the establishment of identity as well as its variability [11].

The genetic variability of citrus pathotypes of *Alternaria alternata* is associated with the conditions of the *in vivo* or *in vitro* environment in which these fungi develop. Indeed, environmental conditions are a major cause of genetic variation within a population and exert an influence on the emergence of new genotypes and new combinations involving the fusion of gametes, the recombination of alleles during the pairing of homologous chromosomes, segregation and the generation of viable descendants [11].

In the state of Paraíba (Northeast Brazil), the occurrence of alternaria brown spot on Dancy tangerines (*Citrus tangerina* hort. ex Tanaka) caused by the tan-

gerine pathotype of *A. alternata* is associated with environment factors, such as humidity, luminosity and high temperatures, which range from 25˚C to 30˚C. These conditions are important to the reproduction and dissemination of the pathogen, which causes serious harm to citrus crops, reducing productivity and the quality of fruit [12]. However, little is known regarding the influence of variations in these environmental aspects on the genetic variability of *A. alternata*. Thus, there is a need for studies involving methodologies in controlled environments for subsequent use of the information acquired to improve the management of Alternaria brown spot on tangerines in the field.

The effectiveness of disease control strategies depends on the understanding of the pathogen and its population dynamics [13]. In this context, the study of variability in fungal populations is an important research tool. From an evolutionary point of view, the genetic variability of a population is important in determining the potential for adaptation of an organism to different environmental conditions. Furthermore, from the epidemiological point of view, pathogenic variability has direct implications for disease management [14]. Therefore, it is of great importance to obtain information on the molecular analyzes of use in combination with morphological studies, which opens new avenues to find evidence of host specificity for this pathogen in the state of Paraíba, Brazil. The aim of the present study was to evaluate the diversity of isolates of the tangerine pathotype of *A. alternata* using morphophysiological and molecular markers.

2. Material and Methods

The morphophysiological analyses were performed at the Phytopathology Laboratory and the molecular analyses were performed in the molecular biology sector of the Animal Product Analysis Laboratory, both of which are located in the Centro de Ciências Agrárias of the Universidade Federal da Paraíba, Campus II, Areia, Brazil.

Thirty-four isolates of the tangerine pathotype of *A. alternata* were collected from citrus production areas in the state of Paraíba. Three isolates were obtained from the collection of the Phytopathology Laboratory of the School of Agrarian and Veterinary Sciences of the Universidade do Estado de São Paulo, Jaboticabal, Brazil, which had been obtained from symptomatic leaves of the "Fortune" tangor (*C. clementina* × *C. tangerina*) in the citrus production region of Pratânia, state of São Paulo, Brazil. One isolate of the tangerine pathotype of *A. alternata* was obtained from Portugal from tangerines (*C. tangerina*) with typical symptoms of alternaria brown spot in the region of Trás os Montes, Portugal, for to comparation with Brazilian isolates (**Table 1**).

Isolates from the state of Paraíba were obtained from leaves fragments measuring 5 mm in diameter disinfected by rinsing with 70% alcohol, 1% hypochlorite and sterilised distilled water (SDW) inoculated in potato dextrose agar (PDA). For the confirmation of morphological identity, three discs with fungal colonies measuring 5 mm in diameter were transferred to calcium carbonate

Table 1. Isolates of *Alternaria alternata* pathotype Tangerine used in this work. UFPB, Areia, Brazil, 2016.

Identification of isolates	Varieties	Local of production
1, 2, 3, 4, 5, 6	Tangerine "Dancy"/(*Citrus tangerina*)	Lagoa Seca
7, 8, 9, 10, 11, 12, 13, 14	Tangerine "Dancy"/(*Citrus tangerina*)	Massaranduba
15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 36, 37, 38	Tangerine "Dancy"/(*Citrus tangerina*)	Matinhas
27, 28, 29	Tangor "Fortune"/(*C. clementina* × *C. tangerina*)	Pratânia
30	Tangerine/(*Citrus tangerina*)	Trás os Montes
31, 32, 33, 34, 35	Tangerine "Dancy"/(*Citrus tangerina*)	Remígio

medium (30 g of $CaCO_3$, 20 g of sucrose, 20 g of agar and 1000 mL of SDW) [15]. After seven days of culture, pigmentation, texture and consistency were determined of the front and back of the colonies developed. Staining was performed with methylene blue and stained microstructures were examined on electronic microscope slides. The identification was confirmed based on macrostructures and microstructures.

Spores were collected from fragments of the $CaCO_3$ medium and transferred to test tubes containing 9 mL of SDW. Serial dilutions were performed by transferring 1 mL of suspension with 1×10^5 spores/mL into the third tube, distributed in three Petri dishes containing 20 g of agar and 1000 mL of SDW and incubated at room temperature (25˚C ± 2˚C) for a maximum of 12 hours or until the germination of the spores. Monosporic cultures were obtained by collecting a single spore with the aid of a light microscope and a bronze rod specifically developed for this purpose. The monosporic isolates were transferred to test tubes containing PDA and incubated for seven days at room temperature (25˚C ± 2˚C) until the time of use.

The mycelial diameter of thirty isolates of the tangerine pathotype of *A. alternata* was measured after cultivation of the fungus under biochemical oxygen demand conditions adjusted to different light regimes (continuous dark, alternating light [12 hours of light and 12 hours of dark] and continuous light) with fluorescent light of 40 watts and lamps distant 30 cm of Petri dishes, temperatures (15˚C, 25˚C and 35˚C) and culture media (oatmeal dextrose agar [ODA—65 g of oatmeal, 20 g of dextrose, 20 g of agar and 1000 mL of SDW], PDA, leaf extract agar [LEA—400 g of citric leaves, 20 g of dextrose, 20 g of agar and 1000 mL of SDW] and V8 agar [100 mL of V8 juice, 20 g of agar, 15 g of $CaCO_3$ and 900 mL of SDW]) [16]. The pH of all media was adjusted to 6.0 and sterilisation was then performed in an autoclave at 120˚C for 20 minutes.

Means of mycelial growth of the treatments were performed at 24 hour intervals, with a graduated ruler the two orthogonal axes were measured until reaching the entire 13 mm diameter Petri dish and the results were expressed in (mm) until the day of evaluation [17]. The sporulation averages of the treatments were

made from spore counts (obtained by the addition of 10 mL of sterile distilled water in Petri dishes with the fungal colony, where the spores were released with the aid of a sterilized spatula and filtered through a double layer of sterile gauze) in Neubauer chamber, where the suspension was adjusted to 10^5 and the results were expressed as (n˚ × 10^5 spores/mL) [18].

Among the 38 monosporic isolates used for the molecular characterisation, five fragments of mycelial discs measuring 5 mm^2 were transferred to plastic Falcon tubes. Each tube contained 30 mL of PDA and was agitated for two hours twice a day for seven days at room temperature (25˚C ± 2˚C). After the mycelial growth of the isolates, the PDA broth was removed with a vacuum pump and the mycelial mass of each isolate was filtered, pulverised in liquid nitrogen and placed into 2 mL microtubes. To obtain inter simple sequence repeats (ISSR), three primers were used that produce greater polymorphism in the bands of the electrophoresis gels with the polymerase chain reaction (PCR) of the isolates analysed [19]. DNA extraction was performed in a microcentrifuge with the cetyltrimethylammonium bromide method [20] adapted for the UltraClean® Microbial DNA extraction kit using the protocol described by the manufacturer (MO BIO Laboratories Inc., Carlsbad, CA, USA). Total extracted DNA was analysed in 1% agarose gel, using GelRed Biotium® for viewing under ultraviolet light and quantified in the NanoDrop 2000 spectrophotometer (Thermo Scientific). The concentration of total extracted DNA ranged from 8 to 20 ng/μL and was adjusted to 10 ng/μL for the ISSR reactions.

Each 25-μL ISSR reaction was made from a mixture of 22.5 μL of PCR Mix (Invitrogen Life Technologies) (22 U/mL of recombinant *Taq* DNA polymerase, 22 mM of Tris-HCL, 55 mM of KCl, 1.65 mM of $MgCl_2$, 220 μM of dGTP, 220 μM of dATP, 220 μM of dTTP, 220 μM of dCTP and stabilisers), 1.5 μL of each primer (10 pmol) and 1.0 μL of DNA from each isolate. The reactions were performed using the Platinum® PCR SuperMix Kit from Invitrogen™ in a Techne thermal cycler (TC-5000, Techne Inc., United Kingdom), with initial denaturation at 94˚C for 4 min, followed by 40 cycles of 94˚C for 40 seconds and an annealing temperature ranging from 45.5˚C to 46.5˚C, depending on the ISSR primer (CA1-5'ACCACACACACACACACA3',
CA2-5'ATCACACACACACACACA3',
CAC3 5'ATGCACCACCACCACCAC3'), with extension at 72˚C for 2 min and final extension at 72˚C for 7 min.

The PCR products were stained with Gel Red®, separated by electrophoresis in agarose gel at a concentration of 1.5% and immersed in TBE buffer (0.45 M of Tris-borate and 0.01 M EDTA). The electrophoretic run was performed with 80 volts for 90 min and the PCR products were viewed in a Gel Logic 212 PRO transilluminator (Carestream Molecular Imaging Software-Version 5.0, Carestream Health, Inc., USA). Analysis of the ISSR products from the isolates was performed using the unweighted pair group method with arithmetic mean (UPGMA). The Jaccard similarity index [21] was used with a tolerance of 2% to

determine the genetic similarity between the groups formed and plotted on the dendogram.

The genetic differentiation among populations was analyzed through the analysis of the genetic diversity in populations subdivided from Nei [22] [23] [24], which allows the comparing the levels of heterozygosity between and within populations, as well as obtaining an estimate of the divergence.

An entirely randomised factorial design was used for the morphophysiological characterisation, with 30 isolates, four culture media (ODA, PDA, LEA and V8), three light regimes (continuous dark, alternating light [12 h of light and 12 h of dark] and continuous light) and three temperatures (15˚C, 25˚C and 35˚C), with 12 repetitions composing an experimental area of 12.960 Petri dishes, with each Petri dish considered one repetition. Analysis of variance (F test) was performed to determine differences in mycelial growth and sporulation between isolates under the different culture conditions. Means were grouped for the analysis of the effects of the different culture media, light regimes and temperatures as well as interactions between these variables on mycelial growth and sporulation of the tangerine pathotype of *A. alternata*. General means were compared using the Scott-Knott test and other means were compared using Tukey's test. Statistical analysis was conducted with the aid of the SISVAR® 5.3 (Ferreira 2010) and SAS/STAT® 9.3 [25] software programs, with the level of significance set to 5% ($p \leq 0.05$).

Genetic diversity among the 38 isolates was determined based on the presence/absence of DNA fragments amplified by three primers and viewed in electrophoresis gels. The gel images were processed using the BioNumerics® software (version 7.1, Applied Maths, Belgium) for groups of isolates and the analysis of genetic similarity based on the Jaccard coefficient with 2% tolerance. The similarity dendogram was plotted from the arithmetic means of the groups formed in pairs of combined data (UPGMA).

3. Results

With regard to collection site, all isolates of *A. alternata* demonstrated differences in terms of mycelial growth and sporulation. The highest mycelial growth was found for isolates 2, 6, 7, 8, 9, 10, 11, 12, 14, 20, 24 and 30 (7.20, 7.63, 7.11, 7.35, 7.29, 7.54, 7.29, 7.75, 7.34, 7.15, 7.26 and 7.29 mm, respectively) and the lowest growth was found for isolates 5, 13, 23 and 26 (2.95, 2.97, 3.05 and 3.09 mm, respectively. The greatest mean spore production occurred with isolates 6, 7, 8, 10, 11, 12 and 30 (2.25, 2.18, 2.25, 2.40, 2.42, 2.53 and 2.68 × 10^5 spores/mL, respectively) and lowest mean production occurred with isolates 1, 9 and 15 (0.30, 0.35 and 0.13 × 10^5 spores/mL (**Table 2**).

With regard to the culture conditions, all isolates differed significantly in terms of mycelial growth. Isolates 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 demonstrated greater mycelial growth (12.47, 12.44, 12.73, 11.19, 10.12, 11.06, 12.30, 12.27, 11.82, 10.17, 10.38 and 10.86 mm, respectively) when cultivated in

Table 2. Mean values of Mycelial Growth (MG) and Sporulation (SP) of *Alternaria alternata* isolates pathotype Tangerine. UFPB, Areia, Brazil, 2016.

Locations	Isolates	MG (mm)	SP (10^5 spores/mL)
Lagoa Seca	1	6.64 e	0.30 f
	2	7.20 b	0.45 e
	3	6.99 c	0.45 e
	4	6.81 d	0.56 d
	5	2.95 f	1.05 c
	6	7.63 a	2.25 a
Massaranduba	7	7.11 a	2.18 a
	8	7.35 a	2.25 a
	9	7.29 b	0.35 f
	10	7.54 a	2.40 a
	11	7.29 a	2.42 a
	12	7.75 a	2.53 a
	13	2.97 f	0.75 d
	14	7.34 b	0.76 d
Matinhas	15	6.66 e	0.13 g
	16	6.86 d	0.39 e
	17	6.55 e	0.45 e
	18	6.87 d	0.48 e
	19	6.89 d	0.51 e
	20	7.15 c	0.60 d
	21	6.95 d	0.67 d
	22	7.05 c	0.89 c
	23	3.05 f	1.00 c
	24	7.26 b	1.02 c
	25	6.76 d	1.02 c
	26	3.09 f	1.15 c
Pratânia	27	4.49 e	0.43 e
	28	6.29 b	0.65 d
	29	6.92 d	1.24 c
Trás os Montes	30	7.29 a	2.68 a

Means followed by the same letter do not differ from one another by the Scott-Knott test ($p \leq 0.05$).

continuous dark, alternating light and continuous light at a temperature of 25˚C in the PDA and V8 media through to the seventh day of evaluation (Table 3).

Isolates 15 to 26 demonstrated greater mycelial growth (10.68 to 10.90 mm) when incubated with continuous light at 25˚C in the PDA and V8 media.

Table 3. Mean values of Mycelial Growth (MG) of *Alternaria alternata* isolates, pathotype Tangerine on different cultive conditions. UFPB, Areia, Brazil, 2016.

Locations	Light Regime	Temperature (˚C)	Culture Media			
			ODA	PDA	LEA	V8
			MG (mm)			
Lagoa Seca	CD	15	5.02 b$a\beta$	5.65 aBa	4.58 b$a\beta$	3.18 cCβ
		25	7.61 bAa	12.47 aAa	9.48 cAa	11.49 aAa
		35	3.74 bCβ	4.98 aCβ	4.13 bBβ	4.09 bBβ
	AL	15	5.91 aCa	5.87 aCβ	4.78 bCa	4.55 bCa
		25	9.82 bAβ	12.44 aAa	9.61 bAa	10.12 aAa
		35	7.02 aBa	6.77 aBa	6.43 aCa	6.47 aBa
	CL	15	5.37 bBβ	6.87 aBβ	4.16 cBβ	4.79 bBa
		25	11.51 bAa	12.73 aAa	8.12 cAβ	11.06 aAa
		35	3.94 abCβ	4.23 aCχ	2.86 cCχ	3.45 bcCχ
Massaranduba	CD	15	5.19 bCβ	6.22 aB$a\beta$	4.13 cCa	4.89 bBa
		25	10.61 bAa	12.30 aAa	8.54 cAa	10.17 aAa
		35	7.58 aBa	4.70 bCβ	5.25 bBβ	3.86 cCβ
	AL	15	5.66 bCβ	6.25 aCa	4.13 cCa	4.53 cCβ
		25	9.60 bAa	12.27 aAa	8.71 dAa	10.38 aAa
		35	6.26 bBβ	7.80 aBa	6.68 bBa	7.44 aBa
	CL	15	7.57 aBa	5.72 bBβ	4.38 cBa	5.49 bCβ
		25	9.90 bAa	11.82 aAa	9.05 dAa	10.86 aAa
		35	5.62 bCχ	4.54 cCβ	4.85 cBβ	7.49 aBa
Matinhas	CD	15	5.57 bCβ	6.34 aBβ	4.99 cBa	4.10 dCβ
		25	9.90 bAβ	11.07 aAβ	7.45 dAa	8.23 cAχ
		35	6.67 aBa	4.62 cCβ	4.40 cCβ	5.68 bBβ
	AL	15	5.59 bCβ	6.64 aCa	4.46 dCβ	5.12 cCa
		25	10.23 bAa	10.73 aAβ	7.67 dAa	9.33 cAa
		35	6.20 bBβ	7.20 aBa	6.29 bBa	6.24 bBa
	CL	15	6.29 aCa	6.36 aB$a\beta$	4.92 bBa	5.21 bBa
		25	10.17 bA$a\beta$	10.68 aAa	7.42 dAa	10.90 aAa
		35	6.65 aBa	4.31 bCχ	3.79 cCχ	4.22 bCχ
Pratânia	CD	15	7.10 aBa	5.86 bBβ	2.37 dCβ	4.93 cCa
		25	12.02 aAa	10.37 bAβ	6.45 dAβ	12.25 aAa
		35	6.58 aCa	2.87 dCχ	3.50 cBχ	5.57 bBβ
	AL	15	5.45 aCβ	5.23 aCχ	4.35 bCa	4.69 bCa
		25	10.32 aAχ	10.30 aAβ	8.90 bAa	8.97 bAβ
		35	6.59 bBa	8.08 aBa	6.63 bBa	6.44 bBa

Continued

		15	7.23 aBα	6.37 bBα	4.67 cBα	5.07 cBα
	CL	25	11.41 aAβ	10.80 bAα	9.12 cAα	9.50 cAα
		35	6.26 aCα	5.65 bCβ	4.22 dCβ	4.72 cBχ
		15	6.74 aBα	6.60 aBα	4.10 bABα	4.56 bBα
	CD	25	10.65 aAα	10.97 aAα	5.78 bAα	10.27 aAα
		35	6.29 aBα	2.61 bCχ	3.96 bB$\alpha\beta$	7.23 bAα
		15	5.59 aB$\alpha\beta$	4.30 abCβ	2.92 bBα	5.01 aBα
Trás os Montes	AL	25	8.71 aAβ	10.39 aAα	5.67 bAα	10.50 aAα
		35	6.51 aBα	7.82 aBα	4.28 bABα	6.86 bAα
		15	4.64 abBβ	6.45 aBα	3.42 bBα	5.30 abBα
	CL	25	6.51 bcAβ	10.63 aAα	5.98 cAα	10.31 aAα
		35	7.45 bAα	4.84 aBβ	2.50 bBβ	2.45 bCβ

Locations	Light Regime	Temperature (˚C)	Culture Media			
			ODA	PDA	LEA	V8
			MG (mm)			
		15	5.02 b$\alpha\beta$	5.65 aBα	4.58 b$\alpha\beta$	3.18 cCβ
	CD	25	7.61 bAα	12.47 aAα	9.48 cAα	11.49 aAα
		35	3.74 bCβ	4.98 aCβ	4.13 bBβ	4.09 bBβ
		15	5.91 aCα	5.87 aCβ	4.78 bCα	4.55 bCα
Lagoa Seca	AL	25	9.82 bAβ	12.44 aAα	9.61 bAα	10.12 aAα
		35	7.02 aBα	6.77 aBα	6.43 aCα	6.47 aBα
		15	5.37 bBβ	6.87 aBβ	4.16 cBβ	4.79 bBα
	CL	25	11.51 bAα	12.73 aAα	8.12 cAβ	11.06 aAα
		35	3.94 abCβ	4.23 aCχ	2.86 cCχ	3.45 bcCχ
		15	5.19 bCβ	6.22 aB$\alpha\beta$	4.13 cCα	4.89 bBα
	CD	25	10.61 bAα	12.30 aAα	8.54 cAα	10.17 aAα
		35	7.58 aBα	4.70 bCβ	5.25 bBβ	3.86 cCβ
		15	5.66 bCβ	6.25 aCα	4.13 cCα	4.53 cCβ
Massaranduba	AL	25	9.60 bAα	12.27 aAα	8.71 dAα	10.38 aAα
		35	6.26 bBβ	7.80 aBα	6.68 bBα	7.44 aBα
		15	7.57 aBα	5.72 bBβ	4.38 cBα	5.49 bCβ
	CL	25	9.90 bAα	11.82 aAα	9.05 dAα	10.86 aAα
		35	5.62 bCχ	4.54 cCβ	4.85 cBβ	7.49 aBα
		15	5.57 bCβ	6.34 aBβ	4.99 cBα	4.10 dCβ
Matinhas	CD	25	9.90 bAβ	11.07 aAβ	7.45 dAα	8.23 cAχ
		35	6.67 aBα	4.62 cCβ	4.40 cCβ	5.68 bBβ

Continued

Matinhas	AL	15	5.59 bCβ	6.64 aCα	4.46 dCβ	5.12 cCα
		25	10.23 bAα	10.73 aAβ	7.67 dAα	9.33 cAα
		35	6.20 bBβ	7.20 aBα	6.29 bBα	6.24 bBα
	CL	15	6.29 aCα	6.36 aB$\alpha\beta$	4.92 bBα	5.21 bBα
		25	10.17 bA$\alpha\beta$	10.68 aAα	7.42 dAα	10.90 aAα
		35	6.65 aBα	4.31 bCχ	3.79 cCχ	4.22 bCχ
	CD	15	7.10 aBα	5.86 bBβ	2.37 dCβ	4.93 cCα
		25	12.02 aAα	10.37 bAβ	6.45 dAβ	12.25 aAα
		35	6.58 aCα	2.87 dCχ	3.50 cBχ	5.57 bBβ
Pratânia	AL	15	5.45 aCβ	5.23 aCχ	4.35 bCα	4.69 bCα
		25	10.32 aAχ	10.30 aAβ	8.90 bAα	8.97 bAβ
		35	6.59 bBα	8.08 aBα	6.63 bBα	6.44 bBα
	CL	15	7.23 aBα	6.37 bBα	4.67 cBα	5.07 cBα
		25	11.41 aAβ	10.80 bAα	9.12 cAα	9.50 cAα
		35	6.26 aCα	5.65 bCβ	4.22 dCβ	4.72 cBχ
	CD	15	6.74 aBα	6.60 aBα	4.10 bABα	4.56 bBα
		25	10.65 aAα	10.97 aAα	5.78 bAα	10.27 aAα
		35	6.29 aBα	2.61 bCχ	3.96 bB$\alpha\beta$	7.23 bAα
Trás os Montes	AL	15	5.59 aB$\alpha\beta$	4.30 abCβ	2.92 bBα	5.01 aBα
		25	8.71 aAβ	10.39 aAα	5.67 bAα	10.50 aAα
		35	6.51 aBα	7.82 aBα	4.28 bABα	6.86 bAα
	CL	15	4.64 abBβ	6.45 aBα	3.42 bBα	5.30 abBα
		25	6.51 bcAβ	10.63 aAα	5.98 cAα	10.31 aAα
		35	7.45 bAα	4.84 aBβ	2.50 bBβ	2.45 bCβ

The cultivation of the fungus adjusted to different light regimes (continuous dark—CD, alternating light—AL [12 hours of light and 12 hours of dark] and continuous light—CL), temperatures (15˚C, 25˚C and 35˚C) and culture media ODA (oatmeal dextrose agar—65 g of oatmeal, 20 g of dextrose, 20 g of agar and 1000 mL of SDW], PDA (potato dextrose agar—200 g of potato, 20 g of dextrose, 20 g of agar and 1000 mL SDW), LEA (leaf extract agar—400 g of citric leaves, 20 g of dextrose, 20 g of agar and 1000 mL of SDW] and V8 agar [100 mL of V8 juice, 20 g of agar, 15 g of $CaCO_3$ and 900 mL of SDW]). Means followed by the same letter do not differ by the Tukey test ($p \leq 0.05$). Greek letter for light regimes, lowercase for culture media and upper case for temperatures.

Isolates 27, 28 and 29 demonstrated greater mycelial growth (12.02 to 12.25 mm in diameter) when incubated with continuous dark at 25˚C in the ODA and V8 media. Isolate 30 demonstrated greater mycelial growth (10.27 to 10.97 mm) when incubated with continuous dark, alternating light and continuous light at 25˚C in the PDA, V8 and ODA media (Table 3).

Alternating light regimes, temperatures of 15˚C and 35˚C were observed, and ODA and LEA media did not influence the mycelial growth of most isolates of

A. alternata until the seventh day of evaluation (Table 3).

Isolates 1, 2, 3, 4, 5 and 6 exhibited greater sporulation (3.09×10^5 spores/mL) when incubated in a continuous dark regime at 25˚C in the V8 medium. The same regime at 35˚C in LEA medium yielded 2.30×10^5 spores/mL and a continuous light regime at 25˚C in the LEA and V8 media yielded 3.60 and 1.65×10^5 spores/mL, respectively. These culture conditions favoured greater sporulation of the pathogen over the other culture conditions through to the seventh day of evaluation (Table 4).

The sporulation of isolates 7 to 14 was favoured when incubated in continuous dark at 25˚C in the V8 medium (1.69×10^5 spores/mL). At 35˚C, these isolates yielded 2.55 and 2.15×10^5 spores/mL in the LEA and V8, respectively. The continuous light regime in the V8 medium stimulated these isolates to produce 1.78 and 1.65×10^5 spores/mL at 25˚C and 35˚C, respectively (Table 4).

The sporulation of isolates 15 to 26 was favoured when incubated in continuous dark at 35˚C in the LEA and V8 media (1.23 and 2.75×10^5 spores/mL, respectively). Continuous light at 25˚C in the LEA and V8 media stimulated the production of 3.83 and 2.19×10^5 spores/mL, respectively (Table 4).

Isolates 27, 28 and 29 exhibited greater sporulation (2.76×10^5 spores/mL) when incubated in continuous dark at 25˚C in the V8 medium. At 35˚C, the LEA and V8 media stimulated the production of 1.56 and 1.42×10^5 spores/mL, respectively. Continuous light in the V8 medium stimulated the production of 2.21 and 2.22×10^5 spores/mL at 25 and 35˚C, respectively (Table 4).

In continuous dark, isolate 30 produced 3.90×10^5 spores/mL when incubated at 25˚C in the V8 medium and 5.35×10^5 spores/mL at 35˚C in the LEA medium. Continuous light at 25˚C in the V8 medium stimulated the production of 3.50×10^5 spores/mL. These culture conditions favoured greater sporulation of the isolate of the tangerine pathotype of *A. alternata* in comparison to the other conditions through to the seventh day of evaluation (Table 4).

It was verified that alternating light regime, temperature of 15˚C, means ODA and PDA did not influence the production spores of most isolates of *A. alternata* until the seventh day of evaluation (Table 4).

Genetic diversity among the isolates demonstrated by the ISSR markers evaluated. Based on approximately 20 polymorphic bands, the isolates formed three groups with genetic similarity greater than 24% and one with 14.1%. Group 1 was formed by isolates 2, 3, 13, 15, 17, 24, 28, 29 and 33 from Lagoa Seca, Massaranduba (state of Paraíba), Matinhas (Paraíba), Pratânia (state of São Paulo) and Remígio (state of Paraíba). Group 2 was formed by isolates 6, 9, 18, 19, 21, 22, 26, 35, 37 and 38 from Lagoa Seca, Matinhas and Remígio in the state of Paraíba. Group 3 was composed of isolates 1, 4, 5, 14, 16, 20, 23, 25, 27, 30, 31, 32, 34 and 36 from Lagoa Seca (Paraíba), Massaranduba (Paraíba), Matinhas (Paraíba), Pratânia (Paraíba), Trás os Montes (Portugal) and Remígio (Paraíba) (Figure 1). Isolates 7, 8, 10, 11 and 12 from the region of Massaranduba (Paraíba) (Group 4).

Table 4. Sporulation of *Alternaria alternata* pathotype Tangerine in different culture conditions. UFPB, Areia, Brazil, 2016.

Localities	Light Regimes	Temperature (˚C)	Culture Media			
			ODA	PDA	LEA	V8
			SP (10^5 spores/mL)			
Lagoa Seca	CD	15	0.02 bAβ	0.25 bB$\alpha\beta$	1.12 aAβ	1.78 aBα
		25	0.09 cA$\alpha\beta$	0.51 bAα	1.16 bAβ	3.09 aAα
		35	0 bAα	0.03 bBα	2.30 aAα	0.81 aCα
	AL	15	0.72 bAα	0.51 cAβ	1.92 aAβ	0.65 bAβ
		25	0.38 bAα	0.004 cAβ	2.35 bcBα	1.17 aAβ
		35	0.006 aBα	0.13 aBα	0.16 aBα	0.05 aBβ
	CL	15	0 cAβ	0.63 bAα	1.30 bB$\alpha\beta$	1.53 aAβ
		25	0.001 cAβ	0.12 cBβ	3.60 aAα	1.65 aAα
		35	0 bAα	0.005 bBα	0.69 aCα	0.005 bBβ
Massaranduba	CD	15	0.41 cAβ	0.91 cAα	0.37 bAα	0.82 aBβ
		25	0.75 cAβ	0.41 cAα	0.69 bAα	1.69 aAα
		35	0.83 bAα	0.41 bAα	2.55 aAα	2.15 aAα
	AL	15	0.13 bAβ	0.13 bAα	0.22 bAα	0.96 ABβ
		25	0.54 cAβ	0.41 cAα	0.32 bAβ	1.13 aAβ
		35	0.77 bAα	0 bAα	0 bBα	0.49 aBβ
	CL	15	0.65 bAα	0.15 cAα	0.46 bcBα	9.167 aBχ
		25	0.50 cAα	0 dBα	1.37 bAα	1.78 aAα
		35	0 aBα	0 aBα	0.04 aCα	1.65 aAα
Matinhas	CD	15	0.22 bBβ	0.03 bAβ	0.048 aBβ	0.064 aBβ
		25	0.16 cAβ	0.07 cAβ	0.089 bAβ	0.01 aAβ
		35	0.09 bBα	0.002 cAα	1.23 aAα	2.75 aAα
	AL	15	0.11 bBβ	0.12 bAβ	0.13 bAα	0.10 aBβ
		25	0.04 cAα	0.17 cAβ	0.14 bCα	0.10 bAα
		35	0.001 B$\alpha\beta$	0.01 bBα	0.02 bBα	0.08 aBβ
	CL	15	0.04 cAα	0.090 bAα	0.09 bBα	1.62 bAα
		25	0.04 cA$\alpha\beta$	0.56 bAα	3.83 aAα	2.19 aAα
		35	0 bBβ	0.02 bBα	0.04 abCα	1.50 aBχ
Pratânia	CD	15	0.13 bcAα	0 cAβ	0.55 bBα	1.36 aB$\alpha\beta$
		25	0.11 cAα	0 cAβ	0.09 bAα	2.76 aAα
		35	0.05 bAα	0 bAα	1.56 aAα	1.42 aAα
	AL	15	0.02 bAα	0.04 abBα	0.01 bAβ	1.23 aBβ
		25	0.23 cAα	1.51 bAα	0.04 cAβ	0.02 cAα

Continued

		35	0 bAα	0.28 abBα	0.14 abAα	0.42 aBβ
		15	0.24 cAα	0.77 bAα	1.56 abAα	0 aBχ
	CL	25	0.06 cAα	0.50 bAα	1.65 bAα	2.21 aAα
		35	0 aAα	0 aBα	0.01 aBα	2.22 aAα
		15	0.50 abBα	0.04 bBβ	0.06 bCα	1.06 aBα
	CD	25	2.03 bAα	1.20 bAα	0.78 bBα	3.90 aAα
		35	0.27 bcAβ	0.85 bAα	5.35 aAα	3.06 bBβ
		15	0.35 bCα	0.35 bB$\alpha\beta$	0.32 bAα	0.14 aBα
Trás os Montes	AL	25	1.47 aBα	0.84 aA$\alpha\beta$	0.82 bAα	1.23 aBβ
		35	1.28 bAα	0 dBβ	1.53 cAβ	0.71 bAα
		15	0.75 abBα	0.97 abAα	0.35 bAα	1.60 bAα
	CL	25	0.76 bBα	0.52 bAβ	1.38 bAα	3.50 aAα
		35	2.03 aAβ	0.80 bAα	0.25 bcAβ	0 cBχ

The cultivation of the fungus adjusted to different light regimes (continuous dark—CD, alternating light—AL [12 hours of light and 12 hours of dark] and continuous light—CL), temperatures (15˚C, 25˚C and 35˚C) and culture media ODA (oatmeal dextrose agar—65 g of oatmeal, 20 g of dextrose, 20 g of agar and 1000 mL of SDW], PDA (potato dextrose agar—200 g of potato, 20 g of dextrose, 20 g of agar and 1000 mL SDW), LEA (leaf extract agar—400 g of citric leaves, 20 g of dextrose, 20 g of agar and 1000 mL of SDW] and V8 agar [100 mL of V8 juice, 20 g of agar, 15 g of $CaCO_3$ and 900 mL of SDW]). Means followed by the same letter do not differ by the Tukey test ($p \leq 0.05$). Greek letter for light regimes, lowercase for culture media and upper case for temperatures.

4. Discussion

There is no consensus in the literature regarding the effect of light on the biochemical mechanisms the formation of mycelial growth and conidiophores of *Alternaria* spp. However, the light regime exerts an important influence on sporulation in different species of phtyopathogenic fungi, such as *Alternaria brassicae* (Berk.) Sacc., *A. solani* and *A. alternata* [26]. In the present study, continuous dark and continuous light stimulated greater sporulation of the isolates of the *A. alternata*. Thus, such culture conditions could be considered ideal for the greater production of spores by this pathogen.

The production of spores of *A. alternata* is high in prolonged periods of dark, which is in agreement with the present findings. Aragaki [27] states that the terminal phase of the sporulation cycle of different fungal species may occur in continuous darkness, since the nucleic acids and sporogenic substances that compose the conidia remain for long periods in the absence of light. Thus, sporulation is induced in filamentous fungi. A long period of light can also exert an influence on the activation of key enzymes [28] responsible for the synthesis of compounds in the culture media that are essential to the sporulation of different fungal species [29].

Based on the present study and inferring what occurs under different environmental conditions, isolates of the pathogen in agricultural crops may sporulate less than in culture media, since tangerines are submitted to an alternating

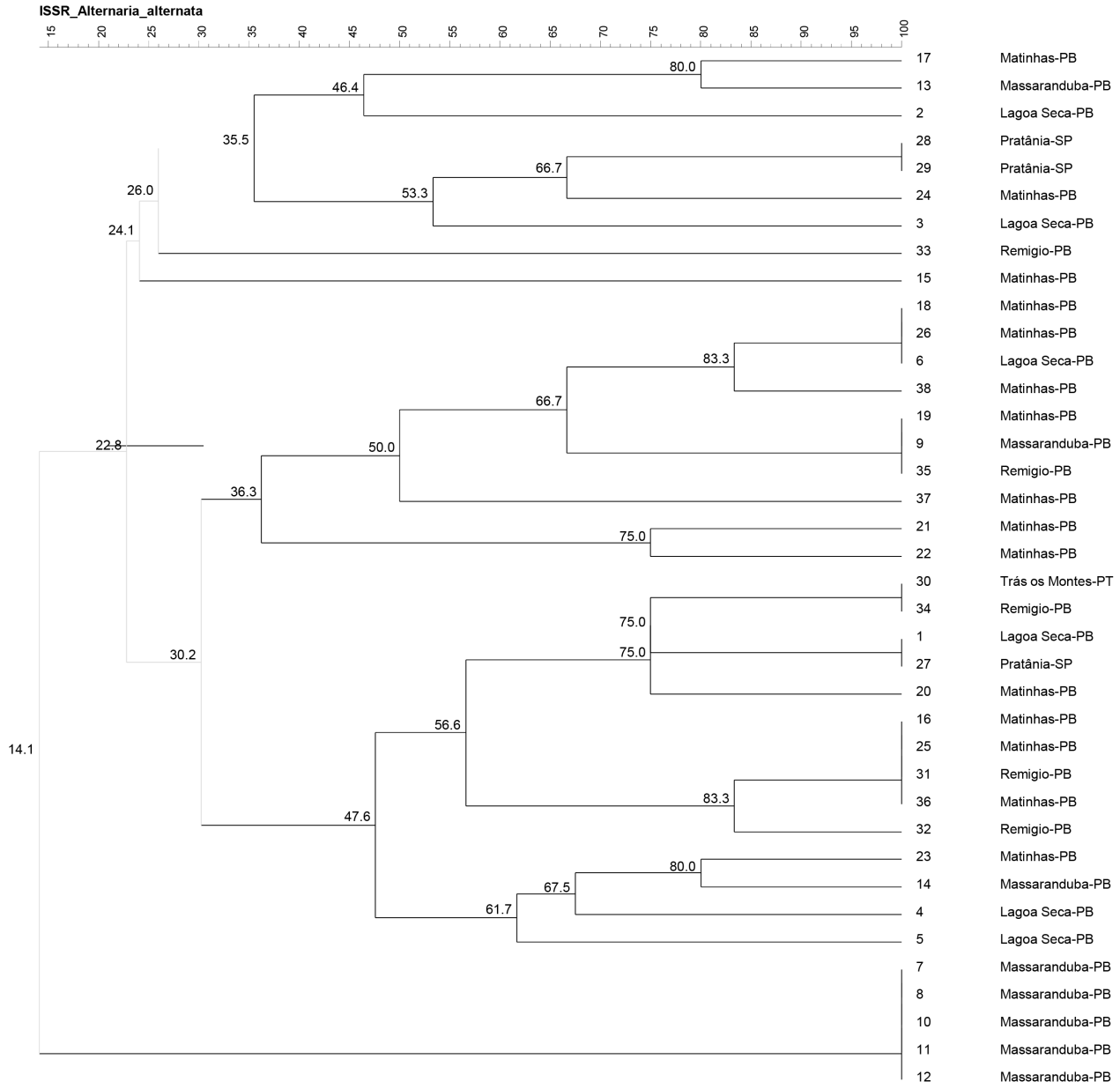

Figure 1. Dendrogram with isolates of *Alternaria alternata* pathotype Tangerine collected in different locations, grouped according to estimates of genetic similarity obtained by the polymorphic bands generated by the ISSR (UPGMA) method. UFPB, Areia, Brazil. 2016.

light/dark cycle rather than continuous dark or continuous light. Thus, the sporulation of the isolates may have been overestimated by the culture conditions used in the evaluations.

Physical agents are capable of either inducing or inhibiting the vegetative and reproductive development of the majority of fungi. Temperature is one of the most important factors, as it modulates the production of proteins and enzymes involved in the maintenance of fungal cells [30] and exerts an influence on cell multiplication. Very low temperatures generally do not stimulate production in the mycelium or sporulation due to the reduction in the metabolism of the pa-

thogen and very high temperatures lead to a reduction in both mycelial growth and sporulation, as some essential enzymes may denature or have their formation altered, resulting in a failure to resume metabolic activity, which can drastically reduce sporulation [12].

According to Meng *et al.* [31] each species of fungus has an ideal temperature range for sporulation, which is reduced at low temperatures and increases with the increase in temperature until reaching a point of maximum sporulation. Thus, a temperature range of 25˚C to 35˚C is favourable to the high production of spores in different species of *Alternaria.*

The highest mycelial growth of the pathotype tangerine *A. alternata* occurred when using potato dextrose agar, since this culture medium is rich in sources of nitrogen, phosphorus, minerals and high sugar content [32] and influence in the increase both mycelial growth. According to Pulz and Massola Júnior [16], this phase requires high concentrations of sugars, which influence the increase of vegetative development in different species of *Alternaria*.

The V8 agar medium exerted an influence on the greater production of spores of *A. alternata*. This medium is composed of eight vegetables (tomato, beet, celery, carrot, lettuce, spinach, parsley and watercress) and is rich in potassium, complex carbohydrates, minerals, fibre and vitamins A, E and C, with only 0.3% sugar, which are essential constituents for the obtainment of high sporulation in different fungal species [29]. According to Brunelli *et al.* [30] for sporulation to occur in different species of *Alternaria*, the medium needs to have low sugar content [32].

The culture medium prepared with citrus leaf extract also provided significant yields of spores, since the leaves contain substances such as calcium, magnesium, zinc, phosphorus and potassium, which are essential to the reproductive phase of pathotype tangerine of *A. alternata*. This finding is in agreement with data described by Satyanarayana and Sadasiva Reddy [33] who report that culture media prepared with plants species that are susceptible to particular pathogens can increase the sporulation rate. Using *in vitro* culture studies, Leão *et al.* [34] found that a watermelon leaf extract medium in a continuous light regime favoured the greater production of spores by *Ascochyta cucumis* in comparison to the other media tested.

The physiological studies performed by Choudhary *et al.* [35] found that the temperature of 25˚C, PDA medium provided the best mycelial growth and sporulation of *Alternaria alternata* under *in vitro* conditions. Pulz and Massola Júnior [16] analysed the effect of 15˚C, 20˚C, 25˚C, 30˚C and 35˚C with continuous light and continuous dark in the V8, ODA and PDA media on the growth and sporulation of *Alternaria dauci* and found greater growth among the isolates analysed when incubated in continuous dark at 20˚C in the ODA medium, whereas a temperature of a 25˚C led to greater spore production in the V8 medium. For *Alternaria solani* (Sorauer), continuous dark at 25˚C in the V8 medium were the conditions that most favoured mycelial growth and sporulation in comparison to the other culture conditions evaluated [16].

Analysing the effect of 20 culture media on mycelial growth and sporulation in *Alternaria brasiliensis* at 25˚C in continuous darkness, Queiroz *et al.* [36] found that the V8 medium was the most efficient. Silva and Teixeira [12] also found that 25˚C was favourable to greater mycelial growth and sporulation rates in *A. dauci* and *A. solani*. In the present study, the V8 media were generally more efficient at sporulation in the isolates of the tangerine pathotype of *A. alternata*.

The present study verified low genetic diversity of the isolates present in Groups 1, 2, 3 (24.1%, 36.3% and 47.6%) and high diversity only in Group 4 (14.01%), however, it can be said that this group presents a tendency for genetic differentiation between isolates group (**Figure 1**).

All isolates of *A. alternata* that affect tangerine trees are genetically close groups with a tendency toward clustering by location or population [37]. Clustering by host has also been reported [13]. The authors cited describe the formation of four distinct groups and obtained an ample profile using AFLP markers. No significant change within groups was found when comparing the RAPD marker, except in the positioning of three isolates. Moreover, only isolates of lime trees were grouped together in both analyses and it was not possible to find groups completely specific to their collection sites or hosts when considering other hosts or plant tissues.

The fact that isolates from the same state are grouped with isolates from another state or even another country may be explained by gene flow. *A. alternata* isolates can be carried to other regions by tangerine seedlings or seeds [38]. If such transportation occurs, isolates of a pathogen carried to a different region or country can favour the breakdown of resistance in tangerine varieties where such isolates did not previously occur [39].

The results of this study corroborate that Ghasemloee and Niazmand [6] when evaluating isolates of *A. alternata*, verified high genetic diversity the isolates collected in southern Iran. Adachi *et al.* [40] found low genetic variation in a population of *A. alternata* with 271 isolates among populations from different producing regions of Japan, which may be explained by genetic drift. Populations of isolates may emerge from small groups that have evolved or are in a process of establishment in response to particular host genotypes or selective pressures from the environment that are common to each region or pathogen. Thus, it is possible that *A. alternaria* tangerine isolates from the present study are evolving to become a genetically distinct population or even a lineage in the near future [41]. However, this information is the first step in obtaining the aetiology of Alternaria brown spot disease. It will serve as a basis for new studies on the control of *A. alternata* in citrus fruits in the state of Paraíba.

The high variability of microorganisms is not only caused by environmental pressure stemming from differences among hosts or geographic areas, but may also be influenced by intrinsic factors of the host or pathogen. In sexual fungi, such as the tangerine pathotype of *A. alternata,* in which sexual reproduction is rare or not observed in nature [13] genetic recombination mechanisms are pre-

valent, such as parasexuality and parameiosis, which may have exerted an influence on the high genetic variability detected in the present study. Likewise, Michereff *et al.* [42] used RAPD markers in isolates of *A. brassicicola* and found high genetic variability as well as variability with regard to mycelial growth and sporulation.

Using ISSR, Meng *et al.* [31] found a diversity of genotypes of *A. solani* in four regions in China. The authors found that 268 isolates demonstrated high genetic variation within populations of the same location and found no genetic differences between collection sites. Evaluating genetic diversity among 45 isolates of *A. alternata* associated with citrus trees in southern Iran using RAPD-PCR, Ghasemloee and Niazmand [38] found high genetic diversity in only 13 isolates, with no genetic divergences found among the other 32 isolates.

5. Conclusion

Groups 1, 2 and 3 presented low genetic variability. Group 4 showed high genetic variability of the isolates obtained from the Massaranduba (state of Paraíba, Brazil) producing region and higher mycelial growth and sporulation of *A. alternata*. The continuous light regime and the temperature 25˚C in PDA and V8 media were the ideal conditions for the mycelial growth and sporulation, respectively, of the isolates of *A. alternata*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Whiteside, J.O. (1976) A Newly Recorded *Alternaria* Induced Brown Spot Disease on Dancy Tangerines in Florida. *Plant Disease Report*, **3**, 326-329.

[2] Canihos, Y., Erkilic, A. and Timmer, L.W. (1997) First Report of Alternaria Brown Spot of *Minneola tangelo* in Turkey. *Plant Disease*, **81**, 1214. https://doi.org/10.1094/PDIS.1997.81.10.1214B

[3] Vicent, A., Armengol, J., Sales, R., GarciaJimenez, J. and Alfaro-Lassala, F. (2000) First Report of Alternaria Brown Spot of Citrus in Spain. *Plant Disease*, **84**, 1044. https://doi.org/10.1094/PDIS.2000.84.9.1044B

[4] Peres, N.A. and Timmer, L.W. (2006) Evaluation of the Alter-Rater Model for Spray

Timing for Control of Alternaria Brown Spot on *Murcott tangor* in Brazil. *Crop Protection*, **25**, 454-460. https://doi.org/10.1016/j.cropro.2005.07.010

[5] Wang, X.F., Li, Z.A., Tang, K.Z., Zhou, C.Y. and Yi, L. (2010) First Report of Alternaria Brown Spot of Citrus Caused by *Alternaria alternata* in Yunnan Province, China. *Plant Disease*, **94**, 375. https://doi.org/10.1094/PDIS-94-3-0375C

[6] Ghasemloee, M. and Niazmand, A.R. (2015) Study of *A. alternata* Associated with *Citrus* spp. *Journal of Pure and Applied Microbiology*, **9**, 473-482.

[7] Akimitsu, K., Peever, T.L. and Timmer, L.W. (2003) Molecular, Ecological and Evolutionary Approaches to Understanding Alternaria Diseases of Citrus. *Molecular Plant Pathology*, **1**, 435-446. https://doi.org/10.1046/j.1364-3703.2003.00189.x

[8] Azevedo, F.A., Martelli, I.B., Polydoro, D.A., Pacheco, C.A., Schinor, E.H. and Batianel, M. (2015) Positive Relationship between Citrus Leaf Miner and Alternaria Brown Spot. *Ciência Rural*, **45**, 1160-1163. https://doi.org/10.1590/0103-8478cr20141248

[9] Bassimba, D.D.M., Mira, J.L. and Vicent, A. (2014) Inoculum Sources, Infection Periods, and Effects of Environmental Factors on Alternaria Brown Spot of Mandarin in Mediterranean Climate Conditions. *Plant Disease*, **98**, 409-417. https://doi.org/10.1094/PDIS-09-13-0956-RE

[10] Dewdney, M.M. (2016) Alternaria Brown Spot, Alternaria Diseases in Citrus, SP 043 Florida Citrus Pest Management Guide. http://edis.ifas.ufl.edu/cg021

[11] Woudenberg, J.H.C., Seidl, M.F., Groenewald, J.Z., Vries, M., Stielow, M.B., Thomma, B.P.H.J. and Crous, P.W. (2015) Alternaria Section Alternaria: Species, Formae Speciales or Pathotypes? *Studies in Mycology*, **1**, 1-21. https://doi.org/10.1016/j.simyco.2015.07.001

[12] Silva, J.L. and Teixeira, R.N.V. (2012) Sporulation and Mycelial Growth of *Fusarium solani* in Different Culture Media and Steady Bright. *Revista Agroambiente*, **6**, 47-52.

[13] Dini-Andreote, F., Pietrobon, V.C., Dini-Andreote, F., Romão, A.S., Spósito, M.B. and Araújo, W.L. (2009) Genetic Variability of Brazilian Isolates of *Alternaria alternata* Detected by AFLP and RAPD Techniques. *Brazilian Journal of Microbiology*, **40**, 670-677. https://doi.org/10.1590/S1517-83822009000300032

[14] Souza, D.C.L. (2015) Molecular Techniques for Characterization and Conservation of Medicinal and Aromatic Plants: A Review. *Revista Brasileira de Plantas Medicinais*, **17**, 495-503. https://doi.org/10.1590/1983-084X/13_071

[15] Canihos, Y., Peever, T.L. and Timmer, L.W. (1999) Temperature, Leaf Wetness, and Isolate Effects on Infection of Minneola Tangelo Leaves by *Alternaria* sp. *Plant Disease*, **83**, 429-433. https://doi.org/10.1094/PDIS.1999.83.5.429

[16] Pulz, P. and Massola Junior, N.S. (2009) Effect of Culture Media and Physical Factors on Growth and Sporulation of *Alternaria dauci* and *A. solani*. *Summa Phytopathologica*, **35**, 121-126. https://doi.org/10.1590/S0100-54052009000200007

[17] Salgado, A.P.S.P., Cardoso, M.G., Souza, P.E., Souza, J.A., Abreu, C.M.P. and Pinto, J.E.B.P. (2003) Fungitoxic Activity Evaluation of Essencial Leaf Oils of *Eucalyptus* on *Fusarium oxysporum*, *Botrytis cinerea* and *Bipolaris sorokiniana*. *Ciência e Agrotecnologia*, **27**, 249-254. https://doi.org/10.1590/S1413-70542003000200001

[18] Gomes-Oliveira, I.V. and Matsumura, A.T.S. (2001) Technique to Obtain of *Alternaria solani* Culture Filtrate. *Tropical Plant Pathology*, **26**, 1.

[19] Waals, J.E.V., Korsten, L. and Slippers, B. (2004) Genetic Diversity among *Alternaria solani* Isolates from Potatoes in South Africa. *Plant Disease*, **88**, 959-964.

https://doi.org/10.1094/PDIS.2004.88.9.959

[20] Raeder, V. and Broda, P. (1987) Rapid Preparation of DNA from Filamentous Fungi. *Letters of Applied Microbiology*, **1**, 17-20. https://doi.org/10.1111/j.1472-765X.1985.tb01479.x

[21] Jaccard, P. (1901) Étude Comparative de la distribuition florale dans une portion des Alpes et des Jura. *Bulletin de la Société Vaudoise des Sciences Naturelles*, **7**, 547-579.

[22] Nei, M. (1973) Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences*, **70**, 3321-3323. https://doi.org/10.1073/pnas.70.12.3321

[23] Nei, M. (1977) F-Statistics and Analysis of Gene Diversity in Subdivided Populations. *Annals of Human Genetics*, **41**, 225-233. https://doi.org/10.1111/j.1469-1809.1977.tb01918.x

[24] Nei, M. (1987) Molecular Evolutionary Genetics. Columbia University Press, New York.

[25] SAS Institute (2011) SAS/STAT Versão 9.3: User's Guide. SAS Institute Inc., 8621.

[26] Lukens, R.L. (1963) Photo-Inhibition of Sporulation in *Alternaria solani*. *American Journal of Botany*, **50**, 721-724. https://doi.org/10.2307/2440051

[27] Aragaki, M. (1962) Quality of Radiation Inhibitory to Sporulation of *Alternaria tomato*. *Phytopathology*, **52**, 1227-1228.

[28] Trione, E.J. and Leach, C.M. (1969) Ligth-Induced Sporulation and Sporogenic Substances in Fungi. *Phytopathology*, **59**, 1077-1083.

[29] Hanada, R.E., Gasparotto, L. and Pereira, J.C.R. (2002) Sporulation of *Mycosphaerella fijiensis* in Different Culture Media. *Tropical Plant Pathology*, **27**, 170-173.

[30] Brunelli, K.R., Fazza, A.C., Athayde Sobrinho, C. and Camargo, L.E.A. (2006) Effect of Culture Media and Light Exposure on the Sporulation of *Cercospora zeae-maydis*. *Summa Phytopathologica*, **32**, 92-94. https://doi.org/10.1590/S0100-54052006000100016

[31] Meng, J.W., Zhu, W., He, M.H., Wu, E.J. Yang, L.N., Shang, L.P. and Zhan, J. (2015) High Genotype Diversity and Lack of Isolation by Distance in the *Alternaria solani* Populations from China. *Plant Pathology*, **64**, 434-441. https://doi.org/10.1111/ppa.12275

[32] Dhingra, O.B. and Sinclair, J.B. (1995) Basic Plant Pathology Methods. 2nd Edition, CRC Press, Boca Raton.

[33] Satyanarayana, K. and Sadasiva Reddy, C. (1986) A New and Cheap Medium Supporting the Sporulation of *Pyricularia oryzae*. *Indian Journal Mycological Plant Pathology*, **163**, 329-330.

[34] Leão, E.U., Santos, G.R., Sarmento, Reis, M.R. and Chagas Júnior, A.F. (2012) Mycelial Growth and Conidial Production of *Ascochyta cucumis* in Different Culture Media and Light Regimen. *Bioscience Journal*, **28**, 325-331.

[35] Choudhary, S., Ghasolia, R.P., Yadav, A.L., Kakraliya, G.L. and Nasanwa, R. (2017) Effect of Physical Factors on Growth of A*lternaria alternata* Inciting Leaf Blight of Isabgol. *Chemical Science Review and Letters*, **22**, 857-863.

[36] Queiroz, F.M., Batista, U.G. and Brommonschenkel, S.H. (2004) Evaluation of Culture Media on the Mycelial Growth and Sporulation of *Alternaria brasiliensis*. *Tropical Plant Pathology*, **29**, 541-543.

[37] Carvalho, D.D.C., Alves, E., Camargo, R.B., Oliveira, D.F., Scolforo, J.R.S., Carvalho,

D.A. and Batista, T.R.S. (2011) Plant Extracts to Control *Alternaria alternata* in Murcott Tangor Fruits. *Revista Iberoamericana de Micología*, **28**, 173-178. https://doi.org/10.1016/j.riam.2011.05.001

[38] Ghasemloee, M. and Niazmand, A.R. (2014) Genetic Diversity of *Alternaria alternate* Associated with Citrus spp. in Southern Iran Based on RAPD-PCR. *Journal of Pure and Applied Microbiology*, **9**, 10-12.

[39] Souza, B.O., Casela, C.R., Nunes, J.A. and Castro, H.A. (2013) Management of Sorghum Anthracnose through Diversification of Genetic Resistance in Host Population. *Tropical Plant Pathology*, **38**, 20-27. https://doi.org/10.1590/S1982-56762013000100003

[40] Adachi, Y., Watanabe, H., Tababe, K., Doke, N., Nishimura, S. and Tsuge, T. (1993) Nuclear Ribosomal DNA as a Probe for Genetic Variability in the Japanese Pear Pathotype of *Alternaria alternate*. *Applied and Environmental Microbiology*, **59**, 3197-3205.

[41] Nodari, R.O. and Guerra, M.P. (2015) Agroecology: Research Strategies and Values. *Estudos Avançados*, **29**, 183-207. https://doi.org/10.1590/S0103-40142015000100010

[42] Michereff, S.J., Noronha, M.A., Rocha Jr., O.M., Silva, J.A. and Mizubuti, E.S.G. (2003) Variability of *Alternaria brassicicola* Strains in the State of Pernambuco, Brazil. *Tropical Plant Pathology*, **28**, 656-663.