ARTIGOS

Diversity of citrus gummosis in São Paulo State, Brazil

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ABSTRACT

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Citrus gummosis is an important disease because it causes the death of young plants, resulting in their replacement, a costly operation. Its causal agents are different species of *Phytophthora*. Dissemination of these microorganisms occurs mainly by contaminated nursery trees. Differences in aggressiveness, competitiveness and response to fungicides varied among species of this pathogen. Therefore, this study aimed to examine diversity among citrus isolates of *Phytophthora* from two different origins in São Paulo State. Thirty isolates from citrus nursery trees from 13 municipalities and 11 from seven commercial orchards were analyzed. Interspecific diversity was performed by species-specific primers and sequencing of the ITS region. Subsequently, intraspecific diversity was carried out with 16 RAPD primers and clustering analysis of UPGMA, using the Dice coefficient. Our data identified only *P. nicotianae* from either substrates or commercial fields. Isolates clustered into two genetically distinct populations. Genetically similar isolates were also found. The existence of these clonal lineages among isolates from geographically distinct nursery trees suggests an efficient dissemination mechanism. This was the first study to examine the diversity of *Phytophthora* in citrus from substrates of nursery trees in Brazil.

Keywords: *Phytophthora* spp., RAPD, PCR, variability

RESUMO

Urashima, A.S.; Mistura, T.F.; Gonçalves, C.R.N.C. Diversidade de gomose dos citrus no estado de São Paulo, Brasil. ***Summa Phytopathologica,*** v.42, n.3, p.209-215, 2016.

Gomose do citrus é uma doença importante porque causa morte de plantas jovens, resultando em sua substituição, que é uma operação onerosa. Os agentes causais são diferentes espécies de *Phytophthora.* A disseminação destes microrganismos ocorre principalmente por mudas contaminadas. Diferenças na agressividade, competitividade e resposta à fungicidas variaram entre as espécies do patógeno. Portanto, esse trabalho objetivou examinar a diversidade entre isolados de *Phytophthora* de citros advindos de duas origens diferentes do estado de São Paulo. Trinta isolados provenientes de mudas cítricas de 13 municípios e 11 de sete pomares comerciais foram analisados. Diversidade inter-específica foi feita através de iniciadores específicos de espécies e seqüenciamento da região ITS. Em seguida, diversidade intra-específica foi conduzida com 16 iniciadores RAPD e análise de grupamento de UPGMA usando coeficiente Dice. Nossos dados identificaram somente *P. nicotianae,* seja em substratos ou campos comerciais. Os isolados foram divididos em duas populações geneticamente distintas. Isolados geneticamente similares também foram encontrados. A existência dessas linhagens clones entre isolados de mudas geograficamente distintas sugere um eficiente mecanismo de disseminação. Esse trabalho foi o primeiro a examinar diversidade de *Phytophthora* de substrato de mudas cítricas no Brasil.

Palavras-chave: *Phytophthora* spp., RAPD, PCR, variabilidade

Since the 1980's, Brazil has been the leading producer of orange and the world's largest exporter of concentrated juice, and the state of São Paulo accounts for 80% of the national orange production and 98% of the exports of this commodity (10). Citrus also has an important social role in the state since 12,000 out of 13,398 growers are small landowners, making their living with up to 20,000 citrus trees (14).

Citrus, at the same time, hosts several diseases demanding considerable input of chemicals, a high burden for the economy of small growers. The resulting production cost makes a high yield mandatory to keep family-run growers in business, especially because this commodity has faced low income.

One of the most common diseases responsible for low productivity in citrus orchards is gummosis caused by *Phytophthora* spp. This pathogen causes symptoms varying from damping off of seedlings to brown rot of fruits; nevertheless, gummosis of crown rot and citrus root rot are the most common and important disease expressions under Brazilian conditions, especially in young plants when their death results in the replacement of trees in newly established fields, a costly operation (8).

Because of the importance of soil for the dissemination of root rot agents, the state of São Paulo created a regulation on the commercialization of citrus nursery trees, banning the use of soil and its replacement for substrate in nursery tress and making sanitary tests mandatory to avoid the dissemination of pathogens from seedlings to

new commercial orchards (24).

More than ten species of *Phytophthora* are described as causal agents of gummosis worldwide, prevailing in Brazil *P. nicotianae* (syn. *P.* parasitica) and *P. citrophthora* (8). Despite the importance of gummosis for the citrus industry in Brazil, and consequently in São Paulo State, little updated information is available on many important questions such as the diversity of the causal agent. Knowledge regarding the diversity of *Phytophthora* is paramount for the success of disease control strategies because it explained the difference in aggressiveness and competitiveness towards citrus roots between *P. palmivora* and *P. nicotianae* (27), as well as the discrepancy of fungicide performance to suppress lesion formation on tangelo bark between *P. nicotianae* and *P. citrophthora* (16).

One of the few studies on the genetic diversity of *P. parasitica* from different regions of São Paulo State showed high genetic similarity among 14 isolates based on ITS1 and ITS2 of 5.8S gene, suggesting single origin and subsequent spread across municipalities through contaminated seedlings (23). Nevertheless, that study examined isolates from soils collected in 1995, well before the implementation of the state regulation on the use of substrate in 2003; no information is available on pathogen diversity after the replacement of soil with substrate in citrus nursery trees.

Therefore, the present study aimed to examine inter and intraspecific diversity of the causal agent of citrus gummosis in São Paulo State, Brazil, using samples from soil and substrate of nursery trees.

MATERIAL AND METHODS

Oomycete isolates

The microorganisms analyzed in this study came from two different sources. One of the sources was substrate used in nursery of citrus trees, which is composed of coconut fiber and/or vermiculite and/or pinus' chips (26). The other origin was soil of citrus trees from commercial orchards exhibiting gummosis symptoms. Isolation of oomycetes from both sources was done by means of leaf trap as previously described (11). Purification was carried out after sterilization of leaves with sodium hypochlorite. A carrot-agar slant was used for sub-culturing pathogen isolates and for long-term storage, and double distilled water slants were employed according to Castellani's method.

DNA preparation

Isolates for DNA extraction were grown in 100 ml of carrot broth in a shaker at 100 rpm and 24°C for 10-12 days. Fresh mycelia (0.5 to 1.0 g) were ground in liquid nitrogen, and genomic DNA was extracted according to CTAB protocol detailed by Murray & Thompson (19).

Oomycete identification

Identification of *Phytophthora* spp. was performed by adopting molecular tools. For this, primers FMPhy-8b and FMPhy-10b, developed by Grunwald et al. (13) for the identification of *Phytophthora* spp., were employed. A specific marker in the range of 450 bp is expected for a positive isolate. Amplification reaction was performed on ABI 9700 (Applied Biosystems) thermocycler in 25µl volume with 10 mM Tris-HCL, pH 8.8, 50 mM KCL, 3.0 mM $MgCl_2$, 0.4 µM of each primer, 200 µM dNTPs, 1.25 units of *Taq* polymerase and 50 ng of genomic DNA under the following PCR conditions: 1 cycle at 95°C for 3 min, 35 cycles of 1 min at 95°C, 1 min at 65.5°C, 1 min at 72°C, finalizing with 5 min at 72°C. After that, 20 µl of PCR product were electrophoresed in 1% agarose gel stained by ethidium bromide and visualized under UV light.

Furthermore, the identity of pathogens was reconfirmed by direct sequencing rDNA segments, including ITS1, ITS2 and 5.8S rRNA gene, after DNA amplification by universal primers ITS6 and ITS4 (6,29), employing previously described PCR protocols (13). Amplicons were treated with commercial kits *Illustra GFX PCR DNA* and *Gel Band Purification* (GE Healthcare) before sequencing forward and reverse on a 3031X1 Genetic Analyser (Applied Biosystems) and subsequent BLAST search against GeneBank (NCBI).

Identification of *Phytophthora* species was performed by different sets of species-specific primers. Thus, the first attempt employed primers PNIC 1 and PNIC 2 in order to identify *P. nicotianae*, which in case of positive reaction would result in a 737bp fragment (12). Reaction was done in a volume of 25µl with 10 mM Tris-HCL, pH 8.8, 50 mM KCL, 2.0 mM MgCl2, 1µM of each primer, 200µM dNTPs, 2% formamide, 0.5 unit of Taq DNA polymerase and 50 ng of genomic DNA. PCR amplification was performed on a thermocycler GeneAmp PCR Systems 9700 (Applied Biosystems) at 95°C for 3 min, 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and finishing at 72°C for 10 min. Visualization was carried out as previously described.

Intraspecific analysis

Random amplified polymorphic DNA (RAPD) markers were employed to examine intraspecific variation of *P. nicotianae*, employing 34 isolates (26 from substrate and 8 from soil orchards) from citrus. Amplification of DNA was carried out with 16 universal primers (Operon Technologies Inc., Alameda, CA, USA) in a thermocycler PT-100 (MJ RESEARCH Inc) performed with 45 cycles of 94ºC for 1 min, 36ºC for 1 min and 2 min at 72ºC (28). PCR reaction mixtures were performed in a volume of 25µL containing 0.2 mM of primer, 1 unit of *Taq* DNA polymerase (Thermo Scientific), supportive buffer (100 mM Tris-HCl, pH 8.8 + 500 mM KCl, 0.8% (v/v) nonidet P40), 0.8 Mm of $MgCl_2$, 0.8 Mm of dNTPs, 25-50 ng of genomic DNA. A negative control in which DNA template was replaced by autoclaved MilliQ water was included in all PCR reactions. Two to three reactions were carried out on different days for each primer. PCR products were electrophoresed on 2% agarose gel in 1 X TBE buffer, stained with ethidium bromide and visualized under UV light.

Diversity analyses

Diversity among isolates was examined through cluster analysis. All RAPD bands were scored as "1" representing presence and "0" representing absence of fragment of a particular molecular weight. Only reproducible fragments present in at least two gels were considered for analyses. When necessary, a third gel was produced with this particular primer. The resulting binary matrix was subjected to pair-wise Dice similarity coefficient by SIMQUAL program. A dendrogram of similarity was generated with UPGMA (unweighted pair group method with arithmetic mean) in the SAHN program of NTSYS-pc version 2.2 (22). A bootstrap analysis of the data was carried out with the WINBOOT program (30) and the robustness of clusters checked in 1000 replicates.

Pathogenicity

Pathogenicity of isolates was carried out in order to complete Koch's postulates. Isolates of *Phytophthora* were transferred from carrot agar slants to Petri dishes with carrot agar culture and allowed to grow for 14 days at 22°C in 24h fluorescent illumination. Active growing areas of mycelial agar plug were placed in contact with mature fruits of Pera sweet orange [*Citrus sinensis*(L.) Osbeck], which had their surface

sterilized with sodium hypochlorite and were kept in plastic bags for 48h at 22°C in order to achieve saturated atmosphere. Subsequently, the fruits were transferred to the laboratory bench at 24+/-2°C for seven days when they were evaluated for symptoms. Presence of sporangia on lesions was examined under a light microscope. Control consisted of agar plugs only.

RESULTS

Forty-one isolates were employed in this study, 30 from nursery trees from 13 municipalities and the remaining 11 from seven different citrus orchards distributed among five municipalities of São Paulo State. Nomenclature, locality, year of collection, origin, rootstock of samples and their identity are shown in Table 1. All isolates were obtained from samples of Rangpur Lime (*C. limonia* Osbeck) as rootstock.

All samples collected from both sources retrieved sporangium-forming organisms by the leaf trap method used in this study. The trap method for citrus leaf pieces was straightforward and there was no apparent difference in detection efficiency between samples from substrate and field soil, validating this technique for both sources.

All 41 isolates yielded a 450bp fragment by primers FMPhy-8b and FMPhy-10b (Figure 1), confirming *Phytophthora* as the genus of these isolates (Table 1). Besides this 450bp fragment, a non-specific fragment was observed in all positive isolates, but no attempt to eliminate it was done because reconfirmation of these isolates was subsequently performed by PNIC primers and sequencing. No fragment was visualized in negative controls.

Pathogenicity of the *Phytophthora* employed in this study was confirmed by completing Koch's postulates. Three of these isolates were randomly selected and inoculated in the fruit of orange (*C. sinensis*) cv. Pera. Seven to ten days after inoculation, all isolates caused large necrotic lesion of brown color, characteristic of this disease.

All 41 isolates were subsequently subjected to PCR for species identification. All of these pathogens produced a band in the range of 737 bp by primers PNIC1 and PNIC2, confirming their identity as *P. nicotianae* (Figure 2). No fragment was observed in the negative control. Their identity was further reconfirmed when two randomly selected isolates were sequenced and similarity of 100% was verified with *P. nicotianae* of GeneBank accession JQ070168.1, JQ303220.1, HQ665198.1, EU080889.1, EU080722.1, EU080508.1 and DQ361241.1 (Figure 3).

Intraspecific diversity among *P. nicotianae* isolates examined by RAPD markers revealed that the majority of primers were suitable for producing a discernible band with various degree of polymorphism among isolates. The number of amplified fragments varied from one

Table 1. Nomenclature, locality, year of collection, origin, rootstock of samples, and their identity.

Nomenclature	Locality	Date	Origin	Rootstock	Pathogen identified [y]
CiPh03-01	Cajobi	Oct/2010	Substrate	Rangpur Lime	*P. nicotianae*
CiPh04-01;03	M. A. P.[w]	Feb/2010	Substrate	Rangpur Lime	*P. nicotianae*
CiPh05-01	Mogi-Guaçu	Apr/2011	Substrate	Rangpur Lime	*P. nicotianae*
CiPh06-01	Mogi-Guaçu	Apr/2011	Substrate	Rangpur Lime	*P. nicotianae*
CiPh07-02;03	Itirapina	Aug/2009	Substrate	Rangpur Lime	*P. nicotianae*
CiPh09-01	Cajobi	Oct/2010	Substrate	Rangpur Lime	*P. nicotianae*
CiPh10-01	Paraíso	Oct/2011	Substrate	Rangpur Lime	*P. nicotianae*
CiPh11-01;02;03;04	Ibitinga	Jan/2012	Substrate	Rangpur Lime	*P. nicotianae*
CiPh12-01	S. O.[x]	Sep/2007	Field	Rangpur Lime	*P. nicotianae*
CiPh13-01	Tabapuã	Aug/2008	Substrate	Rangpur Lime	*P. nicotianae*
CiPh15-01	M. A. P.	Feb/2009	Substrate	Rangpur Lime	*P. nicotianae*
CiPh17-01	S. O.	Nov/2007	Substrate	Rangpur Lime	*P. nicotianae*
CiPh18-01	Conchal	Aug/2010	Substrate	Rangpur Lime	*P. nicotianae*
CiPh19-01	M. A. P.	Jul/2009	Substrate	Rangpur Lime	*P. nicotianae*
CiPh20-01	Cajobi	Mar/2012	Substrate	Rangpur Lime	*P. nicotianae*
CiPh21-01;02	Pirangi	Jan/2013	Substrate	Rangpur Lime	*P. nicotianae*
CiPh22-01	Conchal	Feb/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh24-01	Limeira	Jan/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh25-01;02	E. C.[z]	Feb/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh29-03;04;05	Limeira	Feb/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh30-01	Araras	Feb/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh32-01;02	Araras	Feb/2013	Field	Rangpur Lime	*P. nicotianae*
CiPh33-01;02	Mogi-Guaçu	Jan/2013	Substrate	Rangpur Lime	*P. nicotianae*
CiPh34-01;02	Bebedouro	Jan/2013	Substrate	Rangpur Lime	*P. nicotianae*
CiPh35-01;02	Rio Claro	Feb/2013	Substrate	Rangpur Lime	*P. nicotianae*
CiPh36-01;02	Mendonça	Apr/2012	Substrate	Rangpur Lime	*P. nicotianae*
CiPh37-01	M. A. P.	Apr/2013	Substrate	Rangpur Lime	*P. nicotianae*

[y]*Phytophthora nicotianae*; [w]Monte Azul Paulista; [x]Sales Oliveira; [z]Engenheiro Coelho

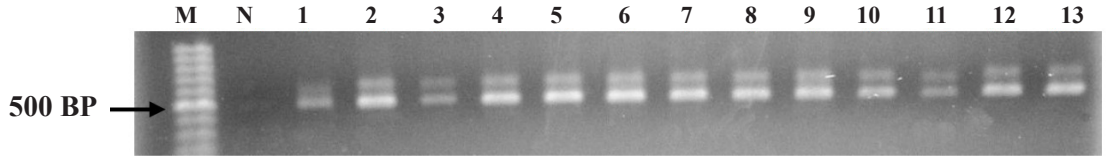

Figure 1. Amplification products of isolates using primers FMPhy-8b and FMPhy-10b. M: 500 bp marker (Fermentas Life Sciences); N: negative control; lane 1: CiPh03-01; lane 2: CiPh04-01; lane 3: CiPh04-03; lane 4: CiPh05-01; lane 5: CiPh06-01; lane 6: CiPh07-02; lane 7: CiPh07-03; lane 8: CiPh10-01; lane 9: CiPh11-01; Lane 10: CiPh11-02; lane 11: CiPh11-03; lane 12: CiPh11-04; lane 13: CiPh13-01.

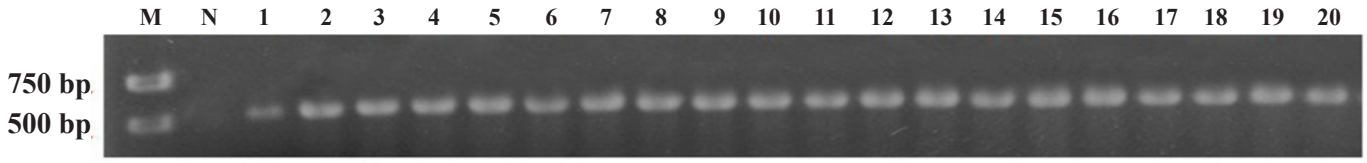

Figure 2. Amplification products of isolates using primers PNIC I and PNIC II. M: 1 kbp marker (Fermentas Life Sciences); N: negative control; lane 1: CiPh03-01; lane 2: CiPh04-01; lane 3: CiPh04-03; lane 4: CiPh05-01; lane 5: CiPh06-01; lane 6: CiPh07-02; lane 7: CiPh07-03; lane 8: CiPh10-01; lane 9: CiPh11-01; Lane 10: CiPh11-02; lane 11: CiPh11-03; lane 12: CiPh11-04; lane 13: CiPh13-01; lane 14: CiPh15-01; lane 15: CiPh17-01; lane 16: CiPh18-01; lane 17: CiPh19-01; lane 18: CiPh20-01; lane 19: CiPh21-01; lane 20: CiPh21-02.

.**Figure 3.** Sequence alignments between *Phytophthora* isolates analyzed in our study and accessions of GeneBank

Table 2. Decamer primer employed for RAPD amplification of *Phytophthora nicotianae* isolates.

Primer	Primer specification	Largest number of amplified fragments	Number of analyzed fragments	Number of polymorphic fragments
OPA 13	5' -CAGCACCCAC-3'	4	3	3
OPA 20	5' -GTTGCGATCC-3'	2	2	0
OPB 15	5' -GGAGGGTGTT- 3'	3	1	1
OPF 08	5' -GGGATATCGG- 3'	2	1	1
OPG 11	5' -TGCCCGTCGT- 3'	2	2	2
OPH 09	5' –TGTAGCTGGG- 3'	1	1	1
OPJ 20	5' -AAGCGGCCTC- 3'	7	2	1
OPL 02	5' -TGGGCGTCAA- 3'	4	3	2
OPL 10	5' -TGGGAGATGG- 3'	3	2	1
OPM 11	5' -GTCCACTGTG- 3'	1	1	1
OPM 16	5' -GTAACCAGCC- 3'	1	1	1
OPM 20	5' -GTAACCAGCC- 3'	4	3	3
OPP 07	5' -GTAACCAGCC- 3'	5	2	2
OPR 07	5' -ACTGGCCTGA- 3'	2	2	2
OPR 15	5' -GGACAACGAG- 3'	2	2	2
OPX 16	5' -CTCTGTTCGG- 3'	2	2	2
TOTAL		45	30	25

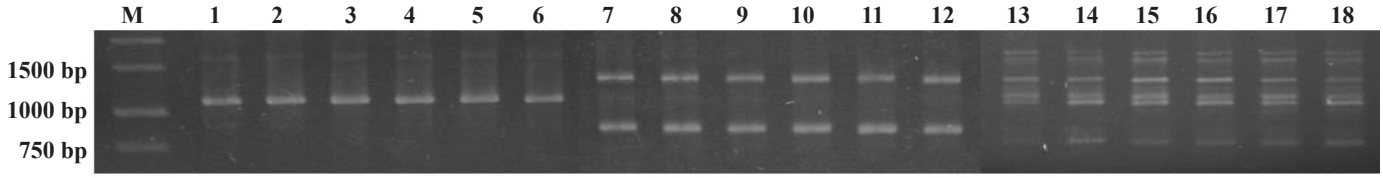

Figure 4. RAPD products amplified by random decamer primer OPM16, OPA20, OPJ20. M: 1 kbp marker (Fermentas Life Sciences); Lanes 1 to 6: primer OPM16; Lanes 7 to 12: primer OPA20; lanes 13 to 18: primer OPJ20. Lanes 1, 7, 13: CiPh17-01; lanes 2, 8, 14: CiPh10-01; lanes 3, 9, 15: CiPh11-03; lanes 4, 10, 16: CiPh07-02; lanes 5, 11, 17: CiPh06-01; lanes 6, 12, 18: CiPh07-03.

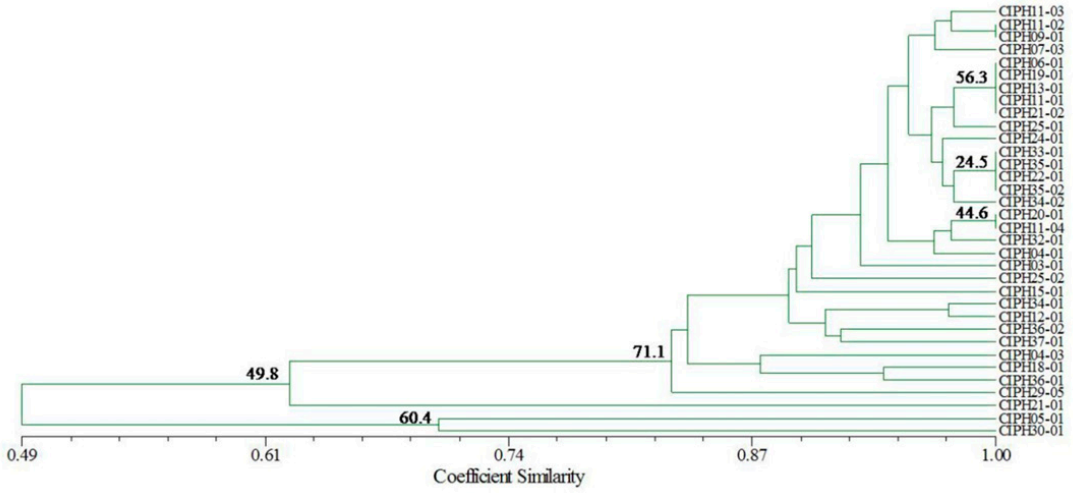

Figure 5. UPGMA cluster analysis of genetic similarity of 34 isolates of *Phytophthora nicotianae* based on 30 fragments obtained with 16 RAPD primers by using NTSYS and Dice index. Boostrap value is indicated at cluster branches based on 1000 replicates.

(primers OPM 11, OPH 09, OPM 16) to two (primers OPA 20, OPF 8, OPG 11, OPR 7, OPR 15, OPX 16) to many fragments (primer OPJ 20) (Figure 4). The total number of bands generated by these markers was 45, of which 30 were distinguishable enough to be evaluated (Table 2). A clear difference of primers to generate information on genetic diversity was observed since primer OPA 20 amplified two bands which were monomorphic, whereas OPG 11 resulted in the same number of fragments but polymorphic; the highest polymorphism was identified with primer OPA 13 with three polymorphic bands (Table 2).

When the data of molecular polymorphism were visualized in the form of genetic similarity in a dendrogram, two distinct subpopulations were detected with 49% of similarity between them (Figure 5). The first subpopulation was formed by two isolates (CiPh30-01 and CiPh05-01) with 70% of relatedness between them and 60.4% of robustness. The other sub-population showed 62% of genetic relatedness among its isolates with the great majority composed by 31 isolates, forming a more homogenous group with 82% of genetic similarity among its isolates and bootstrap value of 71.1%. Interestingly, in the latter group, four different genetic clones were identified, and the group formed by isolates CiPh06-01, CiPh19-01, CiPh13-01, CiPh11-01, CiPh21-02 showed robustness of 56.3%. The low bootstrap value of other branches of clones did not allow any further analysis.

DISCUSSION

The primary aim of this study was to examine interspecific variation of *Phytophthora* spp. (Figure 1), the causal agent of citrus gummosis, using isolates from two different origins, substrate of nursery trees and soil from commercial orchards (Table 1). Our study identified *P. nicotianae* in all samples, regardless of their origin (Figure 2), demonstrating that *P. nicotianae* can be considered the most important causal agent of citrus gummosis in São Paulo State among more than ten species of *Phytophthora* already reported on citrus (8). The same finding was reported in different countries, such as Egypt, where *P. nicotianae* was the most prevalent in two citrus nurseries located at different environments (1), in Taiwan, where it was the most prevalent along with *P. palmivora*, in tropical regions in a survey of 229 orchards from 15 counties on the island (2), as well as in South Africa, where it was present in 20 out of 23 sampled orchards (25). In Brazil, it was also the species identified on commercial citrus fields in the northeastern state of Alagoas (18) and the most prevalent among 21 isolates collected in the southern state of Paraná (4). Therefore, the present finding reinforces the prevalence of *P. nicotianae* in the most important citrus producer state of Brazil, even after the implementation of the state policy of substrate for the production of seedling trees (Figure 3). This was the first study of diversity of *Phytophthora* spp. on substrates from nursery trees.

The widespread prevalence of this species in different countries of the world, as well as in states of Brazil, with great variation of edaphoclimatic conditions, demonstrates its high adaptation and competitiveness since production in screen-house nurseries employed substrates with several composition and proportion such as clean sand, peat moss, coco peat fiber, pinus chips, vermiculite, charcoal, all of which are poor for fungal nutrition (1,26). The pathogen was also identified in different soil types and even though higher percentage of clay favored *P. nicotianae* population, high densities of this microorganism were also found in sand soil of Florida (3). Moreover, *P. nicotianae* is known as a warm-temperature pathogen with more

abundant infection and maximum population level in the summer (9). These can partially explain the prevalence of *P. nicotianae* in this study since all samples originated from regions with high temperature and mild winter, characteristic of São Paulo State. Rootstock might also have played an important role in its prevalence because all samples were gathered from rangpur orange as rootstock (Table 1). This showed best adaptation to the Brazilian Western coastal tableland ecosystem in terms of water balance among five rootstocks (5) and has been the most employed rootstock in Brazil (17) despite its moderate susceptibility to *Phytophthora* spp. (8).

The substrates employed in this study were those sent by growers for sanitary analyses. Detection of *Phytophthora* in these samples demonstrates the efficacy of the policy of pathogen-free certification for commercialization of nursery trees and a remarkable measure to control citrus gummosis in the state of São Paulo. Indeed, *Phytophthora* spp. incidence in citrus produced in substrates under screen-house decreased from 26.2 to 5% during 2000 to 2003, which was much lower compared to 54.7 and 25.5% of plants produced before the adoption of the regulation (24).

Our study also showed two genetically diverse populations of *P. nicotianae* causing gummosis on citrus in São Paulo State (Figure 5). Despite the importance of citrus industry to the Brazilian economy, this was the first comprehensive study to examine intraspecific variability of *P. nicotianae* (Table 2, Figure 4) since previous studies concentrated on interspecific diversity of *Phytophthora* spp. and pinpointed *P. nicotianae* as the most important species in citrus orchards of other states (4,18). There are conflicting findings concerning intraspecific variability of *P. nicotianae* from citrus in the literature since 24 citrus-infecting *P. nicotianae* from India were distributed in different clusters (20), whereas one single clade grouped isolates from Philippines, Italy, Syria, California and Florida (15). The genetic diversity verified in this study contrasting with the high similarity observed by Rosa et al. (23) is another case of discrepancy of *P. nicotianae* (from São Paulo State, this time) and can be credited to different pathogen isolates and a larger number of isolates examined in our study.

Besides the diversity of *P. nicotianae*, this study also identified some isolates with identical genotype within a sub-population. The identification of genetic clones among isolates from Mogi Guaçu (CiPh06-01), Monte Azul Paulista (CiPh19-01), Tabapuã (CiPh13-01), Ibitinga (CiPh11-01) and Pirangi (CiPh21-02), municipalities that are more than 300 km apart in some cases, is intriguing because it suggests a single origin of contamination. Two of the most likely sources for this include substrate of nursery trees and irrigation water. Contamination of substrate by *Phytophthora* spp. was already reported in ornamental nurseries from Oregon, where *P. citricola*, *P. cinnamomi*, and *P. cryptogea* were found in gravel substrates, pots, and soil (21). Disinfestation of substrate for citrus nurseries was efficiently made by methyl bromide in Brazil until its prohibition due to its toxicity to the environment and mammals. Other methods like solarization proved to be efficient to replace this chemical (17) but the current contamination of substrate for citrus nurseries found in this study would suggest that this disinfestation method has not been widely adopted. Therefore, the presence of clones of *Phytophthora* due to contamination of substrate components of common use by these distant growers is a strong possibility. A successful strategy to control gummosis should include efficient substrate decontamination.

Irrigation water is an efficient means of *P. nicotianae* dissemination since it was recovered from naturally infested water reservoirs, harvesting pond and basin water employed for citrus irrigation in India (7). Since the region comprised by these five municipalities of São Paulo State is well provided with rivers, streams, rivulets and brooks that may merge in some instances, the possibility of contamination of substrates from geographically distant nurseries by contaminated irrigation water cannot be ruled out.

Moreover, the fact that these five genetic clones were detected from nursery trees sampled in different years showed that an efficient asexual survival strategy of this single clonal lineage acted along the period from 2008 (CiPh13-01) to 2013 (CiPh21-02) (Table 1). *Phytophthora* spp. possess various survival mechanisms like mycelia, sporangia, zoospores, chlamidospores, but *P. nicotianae* is known to produce abundant chlamydospores and oospores (8) and, due to its asexual nature, the first one is likely the most important mechanism for pathogen survival.

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