

Influence of genome methylation of fig tree accessions on the natural nematode and rust incidence

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Research Article

Keywords: *Ficus carica* L., *Meloidogyne incognita*, *Cerotelium fici*, epigenetics, phenotypic plasticity

Posted Date: November 17th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1005312/v1>

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Abstract

Brazil is the largest fig producer in South America, but the Brazilian commercial fig tree cultivation is based on the planting of a single cultivar, 'Roxo-de-Valinhos', resulting in serious problems related to diseases. Since there are epigenetic variations in the plant-pathogen interaction, mainly through gene regulation, the aim of this study was to carry out the *in vivo* characterization of fig accessions through the analysis of the natural root-knot nematode and leaf rust incidence correlated to its epigenomic profile, in order to support conservation works and genetic improvement. Regarding the analysis of the presence of nematodes, it was observed that all plants were attacked by this pathogen, and the identification of *Meloidogyne incognita* as the root-knot nematode species was confirmed. However, the rust incidence and the global genomic methylation content were statistically different between evaluated accessions. The joint analysis of data showed that methylation and the leaf rust incidence, when observed in the same phenological phase of plants, are correlated, presenting evidences of the same factorial pressure loads in genotypes, with the premise of similar behavior in these genotypes. Biotic factors are also responsible for changes in the DNA methylation of plants, demonstrating a positive role in promoting plant defense.

Introduction

Common fig tree, *Ficus carica* L, commonly known as fig, is a deciduous fruit tree species belonging to the Moraceae family, whose center of origin is the Mediterranean region (Patil 2020), but widely cultivated in all regions of the world with subtropical/temperate climate (Usai et al. 2020).

With important global economic expression, being marketed mainly in the fresh fruit and dried fruit market (Gurung et al. 2021), fig is among the twenty main fresh fruits exported by Brazil, which stands out as the main producer of the southern hemisphere, with production of approximately 23 thousand tons per year in 2,208 hectares of planted area.

However, the Brazilian commercial fig tree cultivation is based on the planting of a single cultivar, 'Roxo-de-Valinhos', resulting in serious problems related to pests and diseases, which reduce production and depreciate fruits, reducing profits, impairing plant development and demanding high expenses with inputs and cultural treatments (Rodrigues et al. 2019).

Among the main diseases affecting fig crops, fig tree rust, caused by the fungus *Cerotelium fici* (Solano-Baéz et al. 2017) and root-knot nematodes (*Meloidogyne incognita*) (Abdel-Sattar et al. 2020) stand out, imposing a major challenge in most cultivated areas around the world.

Thus, studies aimed at finding solutions to some of these problems would bring an enormous contribution to the development of the fig culture, with genotypic diversification being an important strategy for the crop, and genetic diversity being an important tool for the survival of the species and adaptation to biotic and abiotic exposures (Dooren et al. 2020), the latter being characterized by the phenotypic plasticity of plants.

In addition, the strong selection pressures imposed by extreme environments and specialized niches can sometimes lead to the independent evolution of similar morphological and/or physiological characteristics in different lineages (Sackton and Clark 2019), being organisms adapted to extreme environments especially interesting to study the fundamental questions of evolutionary biology.

Given the above, the aim of this study was to carry out the *in vivo* characterization of fig accessions through the analysis of the natural root-knot nematode and leaf rust incidence correlated to its epigenomic profile, in order to support conservation works, genetic improvement and crop production

Results And Discussion

In evaluations carried out in relation to the root-knot nematode incidence, it could be observed that all plants were attacked by this pathogen, showing their susceptibility to it. Analysis regarding the presence of nematodes identified 120-150 adult nematodes per root sample and 15 *Meloidogyne sp* adults per soil sample and 60 *Helicotylenchus sp.* adults per root and soil samples.

The identification of root-knot nematode species (*Meloidogyne incognita*) was confirmed under photonic microscope, by examining and documenting the perineal pattern (females) and the morphology of the labial region of males, as shown in Figure 1.

Ferreira et al. (2015) evaluated the hostability to the *Meloidogyne incognita* nematode in fig tree accessions belonging to the Germplasm Bank of the Seedling Production Center of the Integral Technical Assistance Coordination (CATI) in São Bento do Sapucaí, state of São Paulo and found that diversity did not influenced the hostability of plants to the nematode, since the population of *M. incognita* eggs and juveniles was statistically equal in all accessions evaluated, and the total nematode population ranged from 40,000 to 105,000 per gram of extracted root.

Costa et al. (2015) evaluated 6 fig tree genotypes, including those evaluated in the present study, regarding the response to *Meloidogyne javanica*, *M. incognita* and *M. enterolobii* nematodes, with the possibility of being used as rootstock resistant to this pathogen, and concluded that all genotypes under study behaved as susceptible to them.

Rodrigues et al. (2012) selected mutants in fig plants formed by cuttings irradiated with gamma rays in order to increase their genetic variability in relation to vegetative and reproductive development and found that all plants were carriers of *Meloidogyne incognita* nematode, showing that they are susceptible to it, and it is not possible to select plants resistant to this disease, corroborating data of the present work.

In general, a variety of methods have been used to limit *Meloidogyne* damage, including the development of resistant cultivars (Park et al. 2020). Thus, the identification and characterization of natural sources of resistance are important steps for the development of control strategies for root-knot nematodes. In woody crops, genetically resistant rootstocks different from the cultivar of agronomic interest can be obtained (Saucet et al. 2016); however, in the specific case of fig trees, this strategy is not yet viable.

Other *Ficus* species were selected regarding their resistance to these nematodes to compensate for the current lack of resistant germplasm in the cultivated fig tree. Despite the high resistance and graft compatibility in accessions of *Ficus racemosa* tropical species (Cohn and Duncan 1990), no effective control strategy has yet been implemented, showing that the control of *M. incognita* is a key point for fig tree cultivation (Neugebauer et al. 2018).

Thus, the control of cultivation environments (soil improvement and sterilization by sunlight), the treatment of plant diseases using chemical nematicides and biological control of root-knot nematodes using microorganisms (Du et al. 2020; Sikandar et al. 2020) or plant extracts are prophylactic measures that should be adopted for fig cultivation (Tariq et al. 2017).

Regarding the rust incidence, Table 2 shows the leaf area percentages covered with *Cerotelium fici* pustules, where statistical difference could be observed between evaluated accessions, with emphasis on Calimyrna, Genovesco, Roxo-de-Valinhos A, Stanford, White Adriatic, White Genova A, White Genova B, Smyrna B, Brunswich, PI 214, Accession 33, Accession 35, Accession 46, Caprifigo B, Accession 41, Accession 42, Mini Figo and Turco accessions, which obtained the lower rust incidence averages, indicating good adaptability to the climate conditions of the subtropical cwa climate type (mild and dry winters followed by very hot summers), according to the Köeppen classification (1948).

Table 2

Percentages of area covered with *Cerotelium fici* pustules in new (FFN), median (FFM) and basal (FFB) leaves of fig tree accessions.

TREATMENT	ACCESSION	FFN (%)	FFM (%)	FFB (%)	Mean
1	Calimyrna	0 a	13 a	18 a	10.6 a
2	Nobile	1 a	27 b	25 b	17.4 a
3	Genovesco	0 a	6 a	20 a	8.70 a
4	Roxo-de-Valinhos A	0 a	0 a	12 a	3.9 a
5	Stanford	0 a	1 a	5 a	1.90 a
6	White Adriatic	3 a	19 a	20 a	14.1 a
7	Bonato	6 a	23 a	87 c	38.7 b
8	White Genova A	0 a	8 a	14 a	7.40 a
9	White Genova B	0 a	1 a	23 a	8.20 a
10	Smyrna A	13 a	34 b	77 c	41.3 b
11	Smyrna B	2 a	5 a	14 a	6.80 a
12	Brunswick	1 a	0 a	7 a	2.60 a
13	Caprifig A	20 a	27 b	45 b	30.6 b
14	Pingo de Mel	27 b	0 a	3 a	10.0 a
15	Roxo-de-Valinhos Gigante	43 b	3 a	58 b	34.8 b
16	Palestino	2 a	14 a	44 b	20.0 a
17	Troyano	5 a	14 a	32 b	16.9 a
18	Vermelho	2 a	39 b	70 c	36.8 b
19	Irradiated Plant 440	0 a	65 c	40 b	35.0 b
21	Irradiated Plant 189	60 c	5 a	90 c	51.7 b
22	Irradiated Plant 214	20 a	5 a	15 a	13.3 a
23	Irradiated Plant 301	0 a	25 b	90 c	38.3 b
24	Nazaret	0 a	40 b	60 c	33.3 b
25	Cuello Negro	0 a	50 b	0 a	16.7 a
26	Roxo-de-Valinhos B	7 a	41 b	23 a	23.7 a

*Different letters in the column differ statistically from each other by the Scott-Knott test at 0.05% probability.

TREATMENT	ACCESSION	FFN (%)	FFM (%)	FFB (%)	Mean
27	Accession 27	0 a	15 a	48 b	21.1 a
28	Accession 28	0 a	20 a	80 c	33.3 a
29	Accession 29	5 a	8 a	33 b	15.6 a
30	Accession 30	2 a	43 b	43 b	29.4 b
31	Accession 31	0 a	0 a	35 b	11.7 a
32	Accession 32	0 a	28 b	70 c	32.5 b
33	Accession 33	12 a	8 a	20 a	13.30 a
34	Accession 34	2 a	3 a	37 b	13.90 a
35	Accession 35	0 a	3 a	5 a	2.50 a
36	Accession 36	2 a	15 a	45 b	20.6 a
37	Accession 47	10 a	2 a	43 b	18.3 a
38	Accession 44	0 a	3 a	43 b	15.6 a
39	Accession 46	0 a	3 a	5 a	2.50 a
40	Caprifig B	0 a	7 a	8 a	5.00 a
41	Accession 41	2 a	0 a	0 a	0.60 a
42	Accession 42	0 a	5 a	5 a	3.30a
43	Mini Figo	8 a	23 a	13 a	15.0 a
44	Preto	3 a	37 b	35 b	25.0 b
45	Turco	0 a	0 a	8 a	2.50 a
*Different letters in the column differ statistically from each other by the Scott-Knott test at 0.05% probability.					

“Roxo-de-Valinhos”, considered the base cultivar for the comparison of crop quality in Brazil, presented between 3.9 and 27.3% of rust lesions, corroborating results found by Mezzalira et al. (2015), who compared the efficiency of alternative fungicides and insecticides in relation to conventional products registered for fig tree culture in the state of Paraná and verified the incidence of the disease in this cultivar with approximate values ranging from 4.39 to 30% of incidence for control treatment.

In general, the mean values of the experiment ranged from 0.6 to 51.7%, corresponding to Accession 41 and Irradiated Plant (PI) 189 accessions, respectively. However, even the highest mean value is below the highest degrees of disease severity observed in literature. Sol-Rodríguez et al. (2021) evaluated the

disease incidence and severity in fig trees in Mexico under the moisture conditions similar to those of the present experiment and observed *C. fici* incidence values between 66.2 and 96.2%.

Pastore et al. (2015) evaluated the resistance of fig tree accessions to rust in different locations and observed significant difference between plants and also between locations, concluding that accessions less susceptible to the disease may be associated with greater plant rusticity, but also that the diversity between them can generate genotype-environment interaction and that environmental conditions may be more favorable to the development of fig trees naturalized in this environment in which genotypes had lower incidence.

The ability of a single genotype to generate alternative phenotypes based on changes in the environment - or phenotypic plasticity - is a potential mechanism by which plants can respond quickly to external changes (Arnold et al. 2019), in which growth responses to competition and defense responses to the attack of organisms are two classic examples of adaptive phenotypic plasticity of plants (Fernández-Milmanda et al. 2020).

In this sense, DNA methylation can be considered the best characterized epigenetic mechanism, which is involved in many important aspects of the evolutionary biology of the fig tree, such as in the varietal and behavioral differentiation of plants under environmental pressures (Rodrigues et al. 2019).

DNA methylation is a conserved epigenetic marker that regulates several processes, such as gene silencing, genome stability and genomic imprinting (Zhang et al. 2018). It is also present in gene coding regions in many plant species, leading to their overexpression (Choi et al. 2020).

Figure 2 shows the quantification of global genomic methylation of evaluated fig tree accessions, where dashed horizontal lines show values that were statistically different from the main commercial fig cultivar in Brazil, 'Roxo-de-Valinhos', represented by accession number 4, whose absolute value was 0.172.

Accessions whose absolute values are above the upper limit line, represented by accessions 5, 6, 7, 10, 12, 13, 14, 15, 18, 19, 20, 34, 39 and 40, namely Stanford, Adriático Branco, Bonato, Smyrna, Brunswick, Caprifigo IAC, Pingo de Mel, Roxo-de-Valinhos Gigante, Figo Vermelho, PI 440, PI 189, Accession 35 from Monte Alto, Caprifigo ISA and Accession 41 from Monte Alto, respectively, present global genomic methylation content statistically higher than that of 'Roxo-de-Valinhos', indicating that, in relation to the selected parameter, they are hypomethylated.

The absolute values observed below the lower limit line, represented by accessions 22 and 26, respectively 'PI 433' and accession 27 from Monte Alto, present global genomic methylation content statistically lower than that of 'Roxo-de-Valinhos', indicating that, in relation to the selected parameter, they are hypermethylated, corroborating results found by Rodrigues et al., 2015, who, evaluating the same irradiated plants analyzed in the present work, also observed that accessions numbers 440 and 189 had higher methylation content when compared to 'Roxo-de-Valinhos', and accession 433 had lower content,

revealing that, in this case, irradiation was an external factor capable of changing these patterns in these plants, including in the DNA demethylation process.

Figure 3 presents the principal component analysis using variables percentages of rust incidence in fig accessions and their global genomic methylation content, demonstrating that methylation and rust incidence in young leaves are correlated, with the premise of similar behavior in genotypes.

As for the rust incidence in medium and basal leaves, no correlation with the methylation content was observed, which can be explained by the fact that the DNA of accessions was extracted from new leaves, indicating that, before leaf development, there is correlation positive between methylation and this disease; however, as the leaf matures, the global genomic methylation content possibly changes, making it impossible to correlate the variables observed at different phenological times.

Several studies have established the role of epigenetic variations in the plant-microbe interaction, mainly through gene regulation (Kumar and Mohapatra 2021). Epigenetic mechanisms associated with interactions between plants and pathogens, in particular bacterial and fungal pathogens, demonstrate the positive role they can play in promoting plant defense (Zhu et al. 2015); however, the role of such change in DNA methylation in preparing plants against pests/diseases is not yet known (Wang et al. 2019).

Evidence shows that stress alters the epigenetic profile of plants, which can improve their stress tolerance capacity (Varotto et al. 2020), which may be DNA hyper- or hypomethylation, varying among species.

DNA hypomethylation is reported as a general feature in the promoter of many genes associated with fruit ripening, as they contain binding sites for transcription factors associated with ripening (LANG et al. 2017). However, Huanghuan et al. (2019) analyzed the influence of global DNA methylation on the ripening process of orange fruits and observed that DNA hypermethylation is critical for proper fruit ripening.

Thus, it appears that differences were observed in the rust incidence and in the global methylation content of the DNAs of the different fig tree accessions belonging to the Active Germplasm Bank evaluated in this study. In addition, it was possible to correlate the disease with methylation, when observed in the same phenological phase of the plant, showing initial evidence of same factorial pressure loads in genotypes, indicating that, in addition to the genetic factor, Biotic factors are also responsible for changes in the DNA methylation of plants, demonstrating a positive role in promoting plant defense.

Thus, it is evident that future studies on the gene expression between treatments is an extremely important strategy for the understanding of complex regulatory systems, leading to the identification of genes of agronomic interest for the fig tree crop, enabling its subsequent manipulation and propagation of improved cultivars for commercial purposes.

Methods

The plant material was composed of 44 *Ficus* sp. accessions belonging to the three-year-old Active Germplasm Bank of the Agricultural Sector, Faculty of Agrarian and Technological Sciences FCAT / UNESP, Campus of Dracena-SP.

The experimental design was randomized blocks, consisting of 44 treatments, each accession being considered a treatment, with two blocks and three plants per plot, totaling 270 plants, grown in 1.5 mx 1.5 m spacing, conducted in an open cup format, with 6 productive branches each. Treatments evaluated, with their respective accessions and places of origin, are presented in Table 1.

Table 1

Active Germplasm Bank of fig tree accessions of the Faculty of Agricultural and Technological Sciences (FCAT / UNESP).

TREATMENT	ACCESSION	PLACE OF ORIGIN
1	Calimyrna	São José do Rio Preto
2	Nobile	IAC ¹
3	Genovesco	IAC ¹
4	Roxo-de-Valinhos A	São Sebastião do Paraíso
5	Stanford	IAC ¹
6	White Adriatic	IAC ¹
7	Bonato	IAC ¹
8	White Genova A	São José do Rio Preto
9	White Genova B	IAC ¹
10	Smyrna A	IAC ¹
11	Smyrna B	São José do Rio Preto
12	Brunswich	IAC ¹
13	Caprifig A	IAC ¹
14	Pingo de Mel	Ilha Solteira
15	Roxo-de-Valinhos Gigante	Ilha Solteira
16	Palestino	Campinas
17	Troyano	IAC ¹
18	Vermelho	Piracicaba
19	Irradiated Plant 440	Ilha Solteira
21	Irradiated Plant 189	Ilha Solteira
22	Irradiated Plant 214	Ilha Solteira
23	Irradiated Plant 301	Ilha Solteira
24	Nazaret	Espanha
25	Cuello Negro	Espanha
26	Roxo-de-Valinhos B	Ilha Solteira

TREATMENT	ACCESSION	PLACE OF ORIGIN
27	Accession 27	Monte Alto
28	Accession 28	Monte Alto
29	Accession 29	Monte Alto
30	Accession 30	Monte Alto
31	Accession 31	Monte Alto
32	Accession 32	Monte Alto
33	Accession 33	Monte Alto
34	Accession 34	Monte Alto
35	Accession 35	Monte Alto
36	Accession 36	Monte Alto
37	Accession 47	Bahia
38	Accession 44	Piracicaba
39	Accession 46	Piracicaba
40	Caprifig B	Ilha Solteira
41	Accession 41	Monte Alto
42	Accession 42	Monte Alto
43	Mini Figo	IAC ¹
44	Preto	Piracicaba
45	Turco	Campinas
¹ IAC = Agronomic Research Institute, APTA Fruit Center, Jundiaí / SP.		

Fertilization was performed according to soil analysis in the projection of fig tree canopies, with soil lacking mulching. Whenever necessary, weed control was performed by weeding, and irrigation by drip tape three times a week with average precipitation of 2.0 mm / h and a 50 cm wide strip in the planting row. Phytosanitary treatment was carried out with fortnightly applications of 1% Bordeaux mixture.

For evaluations of nematode incidence, the root system of plants was analyzed in the months of November and April, regarding the occurrence or not of *Meloidogyne incognita* nematodes, characterized by the formation of galls on the roots of infested plants. Thus, plants that presented galls in the root system were considered to be infested by nematodes.

In addition, soil and root samples with galls were sent to the Laboratory of Agricultural Nematology, Department of Plant Protection, Faculty of Agricultural Sciences at FCA/UNESP for identification and quantification of infecting nematodes by gender. In order to identify the species of root-knot nematodes, root samples were sent to the Laboratory of Nematology, Faculty of Agricultural and Veterinary Sciences, UNESP, Campus of Jaboticabal.

Evaluations to quantify the natural rust incidence in the field were carried out in November, coinciding with the beginning of fruit maturation. The experimental design adopted was completely randomized, each material being considered a treatment, with six replicates, with each plant being considered a replicate. Evaluation of the resistance of fig accessions was carried out based on the infection efficiency, which was evaluated through the percentage of area with symptoms, taking into account a 1cm² disk of a specific leaf region (upper right part of the leaf), in three leaves per replicate, each leaf being collected from one part of the plant (new, medium and basal leaves). The injured area was estimated with a 20 x magnifying glass coupled to a digital camera, and then, images were evaluated in computer based on methodology described by Rodrigues et al. (2012).

To quantify the global genomic methylation content of fig trees, young leaves without spots or perforations were collected and washed under running water, and their veins were removed. The extraction of total genomic DNA from plant tissues was performed according to the modified cetyltrimethylammonium bromide technique.

Global DNA methylation was quantified using the Imprint DNA Methylation Quantification kit® (Sigma), following manufacturer's instructions. This kit functions similarly to an ELISA plate assay, using well strips that have been pre-treated with binding methylated DNA, and using DNA methylation sensitive capture antibody and detection allowing colorimetrically detection of relative amounts of DNA methylation at absorbance of 450nm.

Statistical analyses were carried out on results of all accessions in each evaluation data. The averages obtained were compared using Scott-Knott's test at 5% probability significance threshold. The SISVAR software (Ferreira 2019) was used to perform statistical analyses and experimental planning.

Subsequently, with data on variables percentage of rust incidence and global genomic methylation content, principal component analysis was performed to define the clustering pattern between genotypes and the relative contribution of diversity per character in order to allow identifying which variables overlap and that can be discarded through the residual correlation matrix obtained by the joint analysis of variance using the GENES software (Cruz et al. 2013).

Declarations

AUTHOR CONTRIBUTION STATEMENT

M.G.F.R. is the creator of the work, responsible for raising funds and installing the experiment, in addition to being the main author.

A.C.F. holds a Ph.D. in phytopathology and helped in the analysis of rust, in addition to contributing to the writing of the article.

J.J.V. performed the field rust analysis.

B.E.P. holds a PhD in genetics and performed the joint analysis of the data, as well as assisting in the writing of the article and in the statistical analyses.

A.F.A.F. performed the statistical analyzes and developed the graphs for this article.

L.N H.M. assisted in the writing of the article and in the nematoid count analysis in the field as well as Maria Eugênia da Silva Martins.

R.V.G.S. performed the DNA methylation analysis of the accessions in addition to assisting in the writing of the article.

E.S.R. holds a PhD in genetics and assisted in DNA methylation analysis.

All authors reviewed the manuscript

ACKNOWLEDGEMENTS

To the State of São Paulo Research Foundation (FAPESP Process number 2020/07005-1) for the financial assistance granted to the first author and to the Faculty of Agrarian and Technological Sciences FCAT / UNESP.

COMPLIANCE WITH ETHICAL STANDARDS

Funding: This study was funded by The São Paulo Research Foundation, FAPESP (grant number 2020/07005-1).

The present study has been conducted in accordance with the appropriate environmental guidelines of the UNESP campus of Dracena/FCAT and the rules dictated by the Brazilian environmental legislation.

Conflict of Interest: The authors declare that they have no conflict of interest.

The datasets generated during and/or analysed during the current study are available in the DMP Tool repository, <https://dmptool.org/plans/68513>

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Figures

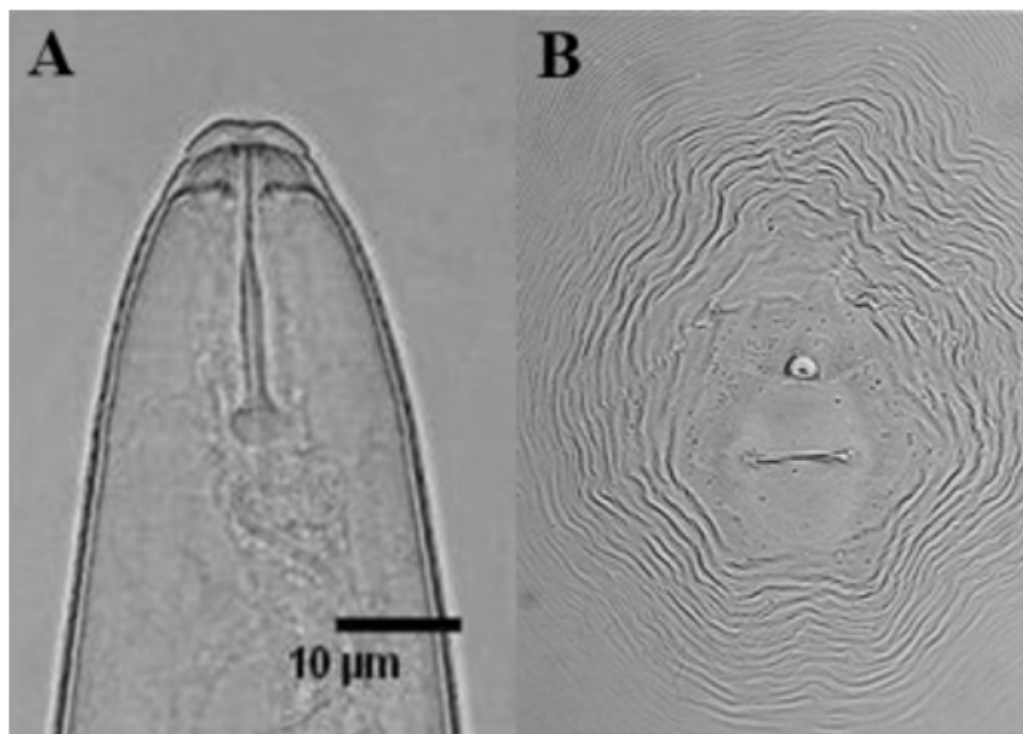


Figure 1

Morphological characteristic to identify the *Meloidogyne incognita* population used in this study, demonstrating the perineal configuration of females (A) and the labial region of males (B).

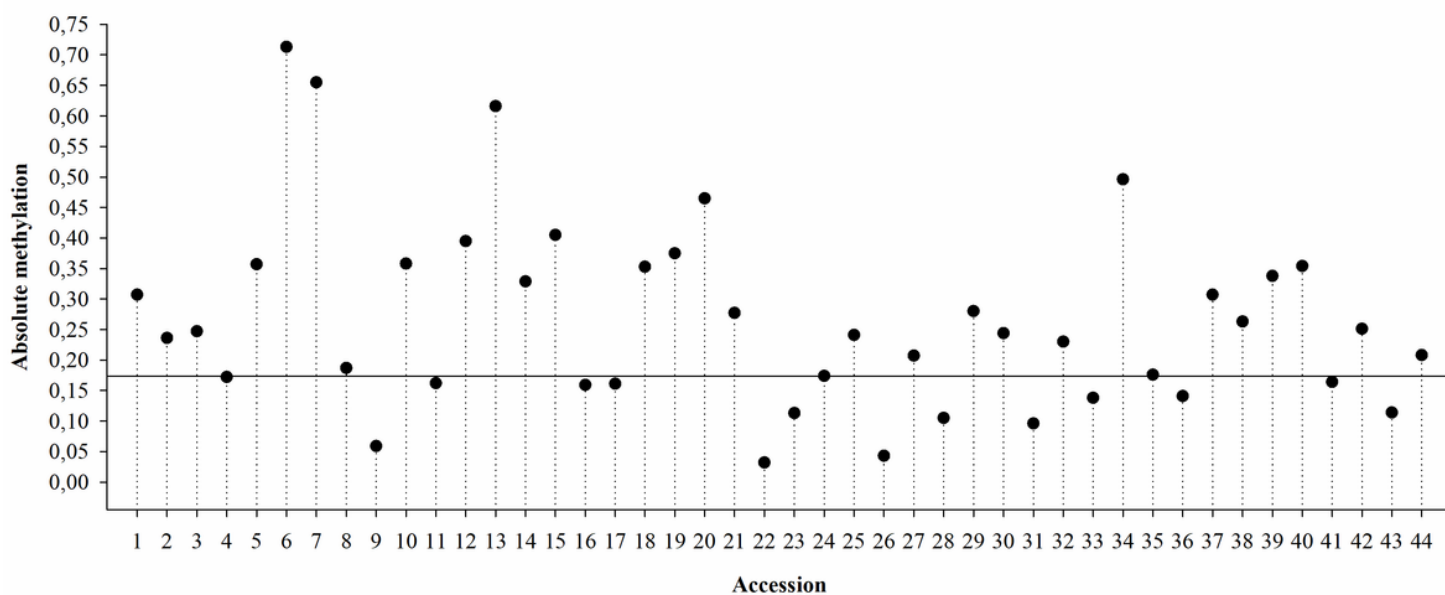


Figure 2

Quantification of global genomic methylation of fig tree accession. Dashed horizontal lines indicate the limits of values statistically different from the 'Roxo-de-Valinhos' pattern, represented by the continuous horizontal line.

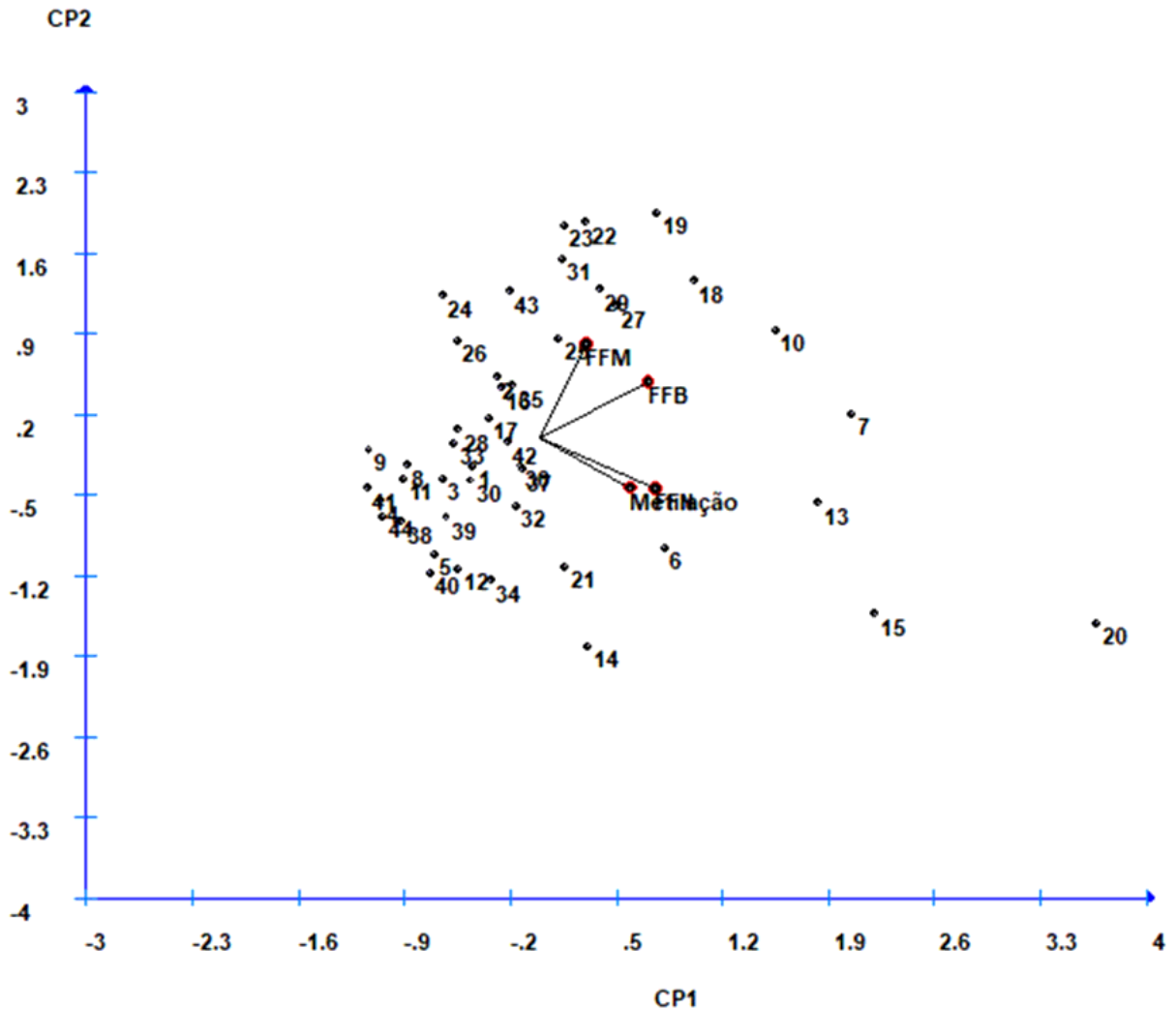


Figure 3

Principal component analysis using percentages of rust incidence and global genomic methylation content of fig tree accessions.