Species of Fusarium fujikuroi complex causing seed and seedling rot of Baru (Dipteryx alata Vogel) in Brazil

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Article

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Abstract

Baru (*Dipteryx alata* Vogel) is a plant native to the Brazilian Cerrado and has numerous uses. In October 2022, Baru seedlings were received at the NPF plant disease diagnosis clinic, Universidade Federal de Goiás. These plants showed symptoms of stem lesions and apical death, in addition to rotten seeds. To investigate the cause of the disease, isolation was initially carried out on potato dextrose agar medium, and 30 conidia were measured. Then, the pathogenicity test was conducted on Baru seedlings and seeds. Molecular characterization was performed by partial sequencing of *act*, *its*, *rpb2* and *tef1-α* markers and concatenated phylogenetic analysis by Bayesian inference. Based on the isolations, it was observed that the mycelium presented a cotton-like appearance in culture, from pink to purple. The microconidia were ellipsoid, unicellular, and ranged from 5.224 to 12.773 µm x 2.250 to 4.532 µm. In the pathogenicity test, apical death of seedlings, lesions and seed rot were observed. In the laboratory test, the appearance of depressed lesions on the seed coat, cotyledons, stem, and roots was observed, from which it was also possible to recover the fungi, fulfilling Koch's postulates. The molecular identification revealed that the isolates belong to the species *Fusarium sacchari* and *F. musae*. To our knowledge, this is the first report of them associated with rot of Baru seeds and seedlings in Brazil.

Introduction

The Baru (*Dipteryx alata* Vogel, Fabaceae) is a tree native to the Brazilian Cerrado that has numerous uses, such as its almond, used in human nutrition and capable of improving the lipid profile in the blood. Furthermore, its biotechnological potential stands out from a microbiological and energy point of view[1]. As it is a native plant, mostly exploited in an extractive way, few diseases associated with it are known. In its seeds, for example, fungi such as those of the genera *Phomopsis*, *Aspergillus* and *Fusarium* have been observed, although the species was not identified at the time[2]. The fungus *Phoma multirostrata* has also been associated with this plant in Brazil, causing leaf spots[3]. Despite these drawbacks, this plant is an excellent option for the crop-livestock-forest integration system, which indicates that its commercial cultivation can expand in the country[4].

Fusarium (Ascomycota, Nectriaceae) is a genus of anamorphic filamentous fungi with great diversity and worldwide distribution, capable of infecting plants, animals and humans[5]. Although difficult to estimate currently, in 2015 there were around 300 phylogenetically distinct species/ complexes[6]. This ascomycete is among the most economically important plant pathogens[7]. Several species of this fungus can be considered as soil-borne[8] and seed-borne pathogens[9], facilitating its survival and dissemination.

Gibberella fujikuroi (Sawada) Wollenw. is a polytypic species complex with anamorphs in *Fusarium*. It is a taxonomically controversial group, especially when using traditional classification methods based only on the morphology of the genus[8]. Its taxonomy evolved considerably after the adoption of DNA-based sequencing identification[6]. It is noteworthy that the correct identification of the species involved in a disease is of fundamental importance for the control of diseases caused by this fungus. The present

study aimed to identify the causal agent responsible for the rot of Baru seeds and seedlings, in the State of Goiás, in the midwestern region of Brazil.

Results

Isolations and pathogenicity tests. Based on the isolations effectuated, four fungi were obtained. For them, it was observed that the mycelia were septate and had a cottony appearance in culture, from pink to purple (Fig. 1A and B). Concerning morphology and morphometry, the microconidia were ellipsoid, unicellular, and ranged from 5.224 to 12.773 µm x 2.250 to 4.532 µm (Fig. 1C), similar to the genus *Fusarium*.

In the pathogenicity test, after 10 days in the shade house, it was possible to observe depressed lesions on the seeds (Fig. 1D), as well as apical death and rot of cotyledons (Fig. 1E). Some seeds, when they did not germinate, exhibited intense fungal colonization and rot (Fig. 1F). In the seed pathogenicity test, the appearance of depressed lesions on the seed coat, cotyledons, stem, and roots was observed (Figs. 1G, H and I). Unlike the in vivo test, all seeds germinated under these conditions. In both pathogenicity tests, it was possible to recover the fungi, fulfilling Koch's postulates.

Molecular identification. With reference to sequencing-based identification, two isolates with distinct cultural characteristics were selected. Among the markers used individually, *act*, *rpb2* and *tef1-α* made it possible to group the NPF FB2 isolate with the references used from *F. sacchari*. Regarding the NPF FB3 isolate, only *tef1-α* grouped separately with the *F. musae* references, while the other genes did not distinguish it from *F. verticillioides* (data not shown).

On the contrary, in the concatenated analysis of the four partially sequenced genes, it was observed that one isolate (NPF FB 2) grouped with the references of *Fusarium sacchari* (CBS 136482 and CBS 147.25), with PP = 1 and the other (NPF FB 3) with the references of *F. musae* (CBS 624.87 and NRRL 25059), with PP = 0.96 (Fig. 2). The sequences of our isolates have been deposited in GenBank under accession numbers OR497260, OR497261, OR567100, OR567101, OR567102, OR567103, OR567104 and OR567105.

Discussion

As previously mentioned, Baru is a nut of great nutritional value, in addition to being part of the Brazilian genetic heritage. A few diseases are known in this plant, as it is exploited in an extractive way. Despite this, interest in planting this species is growing in Brazil, and it is considered an excellent option for the crop-livestock-forest integration system[4]. It is possible that new diseases will be described or discovered in this Brazilian tree species. To date, and from what has been accessed, only the fungi *Phomopsis*, *Aspergillus* and *Fusarium* have been described in Baru seeds, although the species have not been determined[2]. In addition to these, the species *Phoma multirostrata* has also been reported in Brazil, associated with leaf spots[3].

Initially, based on the isolations, the fungi obtained from diseased samples exhibited morphological and morphometric characteristics compatible with the genus *Fusarium*[10]. Regarding the pathogenicity tests implemented, it was possible to prove that the two fungal isolates are pathogenic to Baru. In Koch's postulates, both in vitro and in vivo, the fungi were re-isolated from the induced symptoms, matching what was originally observed and establishing the causal relationship[11].

As it is a soil or seed-borne fungus[8,9], it is postulated that the source of inoculum for infection of Baru seedlings is the substrate used. It is known that the genus *Fusarium* has a wide distribution and host range[5], and it is among the fungi with the greatest economic impact on agriculture[7].

There are more than 300 species or species complexes of *Fusarium*[6] and its taxonomy has evolved greatly since the introduction of DNA sequencing identification[8]. The *Gibberella fujikuroi* (Sawada) Wollenw complex also stands out in this group with more than 50 species of agricultural importance, including *F. musae* and *F. verticillioides*[6].

The phylogeny conducted, based on the concatenated analysis of molecular markers, allowed the identification of two species. The first, *F. musae*, is close to *F. verticillioides*, which was once considered a population of the latter. However, multilocus phylogeny allowed separation into a new species, with a host range more restricted to banana[12]. The other species identified and that was associated with Baru rot was *F. sacchari*. This species was reported causing root rot in corn, sorghum, and rice[13], and the expansion of its range of host plants is considered relevant for the introduction and establishment of these fungi in new cultivated areas[14]. Furthermore, for other species of this fungus, passage through alternative hosts is also an important adaptation mechanism[15], which may explain the occurrence of these species in Baru.

Conclusion

In light of these findings, it is concluded that the fungi in question belong to the *Fusarium fujikuroi* complex and, to our knowledge; this is the first report of them associated with rot of Baru seeds and seedlings in Brazil, which can cause problems, especially in nursery conditions.

The expansion of these species to Baru may be associated with the substrate as a source of inoculum and highlights the phytosanitary importance that these fungi may represent for cultivation of native Brazilian plants.

Materials and Methods

Obtaining Baru samples. In October 2022, Baru seedlings from a school nursery (16°35'48.4"S 49°16'45.1"W) were received at the Phytopathology Research Center (School of Agronomy, Universidade Federal de Goiás). These plants showed symptoms of stem lesions (Fig. 3A), in addition to seed rot

(Fig. 3B and C) and apical death (Fig. 3D). According to a report from the study group, approximately 400 seedlings had already been lost due to this phytosanitary problem.

Isolations and pathogenicity tests. To investigate the cause of the disease, direct and indirect isolations were initially performed on potato dextrose agar medium. After growth, the cultures were purified using the hyphal tip technique. The fungi are kept in the mycotheque at our research center. Based on pure cultures, microscopy was performed, and conidia were measured (n = 30).

In the first pathogenicity test, to fulfill Koch's postulates[11], 5 g of parboiled rice colonized with the isolates was incorporated into pots containing autoclaved soil. Then, superficially disinfested seeds were sown and kept in a shade house. To investigate the effect of fungal isolates on seeds, in the second pathogenicity test, suspensions were prepared at a concentration of 1 x 10^7 conidia/mL (adjusted in a Neubauer chamber), which was used to microbiolize disinfested Baru seeds, and maintained in gerboxes, containing 3 sheets of germitest paper moistened with 2.5x their weight. They were kept in an incubator at 28ºC.

Molecular identification. Starting the molecular identification of the isolates, the DNA of the fungi was extracted using the methodology of Dellaporta et al.[16]. Partial sequences of four markers (*actin*, *rpb2*, *its* and *tef1-α*) were amplified by PCR (Polymerase Chain Reaction) with the primers: act512F/act783R[17], fRPB2-5f/ fRPB2-7cr[18], ITS5/ITS4[19] and EF1/EF2[20]. After amplification, 5 µL of each PCR product was subjected to electrophoresis in a 1.5% agarose gel, stained with GelRed in TBE buffer (Tris Borate EDTA) at 80 volts and visualized using a UV transilluminator. PCR products were purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com). The obtained sequences were analyzed and compared in GenBank/NCBI using the Basic Local Alignment Search Tool (BLAST). The sequences of the isolates in this study were aligned with additional reference sequences (Table 1) using the MEGA v.6 software[21] employing the MUSCLE® algorithm.

Alignments were checked and manual adjustments were made when necessary. The best nucleotide substitution model for each gene/region was determined using the Akaike Information Criterion (AIC) implemented in MrMODELTEST 2.3[22]. Bayesian Inference (BI) analyses employing the Monte Carlo integration method (MCMC), via Markov chain, were performed with the selected sequences, first with each region separately, then with the combined data set. Phylogenetic analysis of each region and the concatenated dataset was conducted in MrBayes in XSEDE v.3.2.6[23] on the CIPRES Science Gateway[24]. Four MCMC chains were run simultaneously, starting from random trees for 10,000,000 generations. Trees were sampled every 1,000 generations, resulting in 10,001 trees. The first 2,500 trees were discarded. Posterior Probabilities (PP)[25] were determined from a consensus tree, which was generated from the remaining trees. Trees were visualized in FigTree v. 1.3.1[26], exported, and edited using graphic programs. Sequences from *Fusarium oxysporum* strain CBS 130302 were used as an outgroup (Table 1).

Table 1

Baru isolates identified in Brazil (bold) and Genbank accession number references of partial sequences of *act*, *its*, *rpb2* and *tef1*-α used in the phylogenetic analysis.

Species	Strain	*act*	*its*	*rpb2*	*tef1*
F. sacchari	**NPF FB2**	**OR497260**	**OR567100**	**OR567102**	**OR567104**
F. musae	**NPF FB3**	**OR497261**	**OR567101**	**OR567103**	**OR567105**
F. acutatum	CBS:131573	KU603809.1	MH865924.1	KU604291.1	KU711682.1
F. andiyazi	CBS:134430	KU603777.1	KC954400.1	KU604232.1	KC954401.1
F. annulatum	CBS:533.95	NA*	NA	MW402817.1	MW402138.1
F. anthophilum	CBS:245.59	KU603818.1	KR071655.1	KU604278.1	KU711686.1
F. bactridioides	CBS:100057	MT010878.1	MH862686.1	MW402490.1	MN533993.1
F. begoniae	CBS:452.97	NA	MH862660.1	MN534243.1	MN533994.1
F. brevicatenulatum	CBS:404.97	MT010873.1	NA	MT010979.1	MT011005.1
F. circinatum	CBS:405.97	KM231215.1	MH862654.1	MW402656.1	KM231943.1
F. concentricum	CBS:102157	NA	NA	MW402728.1	MW401963.1
F. denticulatum	CBS:407.97	NA	NR_138359.1	MN534274.1	MN534000.1
F. ficicrescens	CBS:125181	KU603781.1	KU604032.1	KT154003.1	KP662900.1
F. foetens	CBS:110286	NA	MH862858.1	MW928825.1	NA
F. fujikuroi	CBS:257.52	KU603839.1	MH857023.1	MW402645.1	MW402119.1
F. globosum	CBS:431.97	NA	LT746280.1	MW402816.1	MW402131.1
F. guttiforme	CBS:409.97	NA	KC464629.1	NA	KC514066.1
F. lactis	CBS 420.97	NA	NA	MN534296.1	MN534015.1
F. musae	CBS:624.87	MT010866.1	NA	MT010973.1	MT010991.1
F. musae	NRRL 25059	NA	NA	FN552108.1	FN552086.1
F. napiforme	CBS:673.94	KU603793.1	KR071649.1	KU604237.1	KR071715.1
F. nygamai	CBS:749.97	KU603795.1	MH862671.1	KU604262.1	KU711704.1
F. oxysporum	CBS:130302	MT010876.1	MH865881.1	MT010985.1	MT011000.1
F. proliferatum	CBS:189.38	KM231217.1	MH855944.1	KM232384.1	NA

*NA - Not Available

Species	Strain	*act*	*its*	*rpb2*	*tef1*
F. sacchari	**NPF FB2**	**OR497260**	**OR567100**	**OR567102**	**OR567104**
F. musae	**NPF FB3**	**OR497261**	**OR567101**	**OR567103**	**OR567105**
F. pseudocircinatum	CBS:455.97	NA	NA	MN534276.1	MN534029.1
F. pseudonygamai	CBS:417.97	MT010871.1	MH862656.1	MW402664.1	MT011008.1
F. ramigenum	CBS:526.97	NA	NA	MN534292.1	MN534032.1
F. sacchari	CBS:136482	KU603833.1	KU604028.1	KU604311.1	KU711671.1
F. sacchari	CBS:147.25	MT010858.1	MH854822.1	NA	MW402099.1
F. sublunatum	CBS:189.34	KM231211.1	MH855485.1	KM232380.1	NA
F. subglutinans	CBS:119831	KU603823.1	KR071624.1	KU604281.1	KU711691.1
F. temperatum	CBS:135538	NA	NA	KU604283.1	MN534039.1
F. verticillioides	CBS:576.78	MH861171.1	MH861171.1	KU604216.1	KU604383.1
F. verticillioides	CBS:579.78	KU603773.1	KR071636.1	KU604223.1	KU604390.1
*NA - Not Available					

Declarations

Author contributions

E.M. and M.G.C. designed the experiments. E.M., M.R.S, E.F.B.R., G.G.L., I.G.F.C. and E.R.B.S. conducted the experiments. E.M. and M.R.S analyzed the data. All authors reviewed the manuscript.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

Additional Information

Collection permission Our higher education institution has permission to collect and study Baru.

CorrespondenceRequests for materials should be addressed to E.M.

Ethical approval This article follows the experimental guidelines of the country. No human beings or animal were involved during the study.

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References

1. Lima, D. C. et al. A review on Brazilian Baru plant (*Dipteryx alata* Vogel): morphology, chemical composition, health effects, and technological potential. Future Foods 5, 100146 (2022).
2. Santos, M. F. et al. Fungos associados às sementes de Baru (*Dipteryx alata* Vog.). Revista Brasileira de Sementes 19, 135–139 (1997).
3. Anjos, J. R. N. et al. Mancha foliar em Baru (*Dipteryx alata*) causada por *Phoma multirostrata*. Rev Bras Frutic 31(2), 593–5 (2009).
4. Embrapa. The baru tree is an excellent alternative for use in integrated crop-livestock-forestry systems *Agroindustry Plant production* https://www.embrapa.br/en/busca-de-noticias/-/noticia/64724803/arvore-do-baru-e-excelente-alternativa-para-cultivo-em-ilpf (2021).
5. Sáenz, V. et al. A One health perspective to recognize *Fusarium* as important in clinical practice. J Fungi (Basel) 6(4), 235 (2020).
6. O'Donnell, K. et al. DNA sequence-based identification of *Fusarium*: Current status and future directions. Phytoparasitica 43, 583–595 (2015).
7. Aoki, T., O'Donnell, K., Geiser, D. M. Systematics of key phytopathogenic *Fusarium* species: current status and future challenges. J Gen Plant Pathol 80,189–201 (2014).
8. O'Donnell, K., Cigelnik, E., Nirenberg, H. I. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90(3):465–493 (1998).
9. Chang, X. et al Diversity of the seedborne fungi and pathogenicity of *Fusarium* species associated with intercropped soybean. Pathogens (Basel, Switzerland) 9(7), 531 (2020).
10. Wollenweber, H. W. Studies on the *Fusarium* problem. Phytopathology 3, 24–29 (1913).
11. Koch, R. Methods for the study of pathogenic organisms. Mitteilungen des Kaiserlichen Gesundheits-Amtes 2, 1–48, (1881).
12. Van Hove, F. et al. *Gibberella musae* (*Fusarium musae*) sp. nov., a recently discovered species from banana is sister to *F. verticillioides*. Micology 103, 570–85 (2011).
13. Leslie J. F., Summerell, B. A. *The Fusarium Laboratory Manual*. Oxford, UK: Blackwell Publishing (2006).
14. Costa, M. M. et al. Identification and pathogenicity of *Fusarium* species associated with pokkah boeng of sugarcane in Brazil. Plant Pathology 68(7), 1350–1360 (2019).
15. Akinsanmi, O. A., Chakraborty, S., Backhouse, D., Simpfendorfer, S. Passage through alternative hosts changes the fitness of *Fusarium graminearum and Fusarium pseudograminearum*. Environ Microbiol 9(2), 512–520 (2007).

16. Dellaporta, S. L., Jonathan, W., James, B. H. A plant DNA minipreparation: version II. Plant Mol Biol Rep 1, 19–21 (1983).
17. Carbone, I., Linda, M. K. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91, 553–556 (1999).
18. Liu, Y. J., Sally, W., Benjamin, D. H. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerse II subunit. Mol Biol Evol 16, 1799–1808 (1999).
19. White, T. J. et al. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, pp. 315–322 (1990).
20. O'donnell, K. et al. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum - F. equiseti* and *F. chlamydosporum* species complexes within the United States. J Clin Microbiol 47, 3851–61 (2009).
21. Tamura, K. et al. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30, 2725–9 (2013).
22. Posada, D., Thomas, R. B. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. Syst Bio 53,793–808 (2004).
23. Ronquist, F. M. et al. Mrbayes 3.2: Efficient Bayesian phylogenetic inference and model selection across a large model space. Syst Biol 61, 539–542 (2012).
24. Miller, M. A., Wayne, P., Terri, S. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *2010 Gateway Computing Environments Workshop (GCE)*, New Orleans, LA, USA, 2010, pp. 1–8 (2010).
25. Rannala, B., Yang, Z. Probability distribution of molecular evolutionary trees: A New Method of Phylogenetic Inference. J Mol Evol 43(3), 304–31 (1996).
26. Rambaut, A. FigTree version 1.3.1 [*computer program*]. http://tree.bio.ed.ac.uk (2009).

Figures

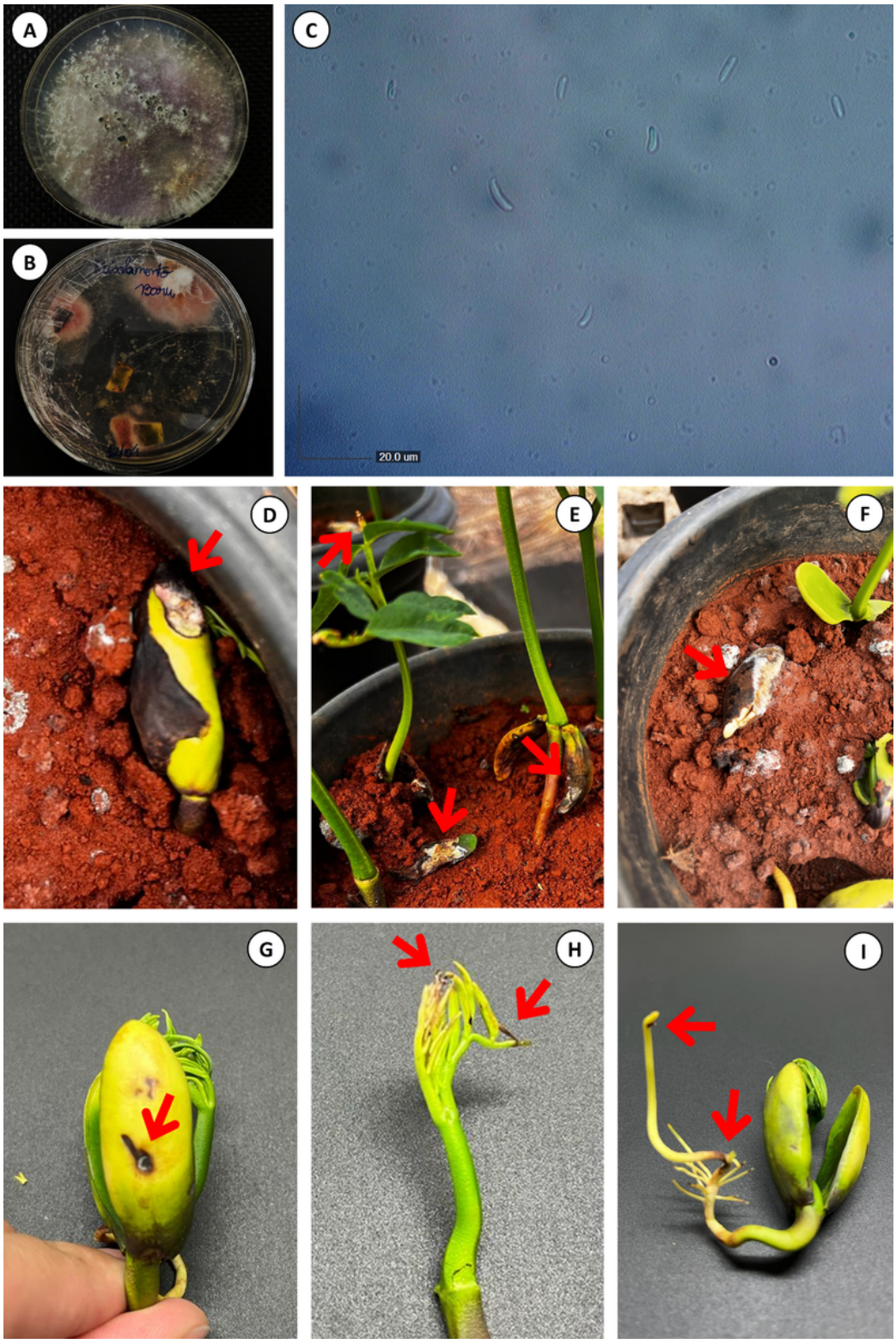

Figure 1

Characterization of fungal isolates from Baru, where A) Pure culture of one of the isolates; B) Reisolations of the pathogenicity test; C) Microscopy showing microconidia; D) Pathogenicity test in shade house with seed showing a large lesion with pink growth, E) Apical death and rot of cotyledons, F) Detail of seed rot; G) Pathogenicity test on seeds in the laboratory, showing a depressed lesion in the cotyledon, H) Apical necroses in the seedling and I) Necroses in the root.

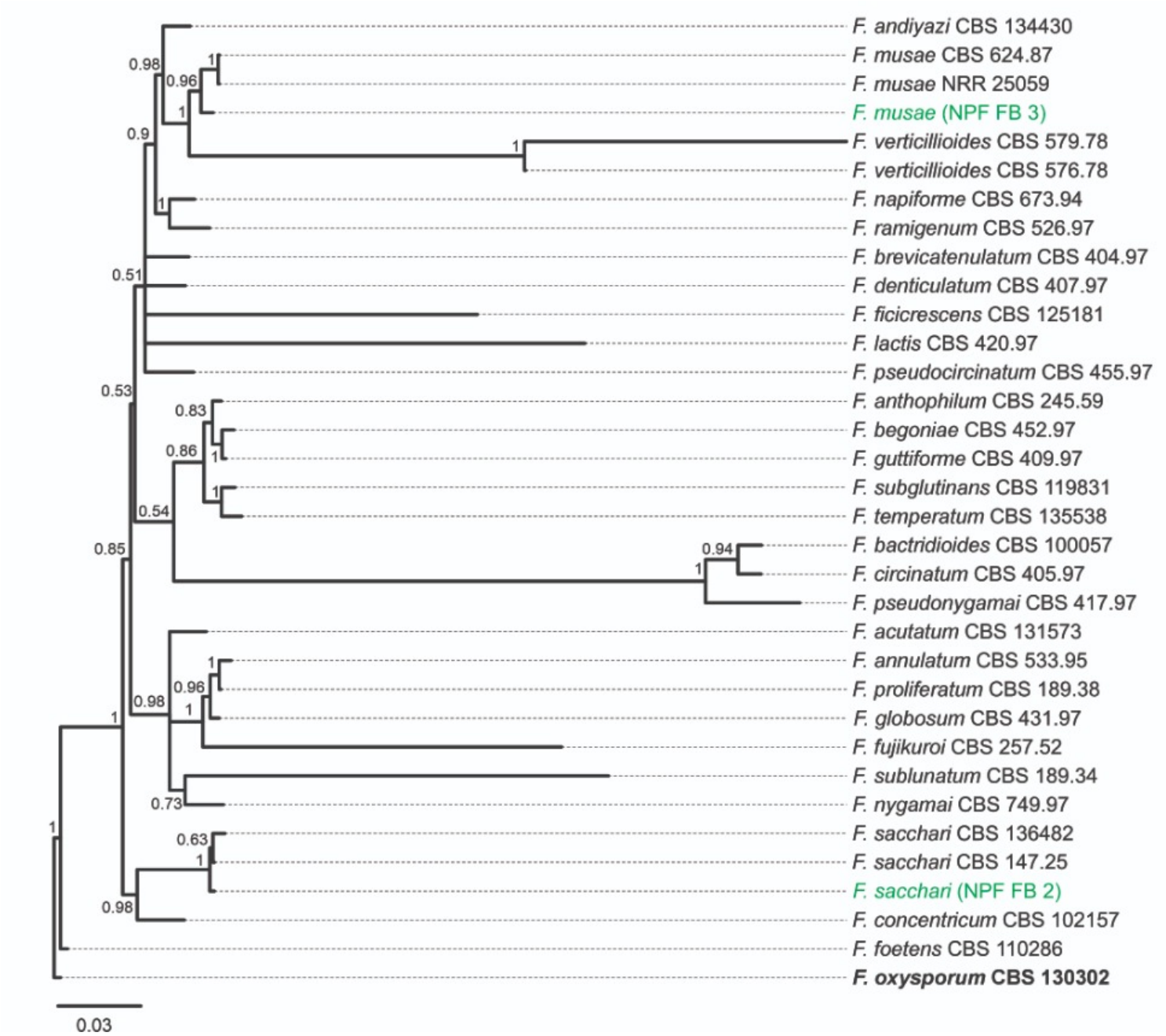

Figure 2

Multilocus phylogenetic tree as inferred from Bayesian analysis based on the combined sequences of *act*,*ITS*, *rpb2* and *tef1-a*. The Bayesian posterior probabilities are next to the nodes. The tree was rooted with *Fusarium oxysporum* CBS 130302. The species in this study are highlighted in green.

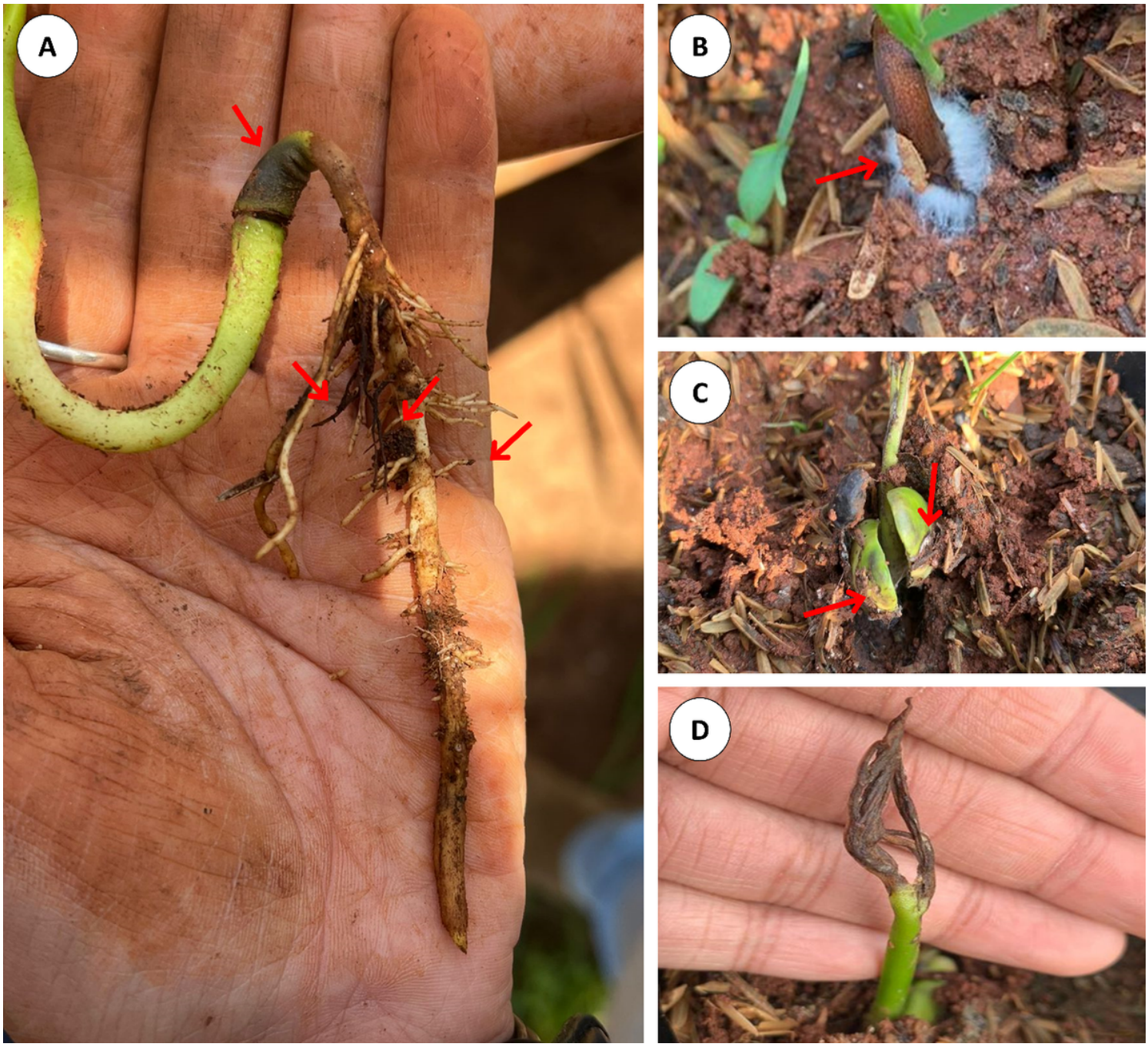

Figure 3

Symptoms observed in Baru seedlings under nursery conditions, where: A) Necrosis in the collar and roots (red arrows), B) Seed exhibiting fungal colonization (red arrows), C) Poorly formed seedling, with lesions on the cotyledon and D) Apical seedling death.