Three species of Colletotrichum are associated with anthracnose of pomegranate in Brazil

Janaíne Rossane Araújo Silva-Cabral*, Lourdes Regina Lopes Batista*, Jaqueline Figueiredo de Oliveira Costa*, Edna Peixoto da Rocha Amorim*, Gaus Silvestre de Andrade Lima*; Iraitides Pereira Assunção*

Federal University of Alagoas, Rio Largo, Brazil
*Corresponding author, e-mail: janaine_rossane@hotmail.com

Abstract

Pomegranate is a fruit rich in vitamins and secondary metabolites used in traditional medicine and industry. However, production losses have been associated with the anthracnose, disease caused by Colletotrichum species. This is an important disease of the pomegranate, as it affects the yield and the quality of the fruits. The present study aimed to investigate which species of Colletotrichum are associated with anthracnose disease in pomegranate in the Northeast region of Brazil, using multi-locus phylogenetic analysis and morpho-cultural characteristics. The total DNA extracted was amplified with GAPDH, TUB2, CAL, ACT genes and the ITS-rDNA region. The sequences obtained were used for the construction of phylogenetic trees of Bayesian inference. The mycelial growth rate, size and shape of the conidia and appressories were evaluated for the morpho-cultural characterization of the species. Six isolates were analysis and three species belonging to the C. gloeosporioides complex were identified in this study. This is the first report of C. theobromicola (2) in pomegranate fruit in Brazil and C. siamense (2) and C. fructicola (2) in the world.

Keywords: Colletotrichum fructicola, Colletotrichum siamense, Colletotrichum theobromicola, Punica granatum, multi-locus phylogeny

Pomegranate (Punica granatum L.) is a tree native to the Middle East but it is well adapted to the climatic conditions of Brazil. Commercial production of pomegranate has expanded internationally as well as within Brazil due to increasing interest from farmers. The fruit is rich in nutrients and active principles that can be used in the cosmetic, pharmaceutical and food industry (Kanetis et al., 2015; Hmid et al., 2017).

However, fungi of the genus Colletotrichum Corda have harmed the yield of this culture, causing the anthracnose disease. Anthracnose fruit rot is considered one of the most important fungal diseases of the pomegranate, both in pre-harvest and post-harvest, because it leads to significant financial losses, mainly due to the rot symptoms that depreciate the fruit and lead to rapid deterioration (Munhuweyi et al., 2016).

Although different species of the genus Colletotrichum cause anthracnose in several hosts, only the species Colletotrichum gloeosporioides (Penz.) Penz. & Sacc had been reported in pomegranate, because its identification was based exclusively on morphological studies, host range and analysis of the internal ribosomal transcribed spacer region (ITS-rDNA) (Lakshmi et al., 2011; Thomidis & Exadaktylou, 2011; Rahimlou et al., 2014).

Nevertheless, the current taxonomy of the Colletotrichum genus has been based on multi-locus phylogenetic studies combined with phenotypic characteristics. Studies using these approaches concluded that C. gloeosporioides is a complex of species (Weir et al., 2012; Jayawardena et al., 2016). In addition, the species C. theobromicola Delacr. Bull and C. tropicale E.I. Rojas, S.A. Rehner & Samuels were reported on pomegranate fruits in India and Brazil, respectively, and C. nymphaeae (Pass.) Aa, C. fioriniae (Marcelino & Gouli) R.G. Shivas & Y.P. Tan, C. simmondsii R.G. Shivas & Y.P. Tan, C. theobromicola, C. siamense Prihastuti L. Cai
Three species of Colletotrichum are... complex obtained from GenBank were included in the analysis (Table supplementary 1). The partial sequences obtained in this study were deposited in GenBank. The alignment and tree were deposited in TreeBASE (http://www.treebase.org; accession number: 23044).

For morpho-cultural characterization, colonies were grown on synthetic PDA incubated at 25°C for 7 days and measured daily. The colour of the colonies was evaluated in the 7th day. Format and size of fifty conidia and appressoria were also measured. Data were submitted to analysis of variance (ANOVA) (p<0.05) by Tukey's test.

Six isolates were obtained from Alagoas (COUFAL0042, COUFAL0043, COUFAL0044), Bahia (COUFAL0040 and COUFAL0041) and Pernambuco (COUFAL0049). All isolates tested caused symptoms at 7 to 10 days after inoculation. The control fruits showed no symptoms. Sequences of the GAPDH gene showed high similarity with sequences of three species from C. gloeosporioides complex deposited in the GenBank: C. siamense, C. theobromicola and C. fructicola. Prihastuti L. Cai & K.D. Hyde. The results of both phylogenetic BI analysis confirmed the prior identification of these three species (Figure 1).

The isolates produced hyaline, straight, cylindrical to clavate conidia with often broadly rounded ends and brown appressoria of irregular format. Colonies varied between grey to white with mean growth of 5.8 mm/day (Table 1). All morpho-cultural features were similar to the species from C. gloeosporioides complex (Rojas et al., 2016; Meetum et al., 2015; Sharma et al., 2015; Liu et al., 2016; Costa et al., 2019). Moreover, the species C. gloeosporioides was not reported in the present study.

These species are widespread in different world locations causing anthracnose on several hosts. However, C. fructicola was not previously reported on pomegranate anywhere in the world, while C. siamense and C. theobromicola were reported only in the USA and the latter also in India, probably because of the lack of studies using multi-locus phylogeny for the identification of species from Colletotrichum in this host (Alaniz et al., 2015; Meetum et al., 2015; Sharma et al., 2015; Liu et al., 2016; Costa et al., 2019). Moreover, the species C. gloeosporioides was not reported in the present study.

Studies carried out in Brazil with other hosts using multi-locus phylogenetic, in which anthracnose had also been attributed exclusively C. gloeosporioides based in morphological characteristics, corroborate with this result, reporting C. fructicola on cashew, pine cone, sourpox and mango; C. siamense on cashew, pine cone, sourpox and mango, C. tropicale on cashew, pomegranate, pine cone, sourpox and mango, C. asianum on mango, C.
Three species of Colletotrichum are... 

![Image](https://example.com/image.png)

**Figure 1.** Multi-locus phylogenetic tree inferred from the Bayesian analysis using GAPDH, TUB2, ACT and CAL genes and ITS region for C. gloeosporioides complex. The combined data set consisted of 2614 nucleotides. The SYM+I model of evolution was used for all gene. The values of posterior probability > 0.5 are indicated above knots. Ex-type cultures are marked with an asterisk. The strains used in this study are emphasized in bold. The tree was rooted with Colletotrichum boninense.

| Species                  | Length | Width | Shape          | Growth rate | Colonies                       |
|--------------------------|--------|-------|----------------|-------------|--------------------------------|
| C. fructicola            | 13.2c  | 4.3b  | Cylindrical    | 4.0c        | Greyish, greendark reverse     |
| COUFAL0040               | (7.72-18.12) | (2.31-6.04) |                |             |                                |
| COUFAL0041               |        |       |                |             |                                |
| C. theobromicola         | 16.7a  | 4.6a  | Cylindrical    | 6.2a        | Gray with white edges, dark gray reverse |
| COUFAL0043               | (13.32-20.95) | (3.18-5.68) |                |             |                                |
| COUFAL0044               |        |       |                |             |                                |
| C. siamense              | 14.2b  | 4.6a  | Cylindrical    | 5.3b        | White, greenish reverse        |
| COUFAL0042               | (12.59-17.62) | (3.68-5.37) |                |             |                                |
| COUFAL0048               |        |       |                |             |                                |

Means followed by the same letter in the column do not differ statistically by the Tukey test at 5% probability.

| Table 1. Summary of morphological and cultural characteristic of Colletotrichum strains. |
|---------------------------------|----------------|----------------|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Species                        | Length | Width | Shape          | Length | Width | Growth rate | Colonies                      |
| C. fructicola                  | 13.2c  | 4.3b  | Cylindrical    | 4.0c   |       |             | Greyish, greendark reverse     |
| COUFAL0040                    | (7.72-18.12) | (2.31-6.04) |                |        |       |             |                                |
| COUFAL0041                    |        |       |                |        |       |             |                                |
| C. theobromicola              | 16.7a  | 4.6a  | Cylindrical    | 6.2a   |       |             | Gray with white edges, dark gray reverse |
| COUFAL0043                    | (13.32-20.95) | (3.18-5.68) |                |        |       |             |                                |
| COUFAL0044                    |        |       |                |        |       |             |                                |
| C. siamense                   | 14.2b  | 4.6a  | Cylindrical    | 5.3b   |       |             | White, greenish reverse        |
| COUFAL0042                    | (12.59-17.62) | (3.68-5.37) |                |        |       |             |                                |
| COUFAL0048                    |        |       |                |        |       |             |                                |

This is the first report of the species C. fructicola causing anthracnose on pomegranate fruits in the world and of C. siamense and C. theobromicola in Brazil. This knowledge is critical to the development of more efficient control measures of anthracnose disease of pomegranate, inasmuch as, little was known about the Colletotrichum species associated with this crop.

**Acknowledgments**

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001. This work also was supported by Fundação de Amparo à Pesquisa do Estado de Alagoas - FAPEAL.
References

Alainz, S., Hernández L., Mondino P. 2015. Colletotrichum fructicola is the dominant and one of the most aggressive species causing bitter rot of apple in Uruguay. Tropical Plant Pathology 40: 265-274.

Costa, J.F.O., Karnei, S.H., Silva, J.R.A., Miranda, A.R.G.S., Nettg, M.B., Silva, S.J.C., Correia, K.C., Lima, G.S.A., Assunção, I.P. 2019. Species diversity of Colletotrichum infecting Annona spp. in Brazil. European Journal of Plant Pathology 53: 169–180.

Doyle, J.J., Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15.

Hmid, I., Elthomani, D., Hanine, H., Oukabli, A., Mehinagic, E. 2017. Comparative study of phenolic compounds and their antioxidant attributes of eighteen pomegranate [Punica granatum L.] cultivars grown in Morocco. Arabian Journal of Chemistry 10: 2675-2684.

Jayawardena, R.S., Hyde, K.D., Damm, U., Cai, L., Liu, M., Li, X.H., Zhang, W., Zhao, W.S., Yan, J.Y. 2016. Notes on currently accepted species of Colletotrichum. Mycosphere 7: 1192–1260.

Kanetis, L., Testempasis, S., Goulas, V., Samuel, S., Myresiotis, C., Karaoglanidis, G.S. 2015. Identification and mycotoxigenic capacity of fungi associated with pre- and postharvest fruit rots of pomegranates in Greece and Cyprus. International Journal of Food Microbiology 208: 84–92.

Lakshmi, B.K.M., Reddy, P.N., Prasad, R.D. 2011. Cross-infection potential of Colletotrichum gloeosporioides Fenz. isolates causing anthracnose in subtropical fruit crops. Tropical Agricultural Research 22: 183-193.

Lima, N.B., Batista, M.V.A., De Morais Jr, M.A., Barbosa, M.A.G., Michereff, S.J., Hyde, K.D., Câmara, M.P.S. 2013. Five Colletotrichum species are responsible for mango anthracnose in northeastern Brazil. Fungal Diversity 61: 75–88.

Liu, F., Tang, G., Zheng, X., Li, Y., Sun, X., Qi, X., Zhou, Y., Xu, J., Chen, H., Chang, X., Zhang, S., Gong, G. 2016. Molecular and phenotypic characterization of Colletotrichum species associated with anthracnose disease in peppers from Sichuan Province, China. Scientific Reports 6: 32761.

Meetum, P., Leksomboon, C., Kanjanamaneesath, M. 2015. First report of Colletotrichum aenigma and C. siamense, the causal agent of anthracnose disease of dragon fruit in Thailand. Journal of Plant Pathology 97: 391-403.

Munhuweyl, K., Lennox, C.L., Meitz-Hopkins, J.C., Caleb, O.J., Opara, U.L. 2016. Major diseases of pomegranate (Punica granatum L.), their causes and management - A review. Scientia Horticulturae 211:126-139.

Phoulivong, S., Cai, L., Chen, H., Mckenzie, E.H.C., Abd-Elsalam, K., Chukeatirote, E., Hyde, K.D. 2010. Colletotrichum gloeosporioides is not a common pathogen on tropical fruits. Fungal Diversity 44: 33-43.

Rahimiou, S., Babaeizad, V., Sayari, M. 2014. First report of fruit spot on pomegranate caused by Colletotrichum gloeosporioides in Iran. Journal of Plant Pathology 96: 603-611.

Rojas, E.I., Rehner, S.A., Samuels, G.J., Van Bael, S.A., Herre, E.A., Cannon, P., Chen, R., Pang, J., Wang, R., Zhang, Y., Peng, Y.Q., Sha, T. 2010. Colletotrichum gloeosporioides s.l. associated with Theobroma cacao and other plants in Panamá: Multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. Mycologia 102: 1318–1338.

Sharma, G., Pinnaka, A.K., Shenoy, B.D. 2015. Resolving the Colletotrichum siamense species complex using ApMat marker. Fungal Diversity 71: 247–264.

Silva-Cabral, J.R.A., Batista, L.R.L., Costa, J.F.O., Ferro, M.M., Silva, S.J.C., Lima, G.S.A., Assunção, I.P. 2019. First report of Colletotrichum tropicale causing anthracnose on pomegranate in Brazil. Plant Disease 103: 583.

Thomidis, T., Exadaktylou, E. 2011. Occurrence of a fruit spot disease of pomegranates caused by Colletotrichum gloeosporioides in the prefecture of Komotini, Greece. Plant Disease 95: 872.

Veloso, J.S., Câmara, M.P.S., Lima, W.G., Michereff, S.J., Doyle, V.P. 2018. Why species delimitation matters for fungal ecology: Colletotrichum diversity on wild and cultivated cashew in Brazil. Fungal Biology 122: 677-691.

Waculicz-Andrade, C.E., Savi, D.C., Bini, A.P., Adamoski, D., Goulin, E.H., Silva Jr, G.J., Massola Jr, N.S., Terasawa, L.G., Kava, V., Glienke, C. 2017. Colletotrichum gloeosporioides sensu stricto: an endophytic species or citrus pathogen in Brazil? Australasian Plant Pathology 46:191-203.

Weir, B.S., Johnston, P.R., Damm, U. 2012. The Colletotrichum gloeosporioides species complex. Studies in Mycology 73: 115-180.

Xavier, K.V., Kc, A.N., Peres, N.A., Deng, Z., Castle, W., Lovett, W., Vallad, G.E. 2019. Characterization of Colletotrichum species causing anthracnose of pomegranate in the Southeastern United States. Plant Disease 103: 2771-2780.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

All the contents of this journal, except where otherwise noted, is licensed under a Creative Commons Attribution License attribution-type BY.