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2 Northwestern Spain

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12 Maize (*Zea mays*) is one of the most important crops worldwide, and fungal diseases are  
13 responsible for major losses in food production. Anthracnose caused by *Colletotrichum*  
14 *graminicola* can infect all maize tissues, although stalk rot and seedling blight cause more  
15 significant economic damage (Munkvold and White, 2016). Anthracnose stalk rot is  
16 characterized by a distinctive external blackening of the lower stalks resulting in large black  
17 streaks. Suspicious maize stems of variety *Tuy* (a locally grown, traditional variety that is used  
18 for flour production for bread and other foods) exhibiting typical symptoms of anthracnose  
19 stalk rot (black lesions and lodging) were collected from a field in Pontevedra, Galicia, Spain  
20 (Geographical coordinates: 42°23'27.1" N - 8°30'46.3" W) between June and December of  
21 2022. In this region, maize fields are typically small comprising a few thousand m<sup>2</sup> and are  
22 planted at an approximate density of 60000 plants/ha. Approximately 200 m<sup>2</sup> in four fields  
23 were sampled resulting in six symptomatic plants from one field, representing about 0.5%  
24 incidence of disease. Stem samples, approximately 50 mm<sup>2</sup>, were dissected and surface-  
25 disinfected for 90 seconds in 20% sodium hypochlorite bleach (v/v) and rinsed three times in  
26 sterile distilled water. The samples were transferred to one half-strength acidified potato  
27 dextrose agar (PDA) supplemented with ampicillin (100 µg/mL) and lactic acid 90% (1.5  
28 mL/L) and incubated for 5 days at 25 °C (Sukno *et al.* 2008). Single spores were transferred to  
29 fresh PDA plates to obtain pure culture isolates. A total of six isolates were obtained, and  
30 among them, two were selected for further characterization (SP-36820-1 and SP-36820-3).  
31 Colonies grown on PDA have dark gray aerial mycelium with orange-colored spore masses.  
32 Conidia are falcate, slightly curved, tapered toward the tips, and are produced in acervuli with  
33 setae, measuring 37.65 to 24.84 x 8.02 to 4.67 µm, respectively (*n* = 100). These morphological  
34 characteristics are in agreement with *C. graminicola* previously described by Bergstrom and  
35 Nicholson (1999). Isolates were grown in potato dextrose broth (PDB) for 3 days at 25 °C and  
36 total genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA,  
37 USA). The internal transcribed spacer region of rDNA and the manganese-type superoxide  
38 dismutase gene (*SOD2*) were amplified using primers ITS4/ITS5 (White *et al.* 1990) and  
39 SOD625/SOD507 (Fang *et al.* 2002) and consequently sequenced. GenBank BLAST analysis  
40 revealed that the sequences were 100% identical to each other and to strains of *C. graminicola*  
41 in GenBank (including the epitype strain CBS 130836). All sequences were deposited in  
42 GenBank (see e-Xtra 1 for accession numbers). To confirm Koch's postulates, plants of a  
43 derivative of maize inbred line Mo940 (developmental stage V3) were placed horizontally in a  
44 tray for inoculation and 20 droplets (7.5 µL) of a suspension of 3 x 10<sup>5</sup> conidia per milliliter  
45 were placed on the surface of the third leaf. The trays were closed to retain moisture and  
46 incubated overnight at 23°C. The next day, the plants were returned to a vertical position and  
47 incubated in a growth chamber at 25°C with 80% humidity and a light cycle of 16 h of light  
48 and 8 h of dark (Vargas *et al.* 2012). After four days inoculated leaves presented brown  
49 elongated lesions with necrotic centers consistent with *C. graminicola* infection, whereas  
50 control plants remained asymptomatic. The strains reisolated from infected leaves were

51 morphologically identical to the original isolates. To our knowledge, this is the first report of  
 52 *Colletotrichum graminicola* causing maize anthracnose in Spain. Recently, maize anthracnose  
 53 was also reported in Bosnia and Herzegovina and China (Duan et al. 2019; Cuevas-Fernández  
 54 et al. 2019), suggesting the pathogen's geographic range is increasing, which may be a threat  
 55 to maize cultivation in locations with optimal humid conditions for disease development.

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90 **e-Xtra 1:** Phylogenetic tree of the *C. graminicola* isolates and closely related sequences.  
 91 Maximum Likelihood phylogenetic tree reconstructed using a multilocus concatenated  
 92 alignment of ITS and *SOD2* genes of *Colletotrichum* strains used in this study and strains  
 93 belonging to the *Colletotrichum graminicola* species complex. The phylogenetic tree  
 94 confirmed the identity of strains isolated from maize as *C. graminicola*. GenBank accession  
 95 numbers of *C. graminicola* sequences generated in this study: ITS - OQ708378, OQ708379,

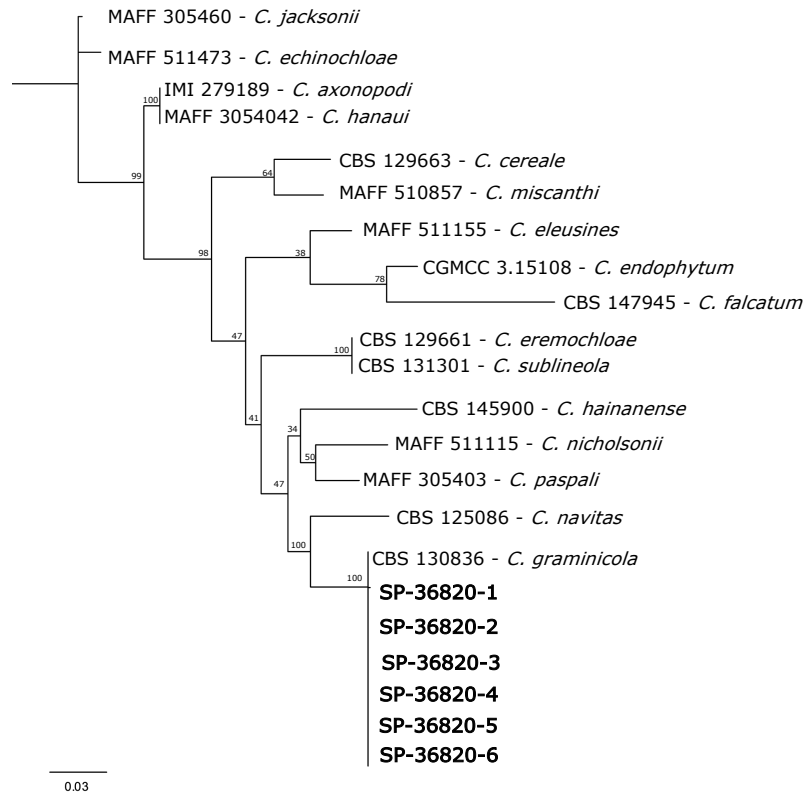
96 OQ708380, OQ708381, OQ708382, OQ708383; *SOD2* - OQ716797, OQ716798, OQ716799,  
97 OQ716800, OQ716801, OQ716802.

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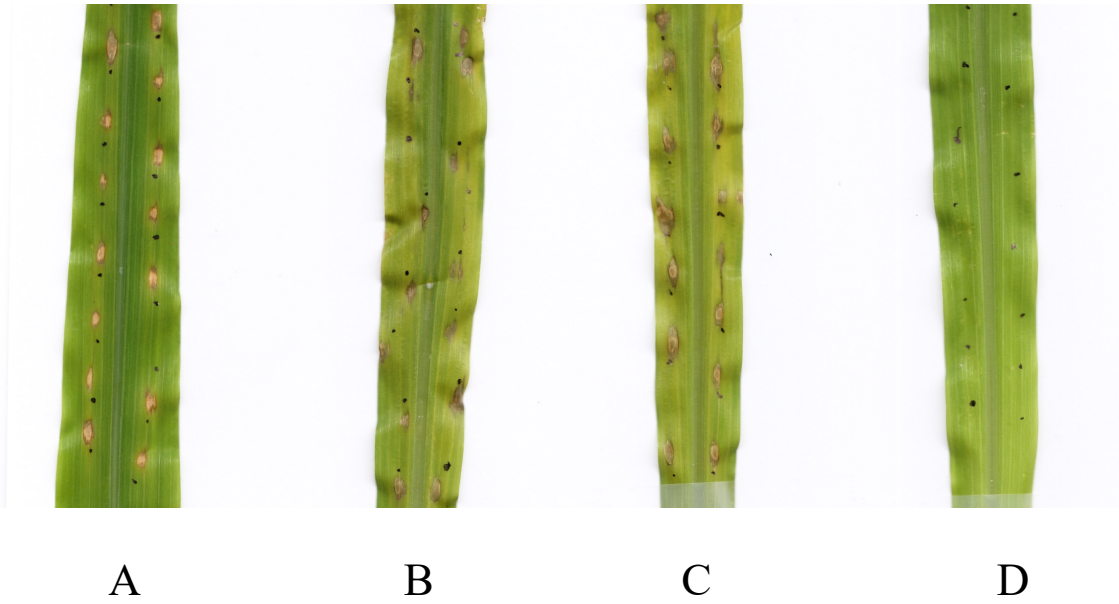
99 **e-Xtra 2:** Leaves of inoculated maize plants.

100 Maize leaves 4 days post-inoculation with conidial suspension from *Colletotrichum*  
101 *graminicola* isolates: M1.001 (A), SP-36820-1 (B), and SP-36820-3 (C), and mock-inoculated  
102 control (C). The black dots indicate the inoculation points. The lesions display the typical  
103 symptoms of anthracnose leaf blight.

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**Figure S1.** Maximum Likelihood phylogenetic tree reconstructed from a multilocus concatenated alignment of ITS and SOD2 genes of *Colletotrichum* strains used in this study and strains belonging to the *Colletotrichum graminicola* species complex. The phylogenetic tree confirmed the identity of strains isolated from maize as *C. graminicola*. GenBank accession numbers of *C. graminicola* sequences generated in this study: ITS - OQ708378, OQ708379, OQ708380, OQ708381, OQ708382, OQ708383; SOD2 - OQ716797, OQ716798, OQ716799, OQ716800, OQ716801, OQ716802.



**Figure S2.** Maize leaves 4 days post-inoculation with conidial suspension from *Colletotrichum graminicola* isolates: (A) M1.001 - positive control, (B) SP-36820-1 and (C) SP-36820-3, and (D) mock-inoculated control. The black dots indicate the inoculation points. The lesions display the typical symptoms of anthracnose leaf blight.