# *A strawberry disease caused by* **Acremonium strictum**

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# A strawberry disease caused by *Acremonium strictum*

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**Abstract** We report *Acremonium strictum* as the causal agent of a new disease in strawberry plants (*Fragaria x ananassa* Duch.) in the Northwest of Argentina. Both the structure of conidiophores and the sequence spanning the internal transcribed spacers 1 and 2 (ITS1 and ITS2) of the nuclear ribosomal DNA (rDNA) allowed confirming the affiliation of the isolate, corresponding to *A. strictum*. An analysis of symptoms and lesions caused by the strain of *A. strictum* in susceptible cultivars showed that the typical symptoms are as follows: in an early stage, small necrotic light-brown spots in leaves and petioles increase in number and size as the disease progresses; in a more advanced stage, dark necrotic areas expand over petioles and leaves causing strangulation of petioles and the plant wilt. Crown rot was not observed even at a very advanced stage of the disease.

**Keywords** Anthracnose · *Fragaria x ananassa* · *Acremonium* ssp · *Colletotrichum acutatum* · *Colletotrichum gloeosporioides* · *Colletotrichum fragariae*

Racedo and Salazar contributed equally to this work.

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Strawberry crops are susceptible to a wide range of diseases caused by viruses, bacteria, fungi and nematodes that are fairly well characterized (Maas 1998). Grey mould caused by *Botrytis cinerea* and anthracnose caused by fungi of the genera *Colletotrichum* are among those that affect strawberry production most adversely (Freeman and Katan 1997). Three species of *Colletotrichum* are known to be the causal agents of anthracnose in strawberry, i.e. *C. acutatum*, *C. fragariae* and *C. gloeosporioides* (Denoyes-Rothan et al. 2003). In Argentina the three species of *Colletotrichum* were detected in the strawberry crop area (Mena et al. 1974; Mónaco et al. 2000; Ramallo et al. 1997, 2000), with *C. acutatum* being the predominant species.

Fungi of the genus *Acremonium* have been found as plant endophytes (Morgan-Jones and Gams 1982), pathogens (García-Jiménez et al. 1994), and mycoparasites of plant pathogenic fungi (Choi et al. 2008). *A. strictum* has been reported as the causal agent of the sorghum wilt disease in Argentina (Forbes and Crespo 1982) and in the United States (Natural et al. 1982); the disease had been detected earlier in sorghum (El-Shafey et al. 1979) and in maize kernels (King 1981), but at that time it was incorrectly attributed to *Cephalosporium acremonium*. Onions and Brady (1987) and Rigotti et al. (2003) reported an association between *Acremonium* and strawberry. Whereas the former reported the isolation of *Acremonium luzulae* (Fuckel) from strawberry fruit, the latter reported the isolation of an undetermined species of *Acremonium* from symptomless frigo plantlets of many strawberry cultivars; however, the genus *Acremonium* had never been associated with a strawberry disease.

One thousand and three hundred fungal isolates were retrieved from 1,000 strawberry plants exhibiting

symptoms of anthracnose (e.g. fruit rot and/or lesions on petioles and runners) cultivated in different areas of Tucumán (Argentina). Isolations were carried out on PDA medium (potato-dextrose-agar) supplemented with streptomycin (300 μg/ml) at 28 °C. Isolates were single-spore propagated and were maintained on PDA slants at 4 °C. Sixteen strains were selected according to the following criteria: i) observable macro- or microscopic differences, and ii) collection site, considering two isolates as different if they were collected in different places. Cultural and microbiological characteristics of each strain were evaluated in triplicate plates. Plates were obtained by transferring a 4-mm diameter mycelia plug from a 3-month-old PDA culture to fresh PDA in a Petri dish under sterile conditions. Growth was estimated by colony diameter after 10 days at 28 °C under continuous fluorescent light (Smith and Black 1990). Conidia and other microscopic features (e.g. perithecia, appresoria and setae) were observed with light microscope (model BXS1, Olympus, Hamburg, Germany) and with a SUPRA 55VP (Zeiss, Hamburg, Germany) scanning electron microscope. By analyzing growth rate, size, type, colour and shape of colonies, conidia shape and size, shape of conidiophores and occurrence of perithecia, 14 strains (M11, F9, MP3, M23, LFG-05, LFC1-05, LFC3-05, EPO-05, EFS-05, TFS2-05, TPS1-05, TPS2-05, TPS3-05 and IPCh-05) exhibited *Colletotrichum*-like conidiophores, but not ascigerous state (e.g. perithecia); a single strain (L9) exhibited *Colletotrichum*-like conidiophores and an ascigerous state; and the strain SS71 did not exhibit ascigerous state but *Acremonium*-like conidiophores (Fig. 1). The occurrence and morphology of both appresoria and setae were not taken into account in this study due to the character variability observed. Only strains L9 and SS71 presented clearly distinctive types of fungal structures. Whereas the former was the only strain that produced perithecia in six- week- old cultures with asci and ascospores typical of *G. cingulata*, the teleomorph of *C. gloeosporioides* (Gunnell and Gubler 1992), the latter was the only one that produced conidiophores typically observed in *Acremonium* sp. (Bandyopahyay et al. 1987; Rivera-Varas et al. 2007) (Fig. 1). The strain M11 was sent to the Commonwealth Mycological Institute (CABI) for determination of species. Results confirmed that the strain M11 corresponded to a *C. acutatum* and was considered our local reference strain. No noticeable difference was detected among conidiophores of *Colletotrichum* isolates including the strain L9.

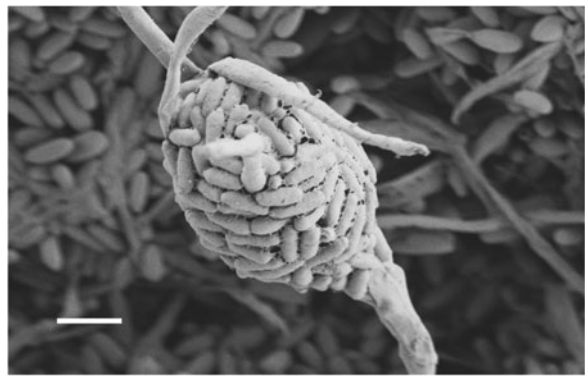

**Fig. 1** Microphotograph of conidiophores observed in the strain SS71 of *Acremonium strictum*. Bar corresponds to 10 μm

Molecular identification of strains was performed by amplification of the ITS1 and ITS2 regions between the small and large nuclear rDNA, including the 5.8S rDNA, according to White et al. (1990). DNA extraction was carried out following the procedures described by Babot et al. (1997). Both Universal PCR primers, ITS1: TCCGTAGGTGAACCTGCGG and ITS4: TCCTCC GCTTATTGATATGC were used. PCR products were removed from the gel (1.5 % agarose), purified using the QIAquickR Gel Extraction Kit (QIAGEN) and sequenced in the Molecular Biology Lab at INTA-Castelar (Argentina). Sequences were analyzed by using the DNAMAN software (ver.7; Lynnon Co 2009) and primer sequences were trimmed. A BLASTN (Altschul et al. 1990) search of the NCBI GenBank database was performed by using individual sequences. Sequence analysis showed that strain SS71 corresponded to *Acremonium strictum* and this sequence was deposited in GenBank under accession number JN098488.

With the aim of confirming the affiliation of strain SS71, a phylogenetic analysis was performed using ITS1-5,8S rDNA-ITS2 sequences of nine strains isolated in this work (Fig. 2), and including the following reference strains: *C. acutatum* type strain STE-U 5292 (GenBank AY376510.1); *C. gloeosporioides* type strain IMI 356878 (GenBank EU371022.1); *A. strictum* type strain CBS 346.70 T (GenBank AY214439.1); and *A. strictum* strains isolated from plants H4471 (GenBank GU595023.1), GCC-603-4 (GenBank DQ279801.1) and UFMGCB 1375 (GenBank FJ605254.1). Alignment was performed with DNAMAN software (ver.7; Lynnon Co 2009). Phylogenetic trees were built by using both the distance matrix method and maximum parsimony algorithm; the construction of the former was based upon the neighbour-joining method with a bootstrap analysis of

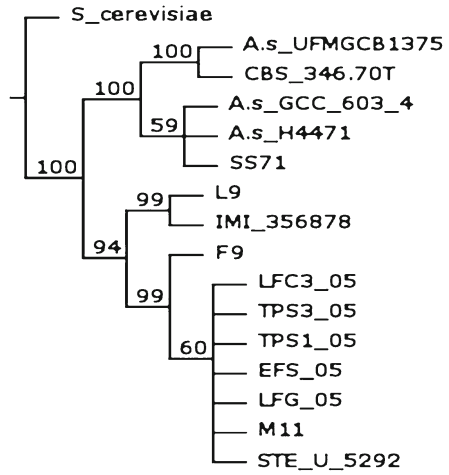


**Fig. 2** Phylogenetic tree built by maximum parsimony algorithm of the ITS1-5,8S rDNA-ITS2 DNA fragment sequences of the strain SS71 of *A. strictum* compared with published sequences of other fungal pathogens isolated and selected closely related species. A.s_UFMGCB1375, CBS 346.70 T (type strain), A.s_GCC_603_4 and A.s_H4471 correspond to sequences of *A. strictum* strains; IMI 356878, corresponds to sequences of *C. gloeosporioides* type strain; STE-U 5292 corresponds to sequences of *C. acutatum* type strain. L9, M11, F9, LFC3-05, TPS3-05, TPS1-05, EFS-05, LFG-05 correspond to sequences of the *Colletotrichum* strains, and SS71 to the *Acremonium* strain isolated in this study. ITS1-5,8S rDNA-ITS2 DNA fragment sequence of the yeast *S. cerevisiae* was used as the out-group species

1,000 random replications with DNAMAN, and the parsimony analyses were performed by using TNT program (Goloboff et al. 2003) with gaps treated as missing. Branch support was assessed by a bootstrap analysis over 1,000 pseudo replicates. Phylogenetic analyses using distance-matrix and maximum-parsimony methods resulted in trees showing similar topology. Figure 2 shows the structure of the consensus tree obtained with the maximum-parsimony analysis, which identified the eight most parsimonious trees (length=477). Strain SS71 clustered with *A. strictum* strains, showing maximum-parsimony with GCC-603-4 and H4471 and grouped in a separate branch of *Colletotrichum* strains suggesting that SS71 belongs to a different genus, a fact which is well supported by a high bootstrap value (100 %). *S. cerevisiae*, used as an out-group species, was separated from the rest of the species, as expected.

Since only one strain was characterized and identified as *A. strictum* by applying the two selection criteria previously described, the occurrence of this pathogen over the 1,300 total isolates was checked. Thirteen strains exhibited macro- and microscopic features similar to the strain SS71 and also identical ITS1-5,8S rDNA-ITS2 sequences, representing 1 % of the total isolates analyzed. It was observed that the 13 *A. strictum* strains came from the same 2-ha field planted exclusively with the cultivar Chandler (10,000 plants/ha). Assessment of the affected area indicated that 8 % of the plants had the disease symptoms. In this field, 85 samples (fruits, leaves and petioles) showing anthracnose-like symptoms were picked at random along five transects that completely crossed the field. The incidence of *A. strictum* was 15.3 % on the 85 samples with symptoms, which was equivalent to 1.2 % of all the plants in the field. In this study *A. strictum* was found only in leaves and petioles lesions, was the only pathogen isolated from lesions, and was not found in fruit lesions.

The pathogenicity of the strains SS71, L9 and M11 was studied on the strawberry cultivars Chandler, Enzed Donna, Pájaro, Oso Grande, Rosalinda, Gaviota, Camarosa, Selva, Sweet Charlie, Milsei, Seascape and Aroma. Plants were obtained in vitro from the strawberry Active Germplasm Bank of the Universidad Nacional de Tucumán. Plants were grown in cabinets at 28 °C, 70 % RH with a light cycle of 16 h/day, watered every other day with 50 ml of sterile distilled water and kept for 15 weeks to confirm that they were free of anthracnose. There were eight plants for each combination of strawberry cultivar and test strain. Four plants were inoculated with a conidial suspension of each strain ($1.5\times10^6$ conidia per ml) by spraying the leaves to run-off, whereas the other four plants received a spray of sterile distilled water. After inoculation, plants were placed in a dew chamber with 100 % RH at 28°C (infection chamber) for 48 h in the dark. Plants were then moved to a growth cabinet (28 °C, 70 % RH with a light cycle of 16 h/day) to incubate for 50 days. The disease severity (DS) was evaluated every 5 days after inoculation up to 50 days and scored on a scale ranging from 1 (healthy plant) to 5 (dead plant), using a disease index proposed by Delp and Milholland (1980). At the end of the experiments Koch's postulates were confirmed. All experiments were repeated three times. Results of the evaluation of disease severity on different strawberry cultivars are shown in Table 1. DS data of repeated experiments were statistically analyzed by the analysis of variance using a Mixed Proportional Odds Model (GLM), and the software InfoStat ver. 2012 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). Differences between means were evaluated by LSD Fisher test ($\alpha \leq 0.05$). To model the longitudinal data, the structure of the residual

**Table 1** Disease severity (DS) of strawberry cultivars infected with the strains SS71 of *A. strictum*, M11 of *C. acutatum* and L9 of *C. gloeosporioides*

| Strain | SS71 | M11 | L9 |
|---|---|---|---|
| Chandler | 5.0 (c) | 4.8 (c) | 2.2 (c) |
| Enzed Donna | 4.7 (c) | 5.0 (c) | 3.0 (b) |
| Pájaro | 1.0 (a) | 5.0 (c) | 5.0 (c) |
| Oso Grande | 1.8 (a) | 4.4 (bc) | 3.0 (b) |
| Rosalinda | 3.3 (b) | 2.6 (ab) | 2.3 (ab) |
| Gaviota | 1.2 (a) | 2.1 (ab) | 1.3 (a) |
| Camarosa | 1.2 (a) | 1.8 (a) | 1.8 (a) |
| Selva | 1.4 (a) | 1.4 (a) | 4.7 (c) |
| Sweet Charlie | 1.8 (a) | 1.4 (a) | 4.7 (c) |
| Milsei | 4.2 (bc) | 4.8 (c) | 4.7 (c) |
| Seascape | 2.2 (ab) | 4.4 (bc) | 4.7 (c) |
| Aroma | 3.8 (b) | 1.5 (a) | 3.5 (b) |

DS were evaluated 50 day after inoculation and scored on a scale based on petiole symptoms (Delp and Milholland 1980). In this 1–5 rating scale, 1 denotes healthy petiole and 5, entirely necrotic petiole and/or dead plant. DS are average values of three independent experiments. Experiments were carried out with four plants per treatment and were repeated three times. Different letters indicate significant differences (LSD Fisher test, $P \leq 0.05$, $n=324$)

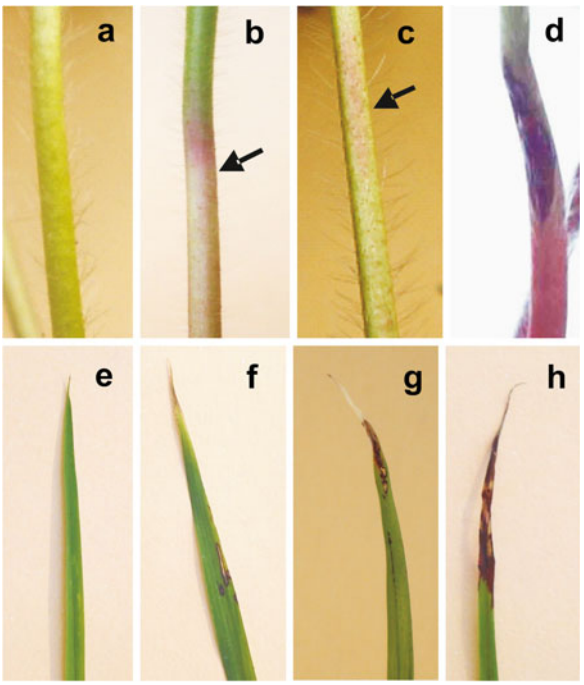


**Fig. 3** Early symptoms of strawberry (**a**–**d**) and sorghum (**e**–**h**) disease caused by the strain SS71 of *A. strictum*. Strawberry petiole of a plant treated with water (**a**); and with SS71, 15 days after inoculation (**b** and **c**), and 30 days after inoculation (**d**). Sorghum leaf of a plant treated with water (**e**); and with SS71, 6 days after inoculation (**f**), 10 days after inoculation (**f**), and 15 days after inoculation. *Arrows* indicate red pigmentation observed in outer (**b**) and inner (**c**) tissues of affected strawberry petioles

covariance matrix and the heteroscedasticity in time were considered. Penalized likelihood (AIC and BIC) was analyzed to choose the best criterion that described the data.

The analysis of the symptoms caused by the strain SS71 of *A. strictum* in susceptible cultivars (e.g. Chandler) showed that these lesions and those caused by the *Colletotrichum* isolates were alike when plants were observed at an advanced stage of the disease (50 days post infection); except that crown and fruit rot were never observed. At that stage, microscopic observation of the necrotic lesions produced by the strain SS71 revealed the appearance of typical *Acremonium* conidiophores as shown in Fig. 1. The first symptoms of the disease were observed in the petioles as a reddish area that grew and became brown as the disease progressed, but they rarely appeared in expanded leaves. In Fig. 3 a typical lesion can be observed in a petiole (Fig. 3b) and in a petiole section (Fig. 3c) in an early stage of the disease. At an intermediate stage of the disease, brown necrotic spots of about 3–5 mm diameter on leaf laminas appeared, and in an advanced stage of the disease, leaves exhibited a severe petiole lesion, necrosis and petiole strangulation (Fig. 3d) resulting in plant wilt.

As *A. strictum* has previously been found as the causal agent of the sorghum wilt disease (Forbes and Crespo 1982; Natural et al. 1982) and infecting maize kernels (King 1981), SS71 pathogenicity was also tested on three maize varieties (Leales 25, Opaco INTA and Perlado INTA) and four sorghum varieties (Kuntur, Nehuen, HQCO and Mishki INTA). Plants were spray-inoculated with a conidial suspension of strain SS71 ($1.5\times10^6$ conidia per ml) as mentioned above. Disease symptoms were evaluated daily for 30 days. At the end of the experiments, Koch's postulates and molecular analysis (PCR amplification of ITS region followed by sequencing of the amplified fragment and comparison with the sequence previously obtained) confirmed the identity of the pathogen. Only varieties Kuntur and HQCO were susceptible to SS71. Symptoms began to appear in inoculated plants after 6 days as yellowish-brown to brown small circular or irregular spots of less than 3 mm diameter, occurring on the minor and lateral veins accompanied with an incipient chlorosis at the tip of the leaves

(Fig. 3f). Ten days after inoculation, plant leaves exhibited brownish specks along the lamina veins with visible necrosis at the tip of the lamina (Fig. 3g). Fifteen days after inoculation, plant leaves had a clear necrosis of nearly half of the lamina surface that caused the plant to wilt (Fig. 3h). Experiments carried out with maize showed similar symptoms to those observed in sorghum but only in cultivar Leales 25; other cultivars remained symptomless including control plants (results not shown).

This study revealed *A. strictum* as a new causal agent of a strawberry disease that can be differentiated from anthracnose at an early stage, and does not produce crown rot. Results of inoculation of healthy strawberry plants with the strain SS71 confirmed the pathogenic feature of this strain of *A. strictum,* a fact which is particularly important because there is no information in literature about the capacity of *A. strictum* to affect strawberries. As the strain SS71 of *A. strictum* proved to be also pathogenic to some sorghum and maize varieties, and local farmers reported that the field had previously been cultivated with these crops, it may be speculated that *A. strictum* has moved from sorghum or maize to strawberry. Although the occurrence of the *Colletotrichum* species in the crop area studied is much more frequent, the disease caused by *A. strictum* is only at the beginning and steps should be taken to prevent or reduce the spread of this new disease. Also, even when the cultivar Chandler of strawberry has been replaced by other more productive varieties, the risk of an outbreak caused by *A. strictum* cannot be completely eradicated because other cultivars of strawberry are also susceptible to this pathogen to a varying degree.

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