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New or Unusual Disease Reports

First report of *Rhodococcus* spp. isolates causing stunting and lateral stem proliferation of *Iresine herbstii* 'Aureo-Reticulata' in Tunisia

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Phytopathogenic isolates of *Rhodococcus fascians,* a Gram-positive bacterium, cause disease on numerous plants by inducing fasciation and leafy galls (Goethals *et al*., 2001). The altered plant morphology resulting from these infections cause serious economic losses to ornamental industries (Putnam and Miller 2007). In April 2017, over 80% of micropropagated *Iresine herbstii* 'Aureo-Reticulata' plants in a greenhouse in northern Tunisia exhibited growth abnormalities similar to those caused by *Rhodococcus* spp. (Stamler *et al*., 2015). These symptoms included stunting and shoot proliferation (Figure 1). To isolate epiphytic populations of *Rhodococcus* spp., affected tissues were placed onto D2 agar (Kado and Heskett, 1970) amended with cycloheximide (2%). To detect endophytic bacterial populations, plant tissues were surfacetreated with 0.5% sodium hypochlorite for 30 s, then for 1 min with 70% ethanol, followed by rinsing three times in sterile distilled water. The tissues were then ground in phosphate buffered saline (pH 7.4) prior to dilution plating. Isolation plates were incubated in the dark at 27°C for 4 d, for detection of epiphytic populations, or 15 d for detection of endophytic populations. Bacterial colonies with colours ranging from cadmium yellow and deep chrome to deep orange were selected for purification and identification. Genomic DNA was extracted from resulting isolates, and diagnostic PCR was used for isolate identification, through 16S rDNA amplification and sequencing (Stamler *et al*., 2015). Representative 16S rRNA sequences from six isolates were deposited in GenBank (MH685556 to MH685561).

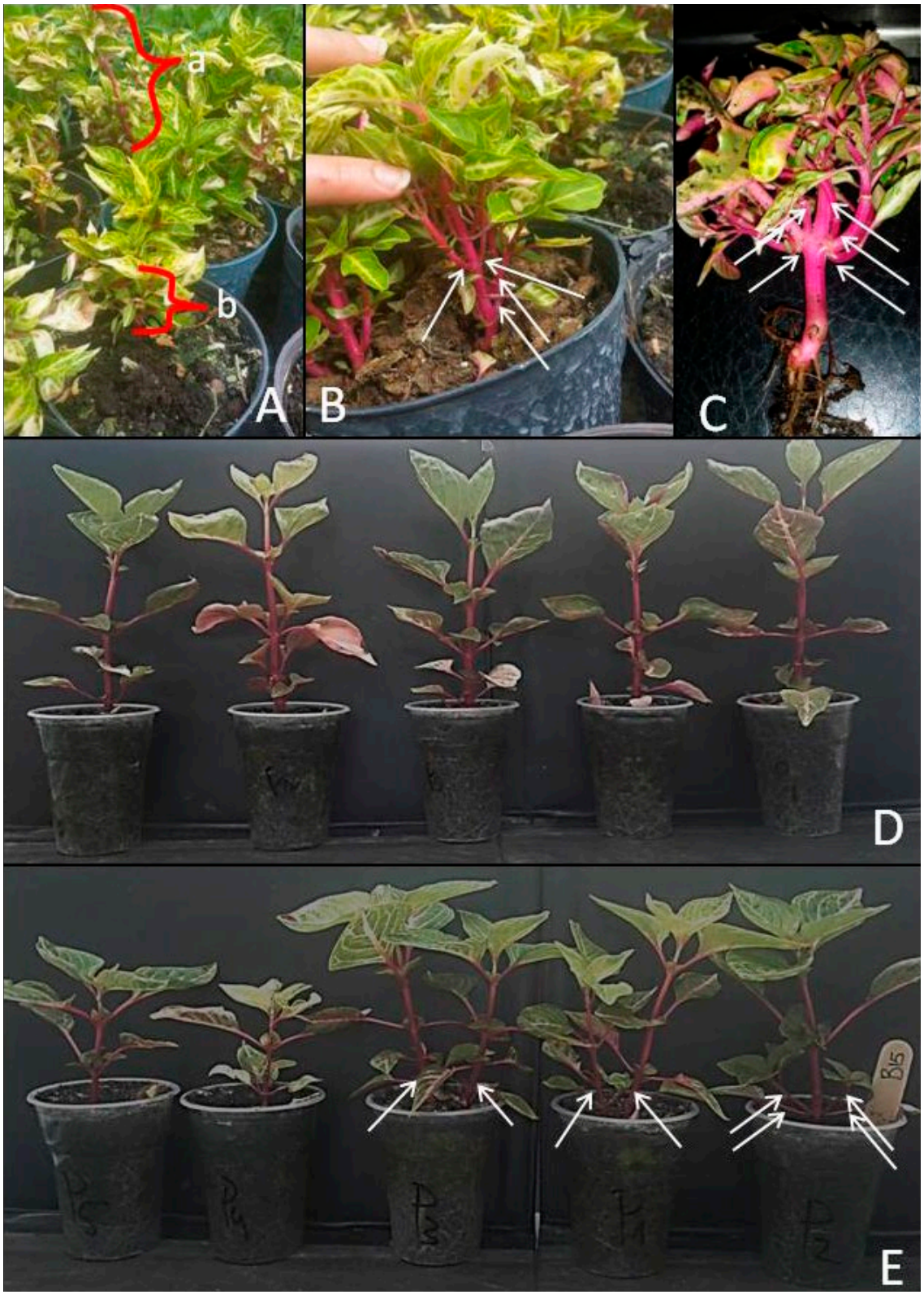

Figure 1. Symptomatic nursery-grown *Iresine herbstii* 'Aureo-Reticulata' plants were stunted (b), as compared to asymptomatic plants (a) (A), and exhibited stem proliferation (arrows) (B and C). Uninoculated control plants (D) developed single stems; plants inoculated with isolate 6 (E) developed multiple stems (arrows).

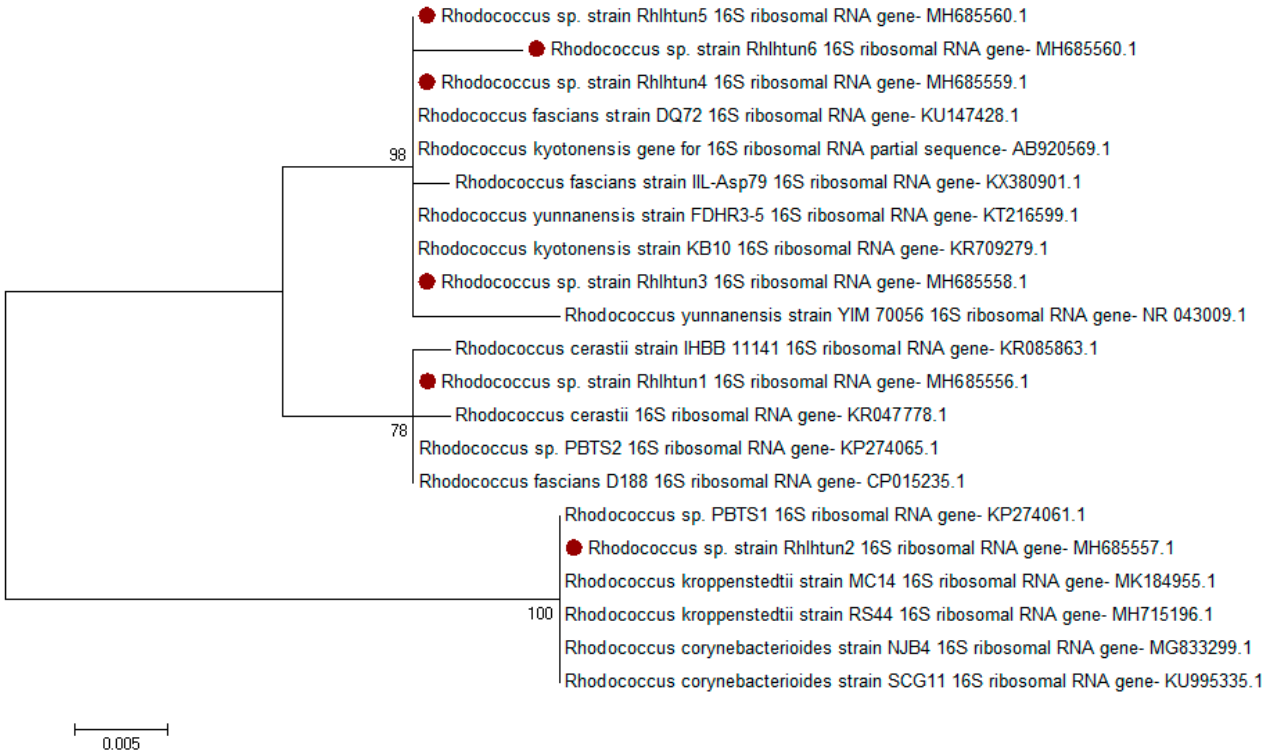

Figure 2. Phylogenetic tree based on the 16S rDNA genes of *Rhodococcus* spp. (labelled in red points), that were isolated from *Iresine herbstii* plants in northern Tunisia. The neighbour joining tree based on 16S rRNA was constructed using the Mega software version 6.06, bootstrapped 1,000× with a support threshold of 70%, corresponding to reference sequences with GenBank accession numbers.

Table 1. Symptom development on *Iresine herbstii* 'Aureo-Reticulata' in response to inoculation with six isolates of *Rhodococcus* spp.

Treatment[S]	Mean plant height (cm)[T]	Incidence of plants with lateral branches (%)[U]	Mean No. of stems per plant[V]	Mean No. of nodes per cm[W]	Mean shoot biomass (g)[X]	Mean root biomass (g)[X]	Mean total plant biomass (g)[Y]
Uninoculated	17.4 a[Z]	0	1.0 b[Z]	0.48 a[Z]	0.5 a[Z]	0.7 a[Z]	1.2 a[Z]
Isolate 1	14.1 ab	0	1.0 b	0.97 b	0.6 a	0.5 a	1.1 a
Isolate 2	9.5 b	0	1.0 b	0.70 b	0.4 a	0.6 a	1.0 a
Isolate 3	15.2 ab	0	1.0 b	0.72 b	0.7 a	0.6 a	1.3 a
Isolate 4	10.2 b	40	1.8 a	1.64 b	0.4 a	0.5 a	0.9 a
Isolate 5	10.1 b	0	1.0 b	1.17 a	0.4 a	0.3 b	0.4 a
Isolate 6	12.5 ab	60	2.6 a	0.97 ab	0.8 a	0.4 b	1.2 a

[S]Micropropagated *I. herbstii* were inoculated by dipping plantlets into bacterial suspensions of each of 6 *Rhodococcus* spp. isolates originating from symptomatic plants; uninoculated control plants were dipped in sterile buffer.
[T]Plant height was determined from the soil surface to the apical meristem 100 d post-inoculation.
[U]The percent of plants (n = 5) producing lateral stems was calculated.
[V]The mean number of stems per plant was determined by adding the main stem and lateral stems for each plant. One stem per plant indicates no lateral stem growth.
[W]Node density was calculated as the number of nodes per unit length of stem. Plants with high node density exhibited compact structure.
[X]Roots were cut from shoots and tissues were dried at 55°C for 48h prior to weighing.
[Y]Total plant biomass was calculated by summation of the root and shoot biomass.
[Z]Means associated with different letters indicate statistically significant differences ($P \leq 0.05$).

The isolates were closely related (>99% similarity) to *Rhodococcus* spp. accessions in GenBank. Isolate 1 was 99% identical to *R. fascians* D188 and *R. cerastii*, isolate 2 was 100% identical to *R. corynebacterioides* and *R. kroppenstedtii*. Isolates 3, 4, 5 and 6 were 99% identical and clustered with the group of *R. fascians*, *R. kyotonensis* and *R. yunnanensis* isolates (Figure 2).

Bacterial suspensions (0.7 OD600) of each isolate were prepared in buffer (10mM $MgCl_2$/MES), and micropropagated *I. herbstii* plants (n = 5) were inoculated by briefly dipping whole plants into the bacterial suspension of each isolate. Uninoculated control plants (n = 5) were dipped in sterile buffer. The plants were then transplanted into potting mix and incubated in a greenhouse maintained at 26°C with a 16 h photoperiod and 70% relative humidity. After 100 d, plant height and degree of lateral branching, and pathogen recovery, were assessed.

The degree of stunting and lateral shoot development varied between isolates. Plants inoculated with isolates 2, 4, and 5 were 41-46% shorter ($P \leq 0.05$) than uninoculated plants. Plants inoculated with isolates 4 and 6 produced more lateral stems ($P \leq 0.05$) than uninoculated plants (Table 1), and plants inoculated with isolate 6 exhibited more stems per plant than for all the other isolates (Table 1; Figure 1D). Epi- and endophytic populations of each isolate were recovered from inoculated plants but not from uninoculated plants, and identities of recovered isolates were confirmed through amplification of the specific virulence *vicA* gene. This completed Koch's postulates for the isolates.

To our knowledge, this is the first report of *Rhodococcus* spp. causing disease on *I. herbstii*, and the first report of pathogenic *Rhodococcus* spp. in Tunisia. These isolates are closely related (>98% similarity) to *Rhodococcus* isolates causing the pistachio bushy top syndrome, a recently-identified disease also associated with micropropagated plants (Stamler *et al.*, 2015). Further studies are necessary to determine genetic factors underlying specific host-isolate interactions.

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