



# Susceptibility of sugarcane to red rot caused by two *Fusarium* species and its impact on stalk sugar level

Fe Dela Cueva<sup>1</sup> · Rachele De Torres<sup>1</sup> · Alyssa de Castro<sup>1</sup> · JayVee Mendoza<sup>1</sup> · Mark Angelo Balendres<sup>1</sup> 

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## Abstract

Red rot of sugarcane is caused by *Colletotrichum falcatum*. A recent survey, however, indicates that other fungal species may be associated with red rot-infected sugarcane leaves. This study 1) investigates the cause of the red rot-like symptoms using a polyphasic approach, 2) determines the pathogenicity of the fungi in sugarcane leaves and infection potential in leaves and stalks in detached-plant assays, and 3) assesses their effect on the stalk sugar level. Based on combined morpho-cultural and molecular characterization, the associated fungi were identified as *Fusarium sacchari* and *F. proliferatum*. The two *Fusarium* species induced red rot in leaves and stalk, as did the *C. falcatum* control isolate. Stalks infected with the two *Fusarium* species had reduced sugar level after 14 days, compared to the healthy stalk checks. To our knowledge, this is the first record of *Fusarium* species associated with red rot in sugarcane. These results suggest that the causal agent of red rot in the field is likely to be considered when developing or implementing disease control measures. Further, sugarcane breeding programs for red rot resistance would be likely aimed at all known causal pathogens.

**Keywords** *Fusarium sacchari* · *Fusarium proliferatum* · *Colletotrichum falcatum* · Sugar brix · Disease etiology

## Introduction

The increasing population and the growing food processing and beverage manufacturing sector demand an ongoing supply and increasing amount of sugar production in the Philippines. Despite the presence of sugar substitutes from extracts of coconut, corn, and beets, sugarcane remains the major cash crop and the main source of refined sugar (PEF 2018). In the Philippines, for the crop year 2014–2015 alone, a total of 416,893 ha of land has been devoted to sugarcane cultivation, producing 2,323,817 metric tons of raw sugar (Delfin et al. 2017). However, a number of insect pests and diseases present an ongoing threat to sugarcane production.

Red rot is one of the popular diseases of sugarcane and in some countries, e.g. in India, it is a major yield-limiting disease (Viswanathan and Rao 2011). Red rot symptoms are longitudinal reddening in stalks that later rots and continuous

or restricted reddish lesions in leaf midribs (Abbott 1938). Historically, red rot is responsible for wiping out elite some sugarcane cultivars and a significant contributor to the reduction in cane yield (Viswanathan and Rao 2011; Sharma and Tamta 2015). Red rot also negatively affects sugar quality (Went 1893; Butler 1906; Lewton-Brain 1908; Edgerton 1910; Viswanathan and Samiyappan 2002). In red rot infected cane stalks, the sugar sucrose is split into fructose and glucose, limiting the availability of sucrose and thus affecting sugar production. This process is commonly known as sugar inversion (Sehtiya et al. 1993). Red rot can reduce sugar by up to 75% in the field (Viswanathan and Samiyappan 1999; Hussnain and Afghan 2006; Viswanathan and Rao 2011). During milling, red rot infected cane mixed with other canes can also result in spoilage (Viswanathan and Rao 2011).

Red rot is caused by the fungus *Colletotrichum falcatum* (Went 1893). Although species belonging to the genus *Colletotrichum* are cosmopolitan pathogens (Cannon et al. 2012), the red rot pathogen is exclusively hosted by plants in the genus *Saccharum* spp. *Colletotrichum falcatum* was previously grouped with *C. sublineola* (Sutton 1968), the sorghum pathogen, but phylogenetic analyses revealed that the sugarcane pathogen is indeed distinct from the sorghum pathogen (Crouch et al. 2009). There are races of *C. falcatum* that

✉ Mark Angelo Balendres  
mobalendres@up.edu.ph

<sup>1</sup> Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, College, 4031 Laguna, Philippines

are associated with cultural characteristics of the fungus. The light-colored isolates were more aggressive compared to the dark-colored isolates (Abbott 1938; Singh 2008). Low disease incidence and severity were recorded in sugarcane fields where dark-colored fungi were isolated (Chona and Srivastava 1960). No other fungal species have yet been associated with red rot in sugarcane.

Most recently, a preliminary report from a field survey has indicated that other fungal species were isolated from red rot infected sugarcane leaves (de Castro et al. 2018). An understanding of the etiology of a disease is important in disease monitoring and plant breeding programs, both of which facilitate the development of disease management measures. This study was aimed at identifying the fungal species, that are not *C. falcatum*, associated with red rot of sugarcane plants using a combination of morphological, cultural, pathological and molecular characterization techniques. Further, the effect of these pathogens on the sugar level (brix concentration) of cane juice was evaluated.

## Materials and methods

### Fungal isolates

Four fungal isolates (PHLB17, PHVI25, PHVI26, and PHVNO21) obtained from a field survey (De Castro et al. 2018) were used in this study. These fungi were isolated separately from sugarcane leaf midribs with conspicuous red rot symptoms during field visits at various sugar milling districts in the Philippines. Isolate PHLB17 originated from Nasugbu, Batangas, Philippines and was collected on April 2017. Isolate PHVNO21 was collected from Brgy. Cambagog, Hinigaran, Negros Occidental, Philippines on May 2017. Isolates PHVI25 and PHVI26 were both collected from Brgy. Talongonan, Passi City, Iloilo on May 2017. All fungal isolates were collected from an unknown sugarcane cultivar. Red rot-infected tissues were taken from randomly selected plants along the imaginary zigzag line within each sampling site. The advancing margin of the red rot lesion was cut into 1 mm × 1 mm using scalpel and then washed with 10% (v/v) sodium hypochlorite solution (Zonrox, Green Cross Inc., Philippines) for 1 min, followed by three series of distilled water washes at 1 min each. The fungi were initially isolated and grown in prepared potato dextrose agar (pPDA) medium comprising 250 g potato, 20 g PTC agar (Pronadisa, Germany) and 18 g dextrose in 1 L of distilled water. The cut-portions were placed in pPDA medium and incubated at 27 °C in a 10/14 h day/night condition. After five days, a 3 mm-mycelial disc was taken from the advancing portion of the fungi and the disc was transferred to a new pPDA medium. Pure isolates were obtained using the single spore isolation technique (Niemeyer and de Andrade 2016). All

fungi used in this study were maintained in both PDA and water agar and were deposited at the Fungal Repository of the Plant Pathology Laboratory, Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños, Philippines.

### Cultural and morphological characterization

Mycelial growth of fungi was assessed in pPDA and synthetic PDA medium (sPDA, Himedia Laboratories Inc., India) at 25 °C with 14 h light exposure in 24 h light/dark cycle. Colony characteristics and radial growth (mm) were assessed 7 days post incubation (dpi). Spore characterization was performed using an agar block set-up. A square block of PDA was placed on a glass slide and sides were inoculated with spores and mycelial fragments of the pathogen. Coverslip was then positioned on the top surface of the block and the set up was moistened with sterile distilled water (in the petri dish). After 3–4 dpi, the coverslip was mounted onto a new glass slide with a drop of cotton blue. Photomicrograph was taken, and spore characteristics (spore shape, pigmentation, ornamentation, and the presence or absence of septa; presence or absence of chlamydospores; if present, the shape, pigmentation, size, single formation or in a chain) of the fungi were microscopically assessed (Olympus CX22, Japan).

### Molecular characterization

The DNA sequences of the fungal isolates internal transcribed spacer (ITS) and translation elongation factor-1  $\alpha$  (TEF) regions were determined by sequencing analyses. Fungal genomic DNA was extracted from a 7-day old culture using a modified CTAB extraction (Doyle and Doyle 1987). The fungal genomic DNA was used as the template for the amplification of the two target gene regions thru polymerase chain reaction (PCR) analysis using primer pairs ITS4/ITS5 (White et al. 1990) and EF1/EF2 (Geiser et al. 2004). For ITS amplification, the PCR was performed in a 15  $\mu$ l total reaction volume containing 1X PCR buffer (Invitrogen, USA), 2 mM EDTA (Invitrogen), 0.2 mM dNTPs (Invitrogen), 0.2  $\mu$ M each primer (Integrated DNA Technologies), 1 U of *Taq* polymerase (Invitrogen), 10 ng of fungal DNA and DEPC-treated water (Invitrogen). The PCR amplification was carried out in C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories). with the following conditions; 5 min of initial denaturation at 94 °C, followed by 25 cycles of denaturation (1 min at 94 °C), annealing (1 min at 66 °C) and extension (1 min at 72 °C) steps, then a final extension step at 72 °C for 1 min. For TEF amplification, the PCR was performed in a 15  $\mu$ l total reaction volume containing 1X PCR buffer (Invitrogen), 2 mM EDTA (Invitrogen), 0.25 mM dNTPs (Invitrogen), 0.3  $\mu$ M each primer (Integrated DNA Technologies), 0.04 U/ $\mu$ l of

*Taq* polymerase (Invitrogen), 10 ng of fungal DNA and DEPC-treated water (Invitrogen). The PCR amplification was carried out in C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories) with the following conditions; 2 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation (1 min at 94 °C), annealing (45 s at 60 °C) and extension (90 s at 72 °C) steps, then a final extension step at 72 °C for 5 min.

PCR products were resolved by gel electrophoresis (PowerPac™ and Sub-Cell GT, (Bio-Rad Laboratories) in 1.5% agarose (Vivantis) dissolved in 0.5X Tris-Acetate-EDTA buffer containing 2 µl GelRed solution (Biotium) and visualized using the Molecular Imager GelDoc™ XR+ with Image Lab software (Bio-Rad Laboratories). PCR products were sent to AIT Biotech for DNA sequencing. A consensus of the forward and reverse sequences of ITS and TEF of each fungus was made using CLUSTW in MEGA software. Following this, a sequence similarity check was performed using the NCBI BLASTn software. The nucleotide sequence data of PHLB17 (ITS, MK252898; TEF, MK252901), PHVI25 (ITS, MK252899; TEF, MK252902), PHVI26 (ITS, MK252900; TEF, MK252903) and PHVN021 (ITS, MK252905; TEF, MK252904) were submitted to GenBank.

### Infection potential

Infection potential of the fungi was performed using a detached leaf and stalk assays. Cut 20-cm long leaves of sugarcane cultivar VMC 86–550 were surface-disinfected by 10% hypochlorite solution (same as above), then washed with distilled water twice before air drying. Leaves were placed in 40 × 30 × 15 cm (L x W x H) transparent plastic container, laid with wet Scott® tissue papers. In each trial, there were five replicate leaves per fungal isolate, with each leaf midrib having 3 points of inoculation (top, middle, and bottom). These inoculation points were wounded by pin pricking. Five (5) mm agar plugs were placed into the pre-determined point of inoculations. The wounded and inoculated portions were sealed with parafilm (3 M) to avoid contamination by saprophytes. Finally, the containers were covered and left at room temperature. Symptom observations and red rot lesion measurement were done 7 days after. The leaf assay was performed three times. Each trial had six leaf-replicates and each leaf had three inoculation sites. Hence, a total of 54 technical replicates for the three trials.

For stalk infection assay, 15 cm-long stalks of sugarcane cv. ‘VMC 86-550’ were collected from the field germplasm, surfaced sterilized and air-dried using the same method as above. Each cane stalk comprises two nodes, and the center of the internode served as the inoculation point. A hole, about 10 mm deep, was made using a 2-mm diameter flame-sterilized nail and 50 µl of the inoculum suspension was injected into the hole. The holes were then sealed with parafilm and then

cane stalks were incubated at 27 °C. Red rot infection was assessed by measuring the vertical lesion length from 14 dpi. Brix or sucrose concentration of infected and non-infected parts of the stalks was measured at 14 dpi using a refractometer (Schmidt + Haensch, Germany). The stalk assay was performed twice, each trial consisted of four replicate stalks.

In all cases, leaves and stalks inoculated with distilled water served as control, healthy, uninoculated check. Leaves and stalks inoculated with *C. falcatum* RR09 served as inoculated positive (red rot) control. After the assessment, fungi were re-isolated from infected leaves and stalks following the same procedure as above to establish Koch’s postulate.

### Pathogenicity assay

The pathogenicity of four fungi was tested by inoculating the fungus in 3-month-old sugarcane cv. ‘VMC 84-524’ grown in soil in the plastic container (10-L capacity). Plants were placed in the greenhouse. The pre-determined inoculation points in the leaf midribs were surfaced sterilized following the same procedure as above, then were pin-pricked. A 5 mm agar plugs of the fungus was placed on top of the pricked portion of the leaf midrib and secured using a transparent tape. Plants were covered with polythene bags for 24 h after inoculation to create humid conditions that are needed for successful inoculation. Red rot development was assessed at 7 dpi. Plants receiving agar plugs without the fungus served as negative/control checks. Re-isolation of the pathogen was performed using the same method used above to establish Koch’s postulate. The test had three (biological) plant-replicates, with each plant having three leaf replicates, and each leaf had three inoculation sites. Hence, there were a total of 27 technical replicates.

### Data analyses

Data from morphocultural characterization, infection potential and pathogenicity experiments were analyzed using the analysis of variance (ANOVA) test. The Tukey’s HSD test was used for multiple comparisons of means. All analyses were performed in IBM SPSS Statistical software.

## Results

### Morpho-cultural characteristics

There were two morphotype groups of fungi identified. At 7 days post incubation (dpi), fungal isolates PHLB17, PHVI25 and PHVI26 growth in pPDA were scanty, mycelia flat and purplish in color. In sPDA, the fungi were profuse, mycelia were aerial that is white with a pinkish center. Their macroconidia were hyaline, slender with smooth walls,

slightly falcate and multiseptated (3–4 septa). Their microconidia were hyaline, slender with smooth walls, constantly ellipsoidal and unicellular to bi-celled (Fig. 1, upper two panels). Isolate PHVN21 had similar morpho-cultural characteristics with the other fungi except that it had a light yellowish center in sPDA, 7 dpi, and the microconidia were hyaline, slender and oval. At 40 dpi (Fig. 1, lower two panels), cultural growth differences were apparent of the two groups. Based on these morpho-cultural characteristics, the four isolates belonged to the genus *Fusarium*.

### Molecular characteristics

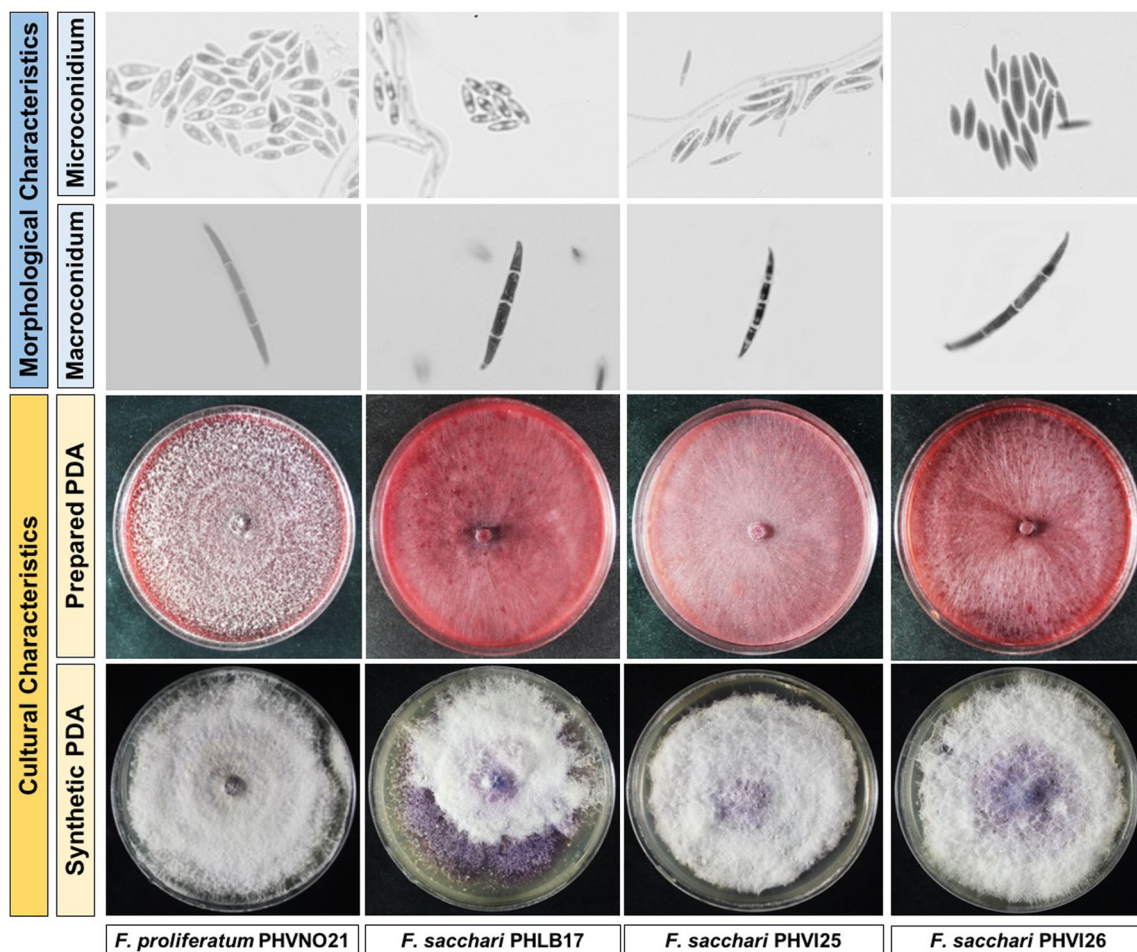
Both the expected bands of the ITS and TEF gene regions (650 bp) were successfully amplified. Sequence comparison and identification using the NCBI BLASTn software showed that the first morphotype group, consisting of the isolates PHLB17, PHVI25, and PHVI26, had 99% similarities of their ITS sequence and 99–100% similarities to the TEF sequence of two *F. sacchari* accessions (Table 1). The isolate PHVNO21, had 100% and 97% similarities, respectively, to

the ITS and TEF sequences of *F. proliferatum* (Table 1). Based on these results, PHLB17, PHVI25 and PHVI26 were identified as *F. sacchari* and PHVNO21 as *F. proliferatum*.

### Infection potential in leaves and stalks

Inoculation of all fungi, separately, in sugarcane leaves elicited a red rot symptom that was comparable to the that produced by *C. falcatum* (Fig. 2). The symptom was observed 2 dpi in all fungi and started out as minute red specks around the inoculation sites then later progressed and elongated, giving the midrib its reddish appearance. No infection was observed in all unwounded inoculated sites and in non-inoculated leaves. *F. sacchari* was the significantly more virulent, followed by *F. proliferatum* and the least was *C. falcatum* ( $P < 0.01$ , Fig. 2). The same fungi were re-isolated from the inoculated sites.

In stalks, symptoms developed 3 dpi in all fungi including the control, *C. falcatum*. The reddening of the internal tissue (Fig. 3) was longer in stalks inoculated with *F. sacchari* PHVI26, but this was not significant in trial 1 ( $P > 0.05$ ). In trial 2, *F. sacchari* PHLB17 was significantly virulent than the



**Fig. 1** Morpho-cultural characteristics of the four *Fusarium* spp. isolates causing red rot in sugarcane. Microconidia and macroconidium were taken at 100X objective and 1000X magnification, respectively

**Table 1** Percent similarities of the Philippines isolates of *Fusarium* spp. associated with red rot of sugarcane to isolates of *F. sacchari* and *F. proliferatum* in GenBank

Isolate	Species	ITS <sup>a</sup>	TEF <sup>b</sup>
PHVN021	<i>F. proliferatum</i>	100% MG562501	97% JX868988 (Masratul et al. 2013)
PHLB17	<i>F. sacchari</i>	99% KC464630	99% KM233195 (Wang et al. 2015)
PHVI25	<i>F. sacchari</i>	99% KJ434046 (Viswanathan et al. 2017)	99% JF740708 (O’donnell et al. 2012)
PHVI26	<i>F. sacchari</i>	99% KJ434046 (Viswanathan et al. 2017)	100% KM233195 (Wang et al. 2015)

<sup>a</sup> ITS, internal transcribed spacer region

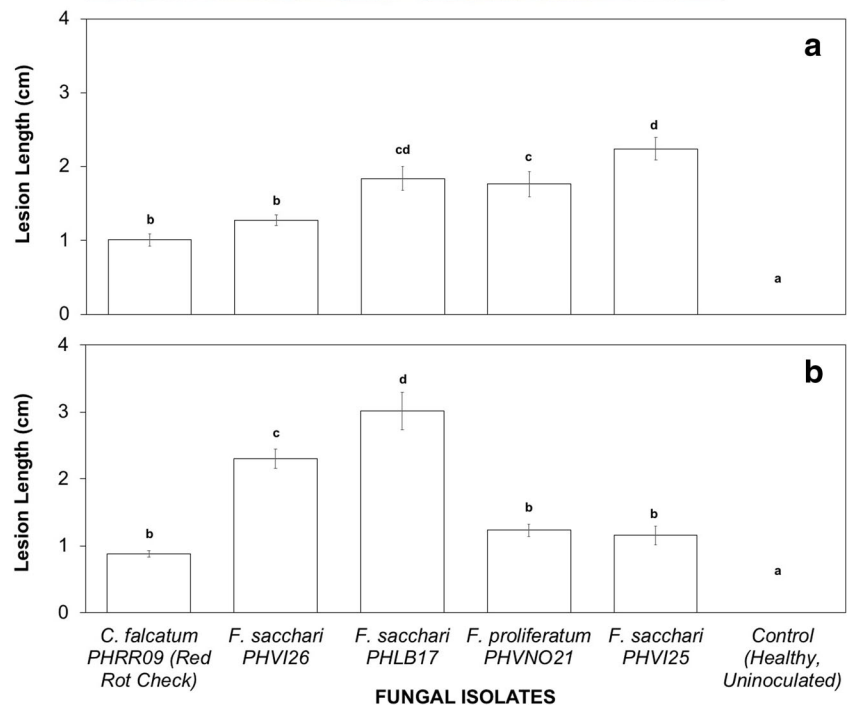
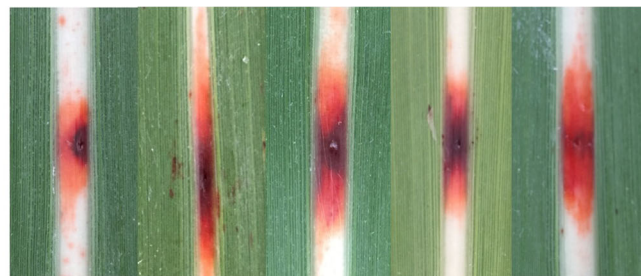
<sup>b</sup> TEF, translation elongation factor

other fungi. Nevertheless, all fungal isolates had comparable or longer lesion size compared to red rot *C. falcatum* check. In the absence of a node, the infection advances and extends throughout the stalk. After 14 dpi, the stalks become extremely discolored, with hues ranging from dark red to black. Hollowing or pitting of the internal tissues of the inoculated stalk then becomes apparent. The same fungi were re-isolated from the inoculated sites.

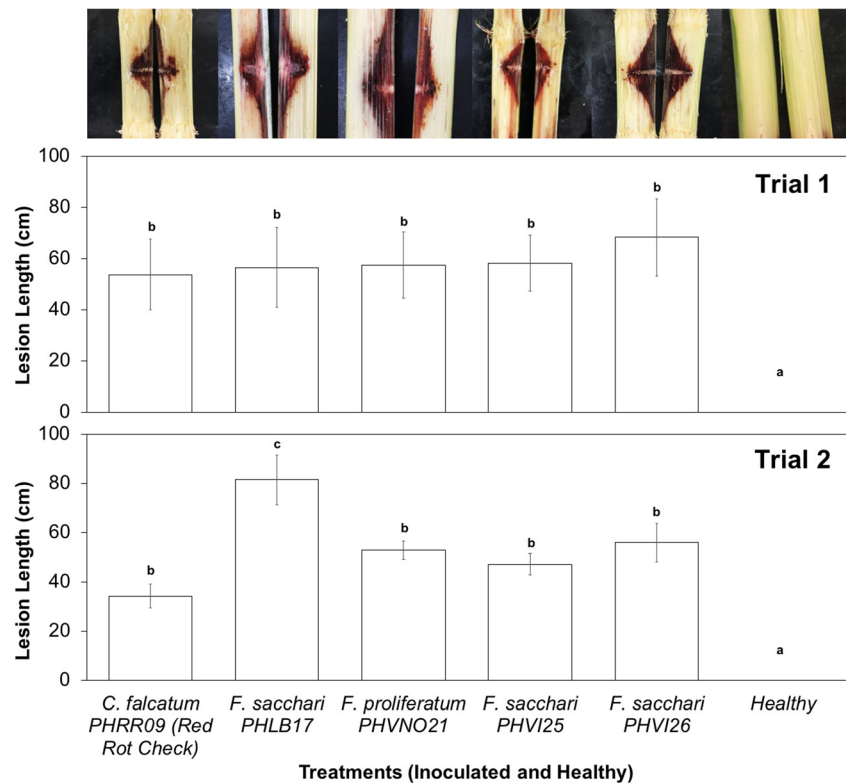
### Pathogenicity in plants

The red rot symptom developed in wounded sites of the leaf midribs. Symptoms observed were similar to those in the detached-leaf assay. *F. sacchari* and *F. proliferatum*, had longer lesions compared to the *C. falcatum* control (Fig. 2). However, this mean difference was only significant in *F. sacchari* isolates VI26 and LB17 ( $P < 0.01$ ). The two

**Fig. 2** Red rot lesion (upper figure) diameter at 7 days post inoculation of different fungi in sugarcane leaves in in vitro detached leaf (a) and in vivo plants (b). Bars with the same letter, within an assay, are not significantly different at  $P = 0.05$  by Tukey’s HSD test. Vertical bars are standard error of means (A  $n = 54$ , B  $n = 27$ )



**Fig. 3** Stalk rot (upper figure) development at 14 days post inoculation of different fungi. Bars with the same letter, within an assay, are not significantly different at  $P = 0.05$  by Tukey's HSD test. Vertical bars are standard error of means (Trial 1  $n = 4$ , Trial 2  $n = 4$ )



*Fusarium* species also induced earlier symptoms (at 2 dpi) than the *C. falcatum* control (at 3 dpi). However, differences between the centers of the midrib lesions are apparent between those inoculated with *C. falcatum* and those inoculated with *Fusarium* species. Lesions caused by *C. falcatum* eventually developed a straw color in the center while those lesions caused by the unknown pathogens have a dark center. The same fungi were re-isolated from the inoculated sites.

### Sugar level reduction

Stalk sugar level (brix) was reduced significantly by the two *Fusarium* species compared to *C. falcatum* in trial 1 (Fig. 4). In trial 2, sugar level was reduced but was only significant in *F. sacchari* PHLB17.

### Discussion

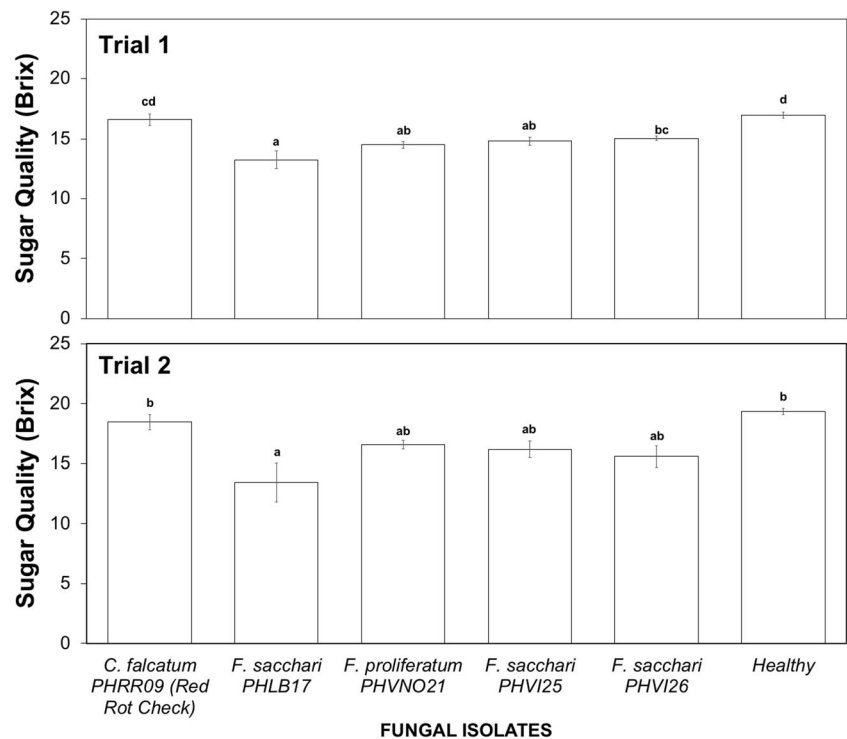
Four fungi were isolated from red rot-infected sugarcane leaves. But, preliminary work (De Castro et al. 2018) indicates that the fungi isolated were not the typical red rot pathogen, *C. falcatum*. In this study, using combined morpho-cultural and molecular characterization, the fungi isolated from the red rot-infected leaves were *F. sacchari* and *F. proliferatum*. The association of these pathogens to red rot symptom in sugarcane was confirmed in both infection potential and

pathogenicity assays under wounded conditions. Infection of these *Fusarium* species also resulted in reduced sugar levels in cane.

Identification of fungal pathogens can be challenging particularly in fungal genera consisting of several species that can be morphologically similar, but genetically different (Cai et al. 2009; Cannon et al. 2012). *Fusarium* and *Colletotrichum* are two fungal genera with species complexes. Thus, alongside morphological and cultural characterization, the addition of molecular characteristics of the fungi helps distinguishing species at a genetic level. This polyphasic approach (Cai et al. 2009) has been successfully used in both genera. In this study, by sequencing the ITS and TEF gene regions, the species identity of the four fungi was determined. The molecular data correspond well to the two morphotype groupings. But, in this study, instead of confusing these two morphotypes with other *Fusarium* species, e.g. *F. moniliforme* and *F. verticillioides*, the molecular data proved to be efficient in elucidating the identity at the species level.

*Colletotrichum falcatum* is the causal agent of sugarcane red rot in the Philippines (Serrano and Marquez 1927) and worldwide where sugarcane is cultivated (Viswanathan and Rao 2011). Red rot is found in leaf midribs and in cane stalks. Leaf infection is considered the primary source of inoculum for stalk infection (Butler and Abdul 1913; Abbott 1938). There is a great deal of work done on the epidemiology of red rot, but none has been reported on other fungal species that may induce similar symptoms caused by *C. falcatum*. Thus, at

**Fig. 4** Sugar quality (concentration in brix) of can stalks 14 days post infection of red rot caused by different fungi. Bars with the same letter, within an assay, are not significantly different at  $P = 0.05$  by Tukey's HSD test. Vertical bars are standard error of means (Trial 1  $n = 4$ , Trial 2  $n = 4$ )



the field level, any grower or technician will likely associate *C. falcatum* to that of a conspicuous red rot symptom observed in the sugarcane leaf. In this study, the four fungi isolated from the red rot-infected sugarcane leaves were not *C. falcatum*. The two *Fusarium* species consistently induced red rot-like symptoms in sugarcane leaves and were able to produce a red rotting symptom in sugarcane stalks. This indicates that infection of the two *Fusarium* species in the leaf may also serve as a source for stalk infection under condition favorable to red rot development. Of note, the lesions incited by *Fusarium* species in leaf midribs were more severe than *C. falcatum*, suggesting that the *Fusarium* species causing red rot may likely become a more pressing concern than *C. falcatum*. Nevertheless, field trials will still need to be performed for stalk infection and additional field surveys for natural stalk infection are warranted. *Fusarium* species, including the ones found in this study, are known to infect a wide host range. In sugarcane, *Fusarium* species causes Pokkah Boeng and specifically *F. sacchari* causes *Fusarium* wilt (Viswanathan et al. 2017), but not red rot. *Fusarium* wilt of sugarcane is unrecorded in the Philippines and hence, investigations on *F. sacchari* as a potential sugarcane wilt pathogen will be needed.

During *C. falcatum* invasion in sugarcane tissue, the enzyme invertase is produced which breaks sucrose into glucose and fructose (Sehtiya et al. 1993). In the US, 33%–50% sucrose reduction has been reported (Bourne 1934; Abbott 1938) and 25–75% in India (Viswanathan and Samiyappan 1999; Hussnain and Afghan 2006; Viswanathan and Rao 2011; Viswanathan 2012). In addition, during milling in

factory, mixing of juices from red rot-infected canes with the healthy stalks juice can result in spoilage (Viswanathan and Rao 2011). Recently, Habib et al. (2016) showed a decrease in percentage brix (10.81%), purity (2.56%) and sugar recovery (15.94%) in red rot infected cane compared to healthy plants, which implies that this problem remains considerably important. Sugar concentration can be reduced by *F. sacchari* PHLB17 (this study). Other isolates were also able to reduce the sugar concentration, though not statistically significant including the red rot control. However, it is important to note that the assessment was made 14 days post infection and thus, as the disease progresses, the level of reduction may be increased by the longevity of infection. Sugar is one of the most important commodities that is sourced mainly from sugarcane. Any factors negatively affecting sugar quality may affect the quantity of refined sugar to be used by many food and manufacturing sectors. Glasshouse and field works following on this study will elucidate the role and impact of these two *Fusarium* species on sugarcane juice quality (and quantity).

To our knowledge, this is the first record of *F. sacchari* and *F. proliferatum* associated with and inducing red rot-like symptoms to sugarcane. This information is important because, prior to this study, red rot is caused exclusively by *C. falcatum*. Further, *Fusarium* species caused more severe red rot lesion in sugarcane leaf midrib than *C. falcatum*, indicating that the former is more likely problematic in an epidemiological perspective. Based on this information,

deployment and development of management strategies for red rot in sugarcane would need to be addressed based on the pathogen associated with red rot in the field. Finally, existing sugarcane varieties will need to be assessed against these two *Fusarium* species.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interests.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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