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Geographic distribution of mealybug wilt disease of pineapple and genetic diversity of viruses infecting pineapple in Cuba

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ABSTRACT

Several species of ampeloviruses and badnaviruses infect pineapple plants around the world. *Pineapple mealybug wilt-associated* ampeloviruses have been associated with mealybug wilt of pineapple (MWP), the major viral disease threatening this crop. Conversely, infection by the badnaviruses Pineapple bacilliform comosus virus (PBCOV) and Pineapple bacilliform erectifolius virus (PBERV) is asymptomatic. To investigate the status of infection of the pineapple crop in Cuba, a diagnostic survey was developed in commercial areas during the period 2009–2012. Incidence of MWP disease was found in up to 100% of the plants in some fields of Central and Eastern regions of the island. Molecular assays revealed the presence of PMWaV-1 for the first time in the Caribbean basin and PMWaV-2, PMWaV-3, either as mixed infections or in combination with PBCOV throughout the country. Furthermore, they revealed for the first time the presence of PMWaV-2 in *Bromelia pinguin* L., a plant commonly used in Cuba as hedgerow. Sequence analysis of partial *heat shock protein 70h* and complete *coat protein* gene of Cuban isolates of PMWaV-1, -2 and -3 showed nucleotide identities above 97% with cognate sequences of viruses isolated from other countries. This work discloses the presence of a complex of viruses associated with the pineapple crop in Cuba, highlights the potential role of *B. pinguin* in the PMWaV-mealybug-pineapple pathosystem and makes available diagnostic tools for the detection of viruses affecting pineapple for a seed certified production system in Cuba.

1. Introduction

Mealybug wilt of pineapple (MWP) is a devastating disease of pineapple (*Ananas comosus* (L.) Merr.) and is currently present in the major pineapple-growing areas of the world (Sether et al., 2010). Disease symptoms include severe tip dieback, leaf reddening, downward curling along the leaf margins and dieback, and wilting of symptomatic leaves, all of them related to a reduced root system size (Sether and Hu, 2002a).

Although MWP etiology is not thoroughly understood, *Pineapple mealybug wilt-associated virus*-1 (PMWaV-1), PMWaV-2 and PMWaV-3 (genus *Ampelovirus*, family *Closteroviridae*) have been identified as the fundamental viral pathogens (Gambley et al., 2008a). In Hawaii, MWP symptoms are caused by the simultaneous presence of PMWaV-2 and mealybugs, but the combination of mealybugs with either PMWaV-1 or PMWaV-3 does not elicit the disease (Sether and Hu, 2002a). On the contrary, in Australia PMWaV-2 is not responsible for 100% of plants showing MWP, suggesting that either other PMWaV species, different strains of PMWaV-2, or additional factors may be involved in the observed symptoms (Gambley et al., 2008a).

Pineapple bacilliform comosus virus (PBCOV) and Pineapple bacilliform erectifolius virus (PBERV) (tentative members of the genus *Badnavirus*, family *Caulimoviridae*) have also been identified in pineapple, but their infections are asymptomatic (Gambley et al., 2008b; Sether et al., 2012). PBCOV has been detected in pineapples from Hawaii, Australia and China, and PBERV has been only identified in pineapples from Australia (Gambley et al., 2008b; Wu et al., 2010; Sether et al., 2012). Although PBCOV and PBERV are not directly involved with MWP, interactions of badnaviruses with the disease cannot be ruled out, considering the experience of badnavirus diseases in other crops (Sether and Hu, 2002a).

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Both pineapple ampeloviruses and badnaviruses are transmitted by mealybugs (*Dysmicoccus* and *Pseudococcus* spp.) and disseminated by the vegetative propagation of pineapple plants (Sether et al., 1998, 2012; Gambley et al., 2008b). In pineapple fields *D. brevipes* and *D. neobrevipes* are generally associated with ants in an interaction of synergism assisting mealybugs population to prosper and spread the disease (Rohrbach et al., 1988).

Control of viral disease in pineapple would demand the use of sensitive and reliable diagnostic systems (Gambley et al., 2008a). Due to clonal propagation of pineapple, healthy-appearing plants could become a source for virus spread to new areas. Tissue blot immunoassay (TBIA) using specific monoclonal antibodies and RT-PCR assay have contributed to the selection of seed production fields and the assessment of ampelovirus spread (Sether et al., 2005; Gambley et al., 2008a; Hernandez et al., 2010a, 2010b). In addition, the presence of characteristic MWP symptoms has permitted epidemiological studies to be carried out (Hughes and Samita, 1998; Awuah and Adzim, 2004).

Losses of pineapple crop associated with MWP fluctuate between 25 and 100% of yield and, when there are outbreaks of the disease during the first three months after planting, average fruit weight is reduced by 55% in comparison to that from PMWaV-free plants (Sether and Hu, 2002b). Particularly, asymptomatic PMWaV-1 infection causes up to 5–15% yield reduction in the secondary crops (ratoon crops) and losses associated with premature or asynchronous fruit ripening reached 30% of the production in Hawaii (Sipes et al., 2002). The high similarity and phylogenetic relation between PMWaV-1 and PMWaV-3 suggest that PMWaV-3 could induce the same deleterious effects as PMWaV-1 on the pineapple crop (Sether et al., 2009).

Pineapple is an economically important fruit crop in Cuba. In 2012, more than 37, 000 tonnes of fruits were harvested from 6172 ha, mainly of the cultivar Red Spanish (Ministry of Agriculture of Cuba, Anonymous, 2012). Even though total production increases every year, continuous declines of yields from pineapple orchards have been detected. Data from recent decades estimated up to 40% of yield losses associated with MWP in the pineapple crop (Borroto et al., 2007). Closterovirus-like particles were first observed in MWP-affected pineapple plants from Ciego de Avila almost 20 years ago (Borroto et al., 1998). Further molecular characterization demonstrated the presence of PMWaV-2 in samples gathered from Ciego de Avila and Isla de la Juventud, and PMWaV-3 in Isla de la Juventud (Borroto et al., 2007; Hernandez et al., 2010a, 2010b). Recently, PBCOV was also detected infecting pineapples showing MWP in the country (Hernandez-Rodriguez et al., 2013). Since the presence of MWP hampers pineapple production, and taking into account the limited extent of unsystematic field surveys previously conducted in Cuba, the aims of this work were to evaluate for the first time in a large scale, the distribution of MWP disease and the presence of PMWaV-1, PMWaV-2, PMWaV-3 and PBCOV in Cuban commercial pineapple fields. This work was conducted as part of an effort to establish a system for production of healthy certified pineapple seed in Cuba.

2. Materials and methods

2.1. Field inspection and plant material collection

The incidence of MWP symptomatic plants was determined by surveys conducted in 24 commercial pineapple plantations of cultivar Red Spanish, during 2009–2012. The number of plants showing characteristic MWP symptoms and total number of plants in the plantation were recorded from each prospected field. MWP symptoms evaluated were foliar reddening, leaves with tips curved down and dieback, and a wilted aspect (Sether and Hu, 2002a). Incidence of disease in a field was calculated based on the percentage of MWP symptomatic plants. In fields with 2 ha or less, 100% of the plants were inspected and, in larger areas, the incidence was estimated by examining 10% of the plants following a systematic sampling method with a W-shaped pathway (Gottwald, 1995). MD2 and Smooth Cayenne fields from Ciego de Avila and San Vicente, Granma, respectively, were inspected only with the aim of collecting diseased plants of those cultivars. Symptomatic plants (110 samples) from all prospected fields were randomly selected and the entire plants or vegetative propagation materials from them (i.e. suckers or crowns) were transplanted to plastic pots kept in a greenhouse at the Research Institute on Tropical Fruit Crops, Havana, Cuba. Asymptomatic *Ananas ananassoides* (Baker) L. B. Sm. Rank plants were collected in private gardens in Havana and Ciego de Avila; meanwhile *Bromelia pinguin* plants showing reddening and die back and used as hedgerows were collected in Banes, Holguin. Regular foliar applications of deltamethrin (Decis® 10 EC, Bayer CropScience) were made in the greenhouse to prevent mealybug and ant infestations and avoid viral transmission among plants.

2.2. Diagnosis and characterization of the ampeloviruses

For total RNA isolation, 100 mg of the basal white portion of pineapple leaves was processed using *TRIzol LS Reagent* kit (Invitrogen, Scotland, UK) and following the manufacturer's instructions. RT-PCR assays for PMWaV-1, PMWaV-2 and PMWaV-3 detection were performed using the *Titan One Tube RT-PCR System* (Roche Diagnostics, Mannheim, Germany) and the 225/226, 223/224 and 263/264 primer pairs, respectively (Sether et al., 2005). ORF5 (open reading frame) of ampeloviruses, encoding the coat protein (*cp* gen), was amplified following the same methodology using the primer pairs as follows: for PMWaV-1 CP227 (5'GAGCTCTTATTTGCGTCCACCCATAAAG3') and CP228 (5'GAGCTCATGGCTGATTCGAGCAAAC3'), for PMWaV-2 CP229 (5'GGATCCCTACCCTGAAACAGCTCCCTGG3') and CP230 (5'GGATCCATGGCTCAGAATTACGTAGCCG3'), and for PMWaV-3 CP231 (5'AAGCTTTCATCTGCGATTACCTG3') and CP232 (5'GGATCCATGAGTACGATTCCAGTAC3'). The annealing temperature for all the assays was 55 °C.

2.3. Detection of Pineapple bacilliform comosus virus

Pineapple DNA purifications were done using the procedure proposed by Murray and Thompson (1980). PBCOV detection was conducted by a non-radioactive dot blot nucleic acid hybridization (DBH) using as probe a fragment of 540 nt of the reverse transcriptase/RNase H region of PBCOV isolate BCuL26 (Hernandez-Rodriguez et al., 2013) and obtained using the PCR DIG labeling and detection kit (Roche). For the DBH, 10 μg of DNA were denatured at 95 °C for 10 min, chilled on ice and applied into nylon membranes (Amersham Pharmacia Biotech, USA) using a commercial device (BIO-RAD BIO-DOT ™ apparatus, Bio-Rad, USA). Nucleic acid extracts were cross-linked to the membrane in a crosslinker device (UVITEC, Cambridge, UK). Pre-hybridization was achieved for 2 h at 55 °C in Church buffer (Sambrook et al., 1989), and hybridized in the same solution at 60 °C for 16 h after adding 100 ng of the DIG-probe denatured at 95 °C during 10 min. After hybridization, membranes were twice washed according to the procedure described in Dig Luminescent Detection Kit (Roche) and the chemiluminescent detection was performed using CPD-star substrate (Roche) and Omat-S film (Kodak).

2.4. PCR product cloning, nucleotide sequencing and bioinformatics

All PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostic) and cloned into pGEM-