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PIV28 - SEGREGATION OF CITRUS TRISTEZA VIRUS (CTV) ISOLATES BASED ON EARLY REMOVAL OF THE INOCULUM SOURCE

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Citrus tristeza, caused by Citrus tristeza virus (CTV), is an endemic disease in Brazil. The control of this disease has been achieved by using tolerant rootstocks, as well as by cross protection in citrus cultivars with certain intolerance to the virus, such as 'Pera' sweet orange (Citrus sinensis L. Osbeck). However, the breakdown of the cross protection has been reported. Further, this breakdown may be related to the segregation of the CTV complex. In this work, we examined the segregation of CTV isolates based on tissue grafting and early removal of the inoculum source. Buds of different clones of 'Pera' sweet orange from the Citrus Active Germplasm Bank of the Instituto Agronômico do Paraná - IAPAR were used as inoculum source and virus free 'Pera Bianchi' sweet orange was used as indicator. A bud of the inoculum source was grafted on Rangpur lime (Citrus limonia Osbeck) tree and a bud of the indicator 'Pera Bianchi' was grafted above them. The buds used as inoculum were removed at 3, 5, 7, 10, 12 and 14 days after grafting. However, the buds were kept in the citrus trees used as positive control. CTV infection was confirmed by RT-PCR and segregation of the viral complex was examined by SSCP of the coat protein gene. Segregating and positive control samples of the CTV were cloned in the pCR®2.1-TOPO® vector and maintained in the Escherichia coli strain DH10B for DNA sequencing. The nucleotide sequences were analyzed by using the DNA Baser Sequence Assembler software and Clustal W. The initial CTV transmission was observed as early as 7 days after bud inoculation. However, partial transmission of the CTV complex was observed for a period ranging from 7 to 12 days after inoculation. The sequences of the segregant isolates, in which inoculum was removed up to 12 days after grafting, reached 99% identity to each other, whereas for the positive control sequences ranged from 92 to 99%. These data confirm the segregation of the viral complex when the inoculum source was removed in early stages. The segregant isolate will be studied in regard to the protective effect for cross protection in the control of citrus tristeza. Financial support: Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Paraná.

PIV36 - COAT PROTEIN PHYLOGENETIC ANALYSIS OF COWPEA APHID-BORNE MOSAIC VIRUS PASSION-FLOWER ISOLATES FROM BRAZIL.

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Cowpea aphid-borne mosaic virus (CABMV) causes woodiness fruit of passion-flower (Passiflora edulis), the main viral disease of this crop in Brazil. In order to evaluate the genetic variability of Brazilian passion-flower CABMV isolates, leaves showing mosaic and blister from passion-flower crops in the states of São Paulo (municipalities of Adamantina, Alvinlândia, Fernão, Garça and Jacupiranga), Paraná (São José da Boa Vista) and Goiás (Planaltina) were submitted to molecular analysis. After total RNA extraction and RT-PCR, DNA fragments correspondent to part of NIb, full-length of CP gene and 3'UTR were successfully amplified, sequenced and deposited into the GenBank as accession codes KC777401 to KC777407. Comparisons with other complete CP nucleotide sequences were done using BLASTn and multiple alignments were done manually. Trees were constructed by maximum parsimony (MP) and by maximum likelihood (ML), TRN+G (0.5689) nucleotide substitution model, using PAUP 4.0b10. Passionfruit woodiness virus sequence was used as outgroup and bootstrap percentage values were computed after 1,000 re-samplings. Phylogenetic analysis showed four well defined clusters. CABMV isolates from São Paulo, including those sequenced in this work, formed a monophyletic group supported by 93% of bootstrapping. Other group was divided into 4 subgroups: Fabaceae isolates from Northeast and Zimbabwe; passion-flower isolates from São Paulo, Espírito Santo and Sergipe. The CABMV passion-flower isolates from Goiás and Paraná clustered in a clade with other Goiás isolate and Northeast CABMV passion-flower isolates. Based on two different phylogenetic analysis methods, we found a high genetic variability among Brazilian CABMV passion-flower isolates. Differently of previously reported, we did not observe a consistent clustering based on geographical origin or host adaptation. The origin of CABMV in Brazil remains to be elucidated. Financial Support: FAPESP (Proc. 2011/11796-5) Fellows of *CAPES and **CNPq

PIV52 - ABSOLUTE QUANTIFICATION OF GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 4 BY TAQMAN REAL TIME RT-PCR IN INFECTED GRAPEVINES

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Grapevine viruses induce reduction of productivity and quality of grapes. Grapevine leafroll-associated virus 4, GLRaV-4 (Closteroviridae, Ampelovirus) causes leaf roll in grapevine. Absolute quantification determines the absolute amount of a target (expressed as a copy number or concentration). The objective of this study was to generate a standard curve for GLRaV-4 absolute quantification in infected grapevines. Reagents and reaction set up for GLRaV-4 amplification were previously described. To generate a standard curve, 5 or 6 different amounts (tenfold diluted) of the standard were quantified by TaqMan real time RT-PCR. Reactions were carried out in triplicates and standard curves were generated by two independent experiments. For quantification of RNA molecules as standard, a fragment containing part of the GLRaV-4 genome (300 bp covering 94 bp hHSP70 DNA fragment amplified by real time RT-PCR) was transcribed in vitro from a previously obtained transcriptional recombinant vector. This clone carries partial sequences of 14 viruses, fused in tandem, including GLRaV-4. After in vitro transcription, plasmid DNA template was removed with DNase and transcribed RNA concentration was measured by spectrophotometry. The use of RNA standard takes the variable efficiency of the reverse transcription reaction into account. The copy number of standard GLRaV-4 RNA molecules was calculated using the formula: Y molecules/µl = (X g/µl RNA / [transcript length in nucleotides x 340]) x 6.022 x 10^23 (Qiagen Handbook, 2011). After a standard curve was generated, 76 infected grapevine samples were evaluated to determine GLRaV-4 titre. The standard curve (plot of CT value, threshold cycle, against log of amount of standard) was generated: y = -1.509ln(x) + 41.202; in which R2 = 0.9999, y = CT value and x = RNA molecules/µl. The CT value of the target was compared with the standard curve (used as a reference in all subsequent reactions), allowing calculation of the GLRaV-4 amount in the samples. The absolute amount of GLRaV-4 nucleic acid in analyzed samples was determined and ranged from ca. 1000 to 150,000 copies of GLRaV-4/µl. This result can improve virus diagnosis by accurately quantifying virus titre variations in grapevines. Financial support: Embrapa

PIV56 - DETECTION AND COAT PROTEIN GENE CHARACTERIZATION OF GRAPEVINE VIRUS B ISOLATES FROM DIFFERENT GRAPEVINE SPECIES

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Corky bark, a component of the grapevine rugose wood complex, caused by Grapevine virus B, GVB (Betaflexiviridae, Vitivirus), induces decrease of production, incomplete ripening of grapes and progressive decline. Cultivars and rootstocks differ in their susceptibility to the corky bark disease. Some are symptomless carriers or exhibit mild symptoms, while others suffer rapid decline. The objective of this work was to characterize partially three isolates of GVB collected from different grapevine species and Brazilian geographical regions: GVB, the isolate named CS, was collected from cv. Cabernet Sauvignon (Vitis vinifera) exhibiting dark red spotted leaves and mild curling down of leaf edges, maintained in Bento Goncalves, Rio Grande do Sul State; the isolate IS-SVF was collected from cv. Isabel (V. labrusca) showing bark swelling and longitudinal cracking of mature canes in Sao Vicente Ferrer, Pernambuco State, and the isolate CO was collected from symptomless cv. BRS Cora (hybrid grapevine) in Jales, Sao Paulo State. The symptoms could not be associated with a single virus, since these plants could be infected by two or more virus. Total RNA was extracted from infected grapevines by capture on silica and the complete coat protein (CP) gene of GVB was RT-PCR-amplified, cloned into pGEM-T Easy vector and sequenced (two clones/isolate). An expected fragment of 594 nucleotides (bp) (coding for 197 deduced amino acids) was amplified by RT-PCR, using specific primers for GVB (6445v and 7038r), from the three different infected grapevine sources. The obtained sequences showed a low variability of coat protein genes among the three GVB isolates. Nucleotide and amino acid identities were higher than 99% among themselves. GVB GenBank accession codes are KF040331 (CO), KF040332 (IS-SVF) and KF040333 (CS). Different grapevine symptoms vary according to combinations of cultivar or host species with viral isolates, strains or species. In this work, high homologous coat protein sequences of three GVB isolates from symptomatic and symptomless grapevines and from distant geographical regions are involved in a