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# DETECTION OF *CHERRY VIRUS A*, CHERRY NECROTIC RUSTY MOTTLE VIRUS AND *LITTLE CHERRY VIRUS 1* IN CALIFORNIA ORCHARDS

**S. Sabanadzovic[1§], N. Abou Ghanem-Sabanadzovic[1], A. Rowhani[1]**
**J.A. Grant[3] and J.K. Uyemoto[1,2]**

[1] *Department of Plant Pathology, University of California, Davis CA 95616, USA*
[2] *USDA-ARS, University of California, Davis CA 95616, USA*
[3] *Cooperative Extension, University of California, Stockton CA 95205, USA*

**SUMMARY**

Leaves were collected during 2003 from trees displaying symptoms of vein necrosis, necrotic rusty mottle, necrotic leaf or marked fruit in surveys of sweet cherry [*Prunus avium* (L.) L. cv Bing] orchards in California. Samples were also taken from trees with leaf necrotic lesion (*P. serrulata* Lindl. cv Kwanzan) and leaf chlorotic spots (*P. avium* x *P. pseudocerasus* cv Colt) from virus disease indexing blocks of *Prunus*. All of the diseased, but none of the healthy, collections yielded high molecular weight double stranded RNA bands following tissue extraction, purification and electrophoreses in acrylamide gel. Positive amplification in reverse transcription polymerase chain reaction (RT-PCR), with two sets of degenerate primers, was used to detect viruses in the families *Flexiviridae* and *Closteroviridae*. Sequence analyses of the RT-PCR products identified *Cherry virus A* (CVA), Cherry necrotic rusty mottle virus (CNRMV), Cherry green ring mottle virus (CGRMV), Little cherry virus 1 (LChV-1) and Plum bark necrosis and stem pitting associated virus (PBNSPaV). This is the first report of the occurrence of the viruses CVA, CNRMV and LChV-1 in California.

*Key words*: cherry, virus, dsRNA, RT-PCR, *Closteroviridae*, *Flexiviridae*.

**INTRODUCTION**

Viruses and virus-like diseases cause serious problems in commercial *Prunus* orchards affecting tree performance. Although one of us (JKU) has researched virus and phytoplasma diseases of cherry trees over several years in California, we have observed leaf and fruit symptoms unlike those seen previously in surveys of commercial orchards and *Prunus* virus indexing blocks. In previous attempts to determine disease etiology, we would have grafted diseased collections to a set of standard indicator plants and waited for symptoms to develop. In this study, we sought to find answers using molecular diagnostic procedures. In that endeavor, leaf samples from symptomatic and asymptomatic trees were collected and analyzed for virus content. The results of our findings are reported and discussed herein.

**MATERIALS AND METHODS**

In commercial sweet cherry [*Prunus avium* (L.) L. cv Bing] orchards in Stockton, Lodi and Placerville, CA, collections were made of leaves from diseased trees exhibiting marked fruit (Fig. 1A), leaf vein necrosis (Fig. 1B), necrotic rusty mottle (Fig. 1C) or necrotic leaf blade (not shown) and apparently healthy trees. Also, collections were made in a couple of virus indexing blocks of *Prunus* at University of California Davis (UC Davis), CA, namely two Colt (*P. avium* x *P. pseudocerasus*) cherry trees, which had been graft-inoculated with a source of Plum bark necrosis and stem pitting associated virus (PBNSPaV) (Marini *et al*., 2002) and two Kwanzan flowering cherry (*P. serrulata* Lindl.) trees graft-indexed with a breeder's advanced selection of sweet cherry. The Kwanzan cherry assay was done to qualify the sweet cherry selection into the California Department of Food and Agriculture's Fruit and Nut Tree Registration and Certification Program. With PBNSPaV, chronic leaf symptoms appeared on both Colt cherry trees. They consisted of chlorotic rings (Fig. 1D) and greasy blotches. With the sweet cherry selection grafted onto indicator trees of Kwanzan flowering cherry, the leaves on both trees exhibited chronic necrotic lesions (Fig. 1E).

All collections were pre-tested by ELISA for *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) (Uyemoto *et al*., 1992). Also, double stranded RNAs (dsRNAs) were isolated from diseased tissues using double phenol-chloroform extractions and CF-11

*Corresponding author*: J.K. Uyemoto
Fax: +530.754.7195
E-mail: jkuyemoto@ucdavis.edu

§ Present address: Department of Entomology and Plant Pathology, Mississippi State University, Mississippi State MS 39762, USA.

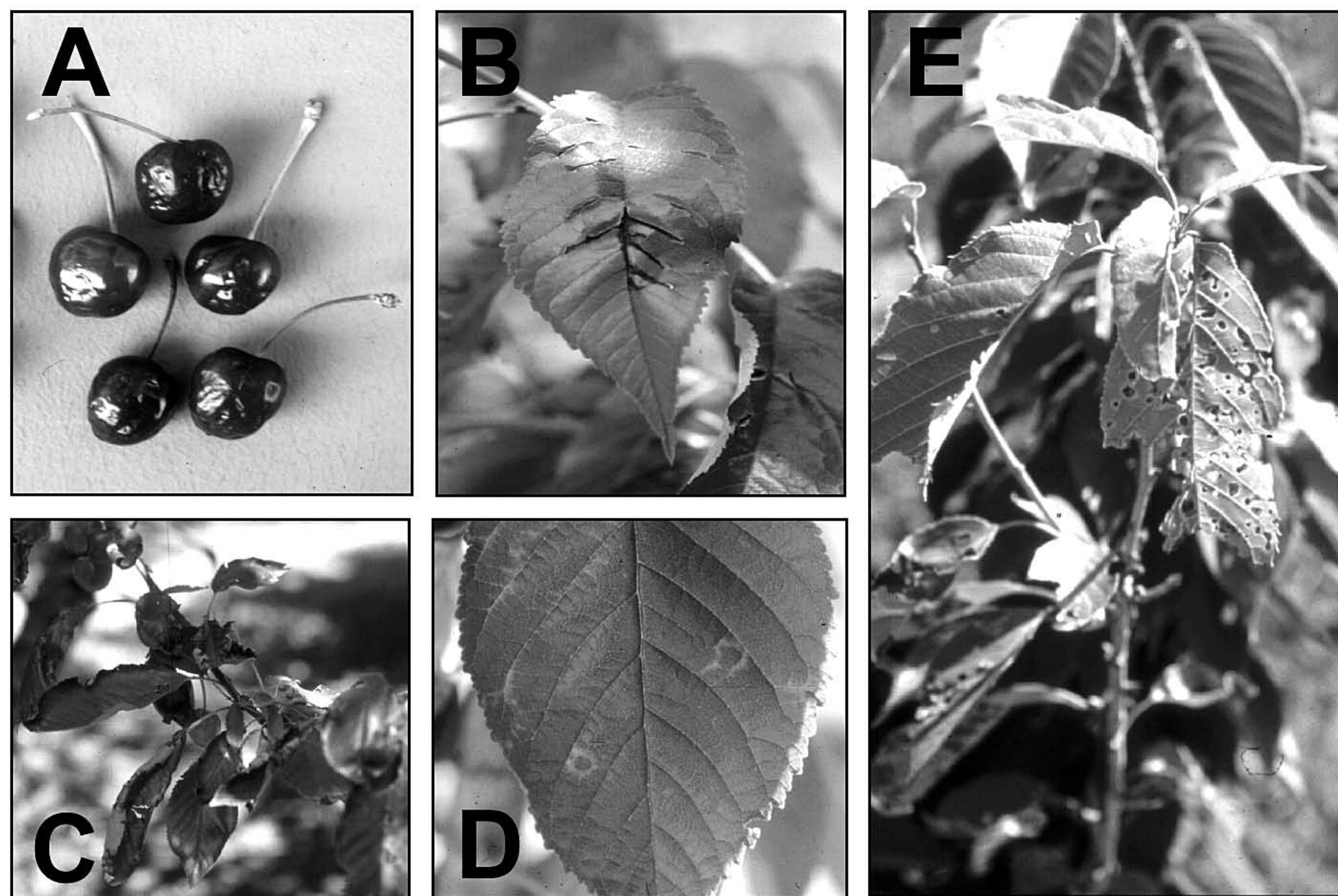


**Fig. 1.** Fruit and leaf symptoms in cherry trees. (**A**) marked fruit; (**B**) leaf vein necrosis; (**C**) leaf necrotic rusty mottle (all Bing sweet cherry); (**D**) leaf chlorotic ring spots (Colt cherry); and (**E**) leaf necrotic lesions (Kwanzan flowering cherry).

column chromatography (Dodds, 1993). The purified preparations were analyzed by polyacrylamide gel electrophoresis (PAGE). Purified dsRNA preparations were reverse transcribed with random primers and amplified by polymerase chain reaction using the degenerate primer sets DRW, designed to amplify a fragment of the RdRp genes of foveaviruses and vitiviruses (Dovas and Katis, 2003) and HSP-P, specific to the phosphate motifs 1 and 2 of the HSP70-homologue gene of viruses in the family *Closteroviridae* (Tian *et al.*, 1996) under the conditions described by the authors. RT-PCR products were cloned into pGEM-T Easy plasmid (Promega Corporation, Madison, USA) according to the manufacturer's instructions. *Escherichia coli* Top10 competent cells were transformed with the resulting recombinant plasmids and selected plasmids (a total 21 plasmids or 3 per PCR-positive assays) were sequenced at the UC Davis DNA Sequencing Facility and sequence data were analyzed using the Lasergene software (DNAStar Inc., Madison, WI, USA). Sequence comparisons and phylogenetic analysis were made by using the ClustalW program (Thompson *et al.*, 1994). One consensus sequence of each virus/isolate was deposited in GenBank as accession numbers AY944062 to AY944067.

## RESULTS AND DISCUSSION

All symptomatic collections tested positive for at least one virus (Table 1). PNRSV was detected by ELISA in extracts of leaves from trees bearing marked fruit, whereas, extracts of leaves with vein necrosis and necrotic lesions reacted with anti-PDV antibodies. All other diseased collections and healthy controls were negative for PNRSV and PDV.

All dsRNA preparations from diseased tissues yielded high molecular weight bands as visualized by PAGE (Fig. 2). In RT-PCR tests, the diseased, but not the healthy, preparations also tested positive as shown by amplification of the expected product(s) with one or both sets of primers.

Five disease sources produced RT-PCR products only with DRW primers, which yielded, as expected, a fragment length of 363 bp (Fig. 3A). Sequence analyses, however, revealed marked differences, indicating that different viruses were present. *Cherry virus A* (CVA, genus *Capillovirus,* family *Flexiviridae*) was identified in Bing cherry with leaf vein necrosis, in Kwanzan flowering cherry with leaf necrotic lesions and in Colt cherry with leaf chlorotic rings. Bing cherry trees with marked

**Table 1.** Symptoms found in cherry trees and viruses identified.

| Host | Symptoms | Viruses detected[a] |
|---|---|---|
| Bing | Leaf vein necrosis | CVA, PDV |
| Bing | Necrotic leaf | LChV-1; and an undetermined virus (*Flexiviridae*?) |
| Bing | Necrotic rusty mottle | CNRMV |
| Bing | Marked fruit | CGRMV, PNRSV |
| Colt | Chlorotic rings, greasy blotches | CVA, PBNSPaV |
| Kwanzan | Necrotic lesions | CVA, PDV |

[a] CVA, *Cherry virus A*; PDV, *Prune dwarf virus*; LChV-1, Little cherry virus 1; CNRMV, Cherry necrotic rusty mottle virus; CGRMV, Cherry green ring mottle virus; PNRSV, *Prunus necrotic ring spot virus*; PBNSPaV, Plum bark necrosis stem pitting associated virus.

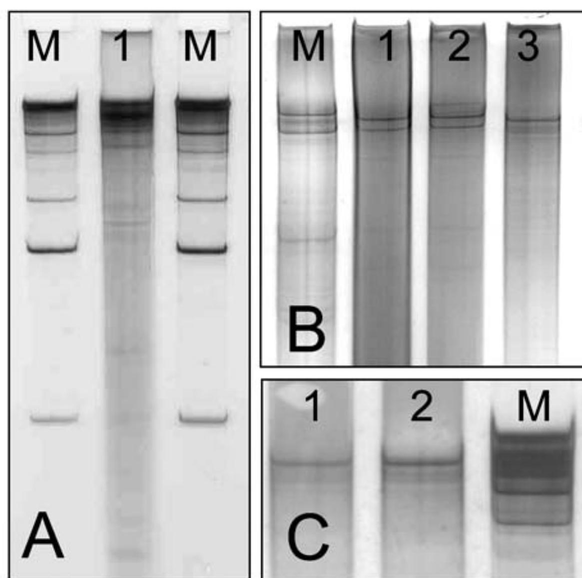


**Fig. 2.** Electrophoretic gel patterns of dsRNA bands extracted from infected samples: Gel pattern (**A**) necrotic leaf (lane 1); gel pattern (**B**) necrotic rusty mottle samples 7 and 8 (lanes 1 and 2, respectively), Rupestris stem pitting associated virus (lane 3); and gel pattern (**C**) vein necrosis and necrotic lesions (lanes 1 and 2, respectively). Lanes marked M contain reference markers. Healthy extracts not shown.

fruit, but not asymptomatic ones, contained Cherry green ring mottle virus (CGRMV, tentative species in genus *Foveavirus*, family *Flexiviridae*); these trees were also co-infected with PNRSV. Similar fruit markings have been associated with PNRSV (Mink, 1995). Cherry necrotic rusty mottle virus (CNRMV, tentative species in genus *Foveavirus*, family *Flexiviridae*) was identified in Bing cherry trees with symptoms of necrotic rusty mottle disease.

Two disease sources yielded RT-PCR products with both primer sets. Bing cherry with necrotic leaf blades yielded two amplicons of 591 and 1,062 bp with HSP-specific primers. Sequence comparison showed that both products were amplified from the same viral template and represented overlapping sequences. The amplified 1,062 bp-long DNA fragment showed 77% nucleotide (nt) and 85% amino acid (aa) homology with sequences of Little cherry virus 1 (LChV-1; an unassigned species in family *Closteroviridae*). Our results confirm previous data on high variability among LChV-1 isolates (Jelkmann *et al.*, 1997; Rott and Jelkmann, 2005; W. Jelkmann, personal communication). A second, DRW-generated product of 363 bp size, showed 83-86% aa homology to CGRMV and CNRMV, whose position in a phylogenetic tree (Fig. 3B) was in-between both viruses, suggesting, perhaps, a possible new species in the family *Flexiviridae* (Adams *et al.*, 2004) or a distinct isolate of CNRMV or CGRMV.

With Colt cherry collections, the preparations also tested positive with both sets of primers. The amplified product using the HSP primers was expected because of the reported association of diseased trees and Plum bark necrosis and stem pitting associated virus (PBNSPaV, tentative species in the genus *Ampelovirus*, family *Closteroviridae*) (Marini *et al.*, 2002). However, the discovery of a second product, amplified using DRW primers, was unexpected. It revealed co-infections by CVA.

The overall sequence comparisons showed that California isolates of CVA shared homologies of 91-93% aa with CVA type isolate (Jelkmann, 1995). Also, the California isolates of CNRMV were identical (99% aa homology) and shared 98% aa homology with CNRMV type (Rott and Jelkmann, 2001). Lastly, the marked fruit isolates of CGRMV were positioned closer to virus isolates from the European Union than to those from the USA (Zhang *et al.*, 1998; Gentit *et al.*, 2002). Sequence homologies of CGRMV isolates that were identified ranged from 82 to 93%, respectively, for EU and USA isolates.

RT-PCR using the primers DRW, designed to detect foveaviruses and vitiviruses (Dovas and Katis, 2003), yielded the predicted genome fragments for CVA, CNRMV and CGRMV confirming the suitability of the

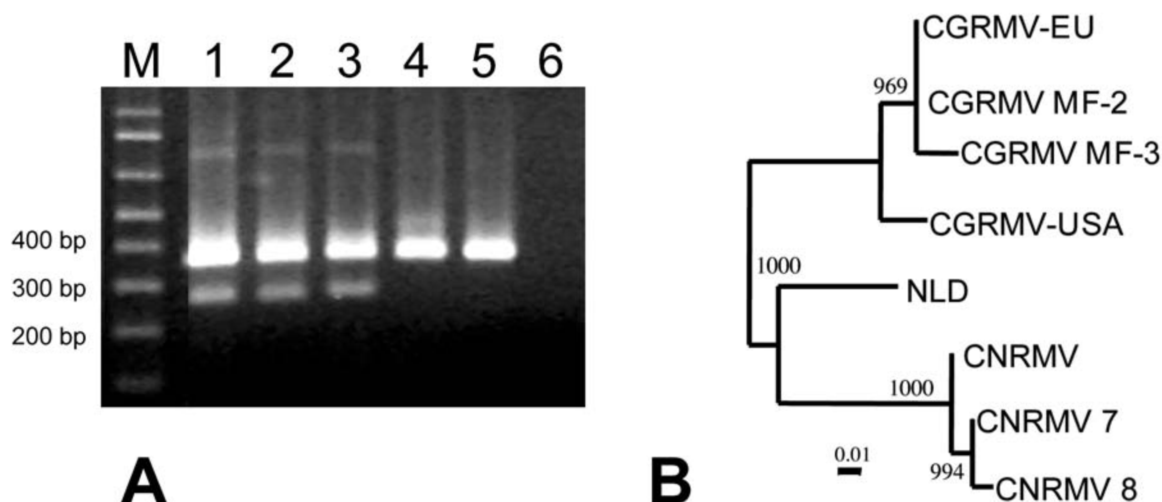


**Fig. 3.** (**A**) Ethidium bromide-stained gel of PCR products generated with DRW primers. CVA-infected Colt, Kwanzan and Bing cherry preparations in lanes marked 1, 2 and 3, respectively; CGRMV in lane 4, CNRMV-7 in lane 5, and a negative control in lane 6. Reference DNA markers in lane M. (**B**) phylogenetic comparisons of a 120 amino acid portion of the viral RdRp genome contained in Bing cherry preparations of necrotic leaf and different isolates of CNRMV and CGRMV (reported herein and elsewhere). EU, European Union; MF, marked fruit and NLD, necrotic leaf disease.

primers for detecting multiple members of the family *Flexiviridae.*

*Cherry virus A*, originally found in a cherry tree affected by little cherry disease (Jelkmann, 1995) and later detected in sweet cherry, peach and apricot trees of different disease status (and in symptomless samples), could not be ascribed to any of the currently known diseases in sweet cherry (Eastwell and Bernardy, 1998; James and Jelkmann, 1998). It was hypothesized that CVA infection may not be significant individually, but may enhance the severity of symptoms when combined with other viruses (James and Jelkmann, 1998). Hence, the necrotic symptoms in CVA-infected trees of Bing cherry with leaf vein necrosis and Kwanzan flowering cherry with leaf necrotic lesions (both co-infected with PDV) pose an intriguing association of virus and tissue necrosis. Although PDV is known to cause shock symptoms a year after infection, consisting of chlorotic and necrotic lesions in various *Prunus* spp. and bark necrosis on Shirofugen flowering cherry, the PDV isolate in Kwanzan flowering cherry was unusual in that it was latent in Shirofugen flowering cherry, while the PDV isolate in the Bing cherry induced the typical necrotic reaction (J. Uyemoto, unpublished data). A non-necrotic strain of PDV had already been reported by Ramaswamy and Posnette (1971). Even though a third undetected virus or viruses may still be present in diseased trees, an association of CVA and leaf necrosis should not be excluded.

Our surveys identified for the first time in California, the presence of CVA, CGRMV, and LChV-1. Although necrotic rusty mottle disease was reported in California (Wadley and Nyland, 1976), the detection and identification of CNRMV was confirmed in this work, which further support the causal relationship of virus and disease symptoms.

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