

## ABSTRACT

**GUZMAN-BAENY, TANIA LINA. Incidence, Distribution, and Symptom Description of Viruses in Cultivated Blackberry (*Rubus* subgenus *Eubatus*) in the Southeastern United States. (Under the direction of Gina Fernández and Zvezdana Pesic-VanEsbroeck).**

During the summer of 2000, an alarming number of blackberry plants with virus-like symptoms were found in commercial plantings and research plots in North Carolina. To determine the nature of these symptoms, a survey and related studies were initiated in 2001 to document the virus situation in North Carolina as well as South Carolina and Virginia. The survey revealed that *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV) and *Impatiens necrotic spot virus* (INSV) were the predominant viruses present in blackberry plantings we tested. In North Carolina, South Carolina and Virginia, the combined incidence of TRSV, ToRSV and INSV in the locations surveyed were 49, 45.2 and 33.1% respectively. In most instances, TRSV, ToRSV and INSV were present as multiple infections in one plant. Detection of viruses varied by plant part tested. Roots generally possessed a higher virus titer and therefore the presence of a virus was more easily detected. *Impatiens necrotic spot virus*, a virus that had not been previously known to occur in blackberry, had been detected for the first time (completion of Koch's postulates is pending). We also determined that TRSV was present in the embryo of blackberry seeds. Visual symptoms varied among viruses and cultivars. Symptoms observed were: chlorotic line patterns, ringspots, vein chlorosis, vein necrosis, necrotic spots, crumbly fruit, yellow blotches, distorted leaves and oak leaf pattern. In many instances, the appearance of virus-like symptoms was concomitant with a decline in plant health. The relatively sudden appearance of symptomatic plants suggests that viral load

reached a critical point not seen before in the region. Plants either came into the fields with infections and/or became infected shortly after they were planted. Many of the plantings we tested were relatively young and therefore, it was likely that infected plants were from commercial nurseries. In addition, these infected plants may have been planted into fields that had viruliferous nematodes and insects. These pests may have been vectors for the introduction of additional viruses. It is likely that other unknown viruses, may be infecting blackberry plantings in the southeastern United States, further complicating the situation. Many questions remain to be answered. For example, systematic studies are needed to determine effects and symptoms of single and multiple infections in each of the cultivars. Unknown viruses and the vectors that introduce them need to be identified. Chemical or biological mechanisms of control for vectors need to be determined. And finally, plant breeders need to identify and utilize germplasm with resistance to vectors in traditional breeding programs or incorporate resistance via molecular techniques. However, it is clear from our studies that some immediate steps can be taken. First, breeding programs need to virus index new releases to ensure that they are not the initial source of infection and second, state nursery certification programs need to be implemented and enforced to insure that the burgeoning blackberry industry remains viable.

**Incidence, Distribution, and Symptom Description of Viruses in Cultivated Blackberry  
(*Rubus* subgenus *Eubatus*) in the Southeastern United States**

by

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‘Para mis amados padres, por tantas cosas...’

‘To my beloved parents for everything...’

## **Biography**

Tania Lina Guzmán-Baeny, a native of Bolivia, South America, spent her formative years in the city of Cochabamba. She was the youngest of six children. As a child, Tania and her family spent the majority of their vacations in some of the tropical regions of Bolivia. At an early age, while surrounded by nature, Tania realized she much preferred outdoor activities to those relating to the indoors.

Tania completed high school in 1988, and the following year she enrolled in the Universidad Mayor de San Simon in Cochabamba. While studying as an undergraduate, she realized she liked to work with fruit crops. In 1995, she graduated from Universidad Mayor de San Simon with a B.Sc. in agriculture. Tania began her thesis research in May of 1995 at the Instituto Boliviano de Tecnología Agropecuaria (IBTA, San Benito). She focused her research on disease control in peaches. In December of 1997, she fulfilled the requirements for a professional certificate and obtained her title as Engineer in Agriculture.

During the summer of 1997, Tania worked as an intern in the Entomology Department at North Carolina State University (NCSU) where she gained experience in pest management and control.

After completion of her degree Tania was employed by Biosis S.R.L., a private company. As an employee with Biosis, she developed fruit projects and provided technical assistance to some of the farmers located in Cochabamba and Chuquisaca.

In 1999, Tania applied for admission to NCSU. In the spring of 2001, she began her Master's Program, under the direction of Dr. Gina Fernández, with a major in Horticultural Science and a minor in Plant Pathology.

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## **Literature Review**

### **Blackberry Botany**

Blackberries, in the genus *Rubus*, subgenus *Eubatus*, consist of a highly variable and complex group of species and hybrids. They are found in Europe, North America, South America, eastern Asia and Africa (Jennings, 1988). The subgenus is virtually absent from Pacific- and Indian-ocean regions (Jennings, 1988). The center of origin of blackberries is not clear, even though, Gustafsson (1942) presented evidence supporting the hypothesis that there were two major centers of origin for the blackberry, namely eastern North America and Europe. Blackberries present perennial crowns and roots and most species biennial canes, which vary from erect to procumbent in growth habit and are usually armed with sharp prickles (Moore and Skirvin, 1990). The fruit develops from a single five-petaled flower, through the adhesion of many separate carpels (drupelets), when ripe, the whole fruit, consisting of receptacle and adhering drupelets, detaches as a unit from the pedicel (Converse, 1970; Ellis et al., 1991).

Ellis et al. (1991), mention that the abundance of wild blackberries exists because of certain traits of their life history. Most are colonizers of habitats disturbed by either natural causes (fire, glaciers, etc.) or human activities (logging, farming, etc.). Sexual reproduction, dispersal of seed by birds, rapid vegetative propagation as well as prolific production of apomictic seed in certain groups have allowed extensive speciation in the *Rubus* subgenus *Eubatus* (Ellis et al., 1991). The variation in the subgenus might best be described as a

network of relatively few sexual species and a great number of polyploid hybrid derivatives (Ellis et al., 1991).

The species of the subgenus range from evergreen subtropical species to to deciduous ones adapted to northern Canada, and the ploidy range extends from diploid ( $2x = 2n = 14$ ) to dodecaploid ( $12x = 2n = 84$ ) (Jennings, 1988). The octoploid species of the *Ursini* section from western North America combine well with raspberries to give hybrids such as the cultivars Loganberry and Tayberry of great economic importance (Jennings, 1988). According to Ellis et al. (1991), the primary species of blackberries in eastern North America seem to have intercrossed more than their European relatives, although higher ploidy types are more common in Europe. Sources of thornlessness, hardiness, disease resistance, flavor, fruit size, early ripening, heat tolerance and productivity have been identified among eastern North American blackberry species, including *R. allegheniensis* Porter, *R. canadensis* L., *R. argutus* Link, *R. cuneifolius* Pursh, *R. frondosus* Bigel, *R. hispidus* L., *R. baileyanus* Britt. and *R. trivialis* Michx. In western North America *R. ursinus* Cham. and Schlechtend. and *R. macropetalus* Douglas are noted for their excellent flavor (Ellis et al., 1991).

Fernandez and Ballington (1999) mention that 11 species of blackberries are either native to North Carolina or were introduced very early to the state. Blackberries have been a favorite native fruit in the southern United States (U.S.) and harvests of blackberries from wild plantings have been a common practice for many generations (Clark, 1999). Native Americans ate wild blackberries for thousands of years, and the early European settlers ate blackberries fresh, dried and as preserves (Fernández and Ballington, 1999).

## **Cultivated Blackberry Production**

Although blackberry species are native to many parts of the world, little domestication and commercial use has been made of them except in North America and Europe (Moore and Skirvin, 1990). There is evidence that they were domesticated by the seventeenth century in Europe and during the nineteenth century in North America (Jennings, 1988).

Moore and Skirvin (1990) classified American blackberry cultivars for ease of grouping as erect thorny, western trailing, semi-erect thornless, dewberries, or raspberry-blackberry hybrids, based on gross morphology. Fernández and Ballington (1999), list cultivars recommended for the eastern U.S. mainly from USDA, the University of Arkansas breeding program and the Scottish Crop Research Institute (SCRI) and have good adaptation in North Carolina. Adapted cultivars include ‘Arapaho’, ‘Chester’, ‘Cherokee’, ‘Kiowa’, ‘Navaho’, ‘Shawnee’, ‘Choctaw’, ‘Cheyenne’, ‘Hull’, ‘Lochness’ and ‘Triple Crown’. The Arkansas cultivars were named in honor of Native American tribes.

The vast majority of cultivated blackberry production in the U.S. is concentrated in the Pacific Northwest; Oregon is the leading state with 2,420 ha in production, with a yield of 21,900 MT and a value of \$18,296,000 in 2002. California and Washington follow Oregon, as the second and third most important producing states respectively (USDA, 2003). Blackberry production in the eastern U.S. has increased during the last five years. Although statistical data are not available for this region, at least 202 ha can be considered in production for 2003 (Fernández, personal communication).

## **Virus and virus-like diseases in *Rubus***

Virus and virus-like diseases have an enormous impact on blackberry production throughout the world. Converse stated that blackberry virology in the U.S. was still in its early descriptive phase (Converse, 1984). Many blackberry virus diseases also occur on wild and cultivated raspberries (*Rubus* spp.). Diseases of the latter have been more extensively investigated; therefore, the reader is referred to review an extensive description of *Rubus* viruses and their detection (Converse, 1984).

Worldwide 33 viruses and virus-like diseases that infect wild and cultivated members of the genus *Rubus* have been reported; 15 are found in cultivars in North America, and some of them are quite serious (Converse, 1991; Martin, 2002). Crandall (1995) mentions that viruses more seriously damage black (*R. occidentalis* L.) and purple raspberries (*R. negléctus*) than either red raspberries (*R. idaeus* L.) or blackberries (*Rubus* spp). There are also substantial differences in susceptibility among cultivars. Not all *Rubus* viruses cause serious yield losses. Some cause damage to plants, but are unimportant because of their limited geographic distribution (Converse, 1970). Others are not serious because of the tolerance of their hosts. However, some *Rubus* virus diseases are both very widespread and destructive, and result in significant yield reduction and plant decline (Converse, 1970).

Based on the method of transmission blackberry viruses are grouped as follows: 1) insect-transmitted, 2) pollen-transmitted and 3) nematode-transmitted (Converse, 1984; Converse, 1991; Martin, 2002).

This research focused on the identification, distribution and symptom expression of

two Nepoviruses: *Tomato ringspot virus* (TRSV) and *Tobacco ringspot virus* (TRSV); two seed and pollen-borne virus, *Raspberry bushy dwarf* (RBDV) and *Tobacco streak virus* (TSV). All these four viruses previously mentioned are known to occur in *Rubus*. Two Tospoviruses that have not been reported in *Rubus* previously *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV) but were included in this research due to their wide host range. Therefore this part of the literature review will concentrate in these six viruses.

### ***Tomato ringspot virus***

According to Converse (1987; 1991) *Tomato ringspot virus* (ToRSV) is the most important and widespread of the nematode-transmitted viruses affecting cultivated *Rubus* in North America. It occurs in *Rubus* and in some other crops in South America but is not known to naturally occur outside the Western hemisphere (Converse, 1991). *Tomato ringspot virus* has a large natural and experimental host range; species in more than 35 dicotyledoneous and monocotyledoneous plant families are susceptible (Stace-Smith, 1987a). It is of minor importance to tomato (*Lycopersicon esculentum* Mill.) production (Agrios, 1997), and in nature, the virus occurs mostly in ornamentals and woody or semi-woody plants (Converse, 1987). Fruit crops affected include strawberry (*Fragaria vesca* L.) apple (*Pyrus malus* L.) and peach (*Prunus persica* L.), in the *Rosaceae*; blueberry (*Vaccinium* spp.), in the *Ericaceae*; and many common weeds, such as dandelion (*Taraxacum officinale* Wigg.) and chickweed (*Stellaria media* (L.) Cirillo) (Converse, 1991).

Among *Rubus* cultivars, ToRSV is most serious in red raspberry but also occurs in some cultivated and wild blackberries and raspberry-blackberry hybrids, but not in black

raspberry (Converse, 1991). The ringspot disease, caused by ToRSV, is the most common virus disease seen in field-grown red raspberries in Oregon and Washington (Converse, 1987). Finn and Martin (1996) did not detect ToRSV infections in clonal and seed propagules of *R. ursinus*, the trailing blackberry, and seeds of *R. leucodermis* Dougl. the Western black raspberry, throughout the Pacific Northwest. Martin (1998) did not find ToRSV in raspberry production fields in the Fraser Valley of British Columbia and it was present sporadically in red raspberry in southern Washington and Oregon. The virus is also prevalent in eastern U.S., where it is associated with crumbly berry in red raspberry (Converse, 1987).

In red raspberry, symptom expression varies with cultivar, time of the year, and plant part infected (Converse, 1991). The most striking symptoms occur when the plant is first infected. When shock symptoms appear at the beginning of the growing season, production of foliage and new suckers may be delayed by 1-2 weeks. In some cultivars this new foliage has a distinctive bronze cast, which disappears as the foliage matures. Spring foliage may show yellow rings, line patterns or vein chlorosis. As the summer progresses, new leaves are usually symptomless, and chronically infected plants often lack distinctive leaf symptoms although the plants may be dwarfed and weak. Because the virus is vectored by viruliferous dagger nematodes (*Xiphinema americanum* Cobb), chronically infected plants usually occur in slowly widening, circular to oval patches in the field. These patches may be mistaken for plants infected by Phytophthora root rot caused by *Phytophthora megasperma* Drechs, *P. cryptogea* Pethybridge and Lafferty, *P. citriicola* Sawada and *P. cactorum* (Agrios, 1997). In the spring, newly infected plants at the edges of such patches will usually develop characteristic symptoms (Converse, 1987; 1991).

Crop loss is difficult to assess because the reduction of fruit yield and quality in red raspberry caused by ToRSV infection varies greatly with cultivar and duration of infection. The cultivar Meeker showed a significant yield reduction but not in drupelet set (crumbly fruit) in the field when infected by ToRSV. The reverse was true for the cultivar Puyallup and neither fruit yield nor drupelet set were significantly depressed in the cultivar Canby (Converse, 1991).

Symptoms on blackberry have not been investigated, but transmission experiments were done with a 'Himalaya' blackberry (*R. discolor* Weihe & Nees) plant that was thought to be infected with ToRSV plus a virus causing feather-vein symptoms (Converse, 1987). These experiments indicated that ToRSV may cause a variety of leaf symptoms, including small chlorotic spots scattered over the leaf blade, large yellow blotches at the base of the leaflets, conspicuous veinal chlorosis and oak leaf patterns. The absence of these symptoms in grafted plants of 'Boysen', 'Nectar', 'Youngberry' and the native trailing blackberry suggests that these cultivars could be immune (Converse, 1987).

*Tomato ringspot virus* is seedborne in *Rubus* and in common weeds, such as chickweed and dandelion. Plants developing from infected seeds are usually symptomless and can serve as foci for new infestations of the virus in fields where dagger nematodes are present (Converse, 1991). Most annual weeds are symptomless when naturally infected by ToRSV in the field by seed transmission or by dagger nematodes. Several herbaceous greenhouse test plants developed characteristic symptoms when sap-inoculated. Some of the most useful of these include *Chenopodium quinoa* Willd., cucumber (*Cucumis sativus* L.) and tobacco (*Nicotiana tabacum* L.) (Converse, 1991).

*Tomato ringspot virus* is naturally vectored by several species of the dagger nematode, including *Xiphinema americanum* Cobb and *X. rivesi* Dalmasso (Converse, 1991; Forer and Stouffer, 1982). They can transmit this virus for several months. Vector specificity involves specific attachment of ToRSV virions to portions of the lining of the alimentary canal of the nematode (Converse, 1991). Speciation in this genus is a matter of current study, and other *Xiphinema* spp., but not all are vectors. Where vector dagger nematodes are present in a red raspberry field, ToRSV spreads along rows at a rate of approximately 2 m per year. New infections mostly occur next to previously infected raspberry plants, resulting in expanding oval patches of infected plants in a field (Converse, 1991).

*Tomato ringspot virus* is a member of the *Nepovirus* genus. It has three types of isometric particles 28 nm in diameter; the three types of particles contain 0, 40 and 43% single-stranded RNA (ssRNA). Total genome size 15.8 kb and the size of ssRNA species of this bipartite genome are 8.5 kb and 7.3 kb respectively and both ssRNA species are required for infection. Each virion has 60 coat protein subunits of  $M_r$  55 kDa (Converse, 1991; Murphy et al., 1995).

### ***Tobacco ringspot virus***

*Tobacco ringspot virus* (TRSV) has a wide natural and experimental host range of important economic crops and weeds outside the genus *Rubus*. The virus causes a ringspot disease of tobacco, cucumber, Easter lily (*Lilium longiflorum* var *eximium* (Dourtois) Baker), hydrangea (*Hydrangea* sp.), iris (*Iris* sp.) and geranium (*Pelargonium* L'Hér). It also affects blueberry, soybean (*Glycine max* (L.) Merr.), and many other annual and perennial crops (Converse, 1991; Stace-Smith, 1987b).

According to Jennings (1988), TRSV is not common in *Rubus*. Knowledge of its occurrence is limited to occasional reports in blackberries near tobacco fields in North Carolina. Where it was found in four native *Rubus* species: *R. allegheniensis*, *R. argutus*, *R. flagellaris* Willd. and an unidentified *Rubus* sp. (Converse, 1987). The only record of the virus being isolated from a cultivated blackberry is from British Columbia (Stace-Smith and Hansen, 1974); although, Clark (2000) mentions that Gergerich has confirmed the presence of TRSV in blackberry cultivars in Arkansas.

Converse (1987) stated that the virus had no economic importance, because there were essentially no commercial raspberry or blackberry plantings in those areas of North America where TRSV and its nematode vector were endemic.

Wild blackberries in North Carolina infected with TRSV had stunted, distorted foliage that exhibited faint to severe ringspots, chlorotic line patterns, mottling and mosaic (Converse, 1987).

*Tobacco ringspot virus* is a polyhedral virus, with a bipartite genome. The isometric virions are 28 nm in diameter. Total genome size 11.2 kb. The two ssRNAs have a size of 6.8 kb and 4.4 kb respectively. Both RNAs are needed for infection (Converse, 1991; Murphy et al., 1995).

### ***Raspberry bushy dwarf virus***

*Raspberry bushy dwarf virus* (RBDV) has a unique genome organization among plant viruses and has therefore been classified in its own viral genus *Idaeovirus*. The name *Idaeovirus* comes from the scientific name for red raspberry, *R. idaeus*. *Raspberry bushy*

*dwarf virus* occurs naturally worldwide in many *Rubus* species and cultivars. The virus is common in Europe, Asia, North and South America and Australasia (Converse, 1991).

Converse (1991) stated that in North America RBDV naturally infects many red raspberry, black raspberry, blackberry-raspberry hybrids and blackberry. It also occurs in wild *R. idaeus*, *R. occidentalis*, *R. parviflorus* Nutt. and *R. leucodermis*. Experimental hosts (by graft inoculation) include *Cydonia oblonga* Mill., *Fragaria vesca* and seven *Rubus* spp., and (by sap inoculation) *Chenopodium quinoa* and several other *Chenopodium* spp. *Cucumis sativus*, *Nicotiana clevelandii*, *Phaseolus vulgaris*, and plants in some genera. *C. quinoa* is often used for the detection of RBDV by sap inoculation. *Rubus molaccanus* L. and *C. oblonga* 'C7/1' develop diagnostic symptoms when graft-inoculated with RBDV. According to Barnett and Murant (1970), the virus is transmissible to 55 species in 12 dicotyledonous families, most of them symptomless. Kokko et al. (1996) detected three new host plants in *Rubus*, Arctic bramble (*R. arcticus* ssp. *arcticus*), Alaskan arctic bramble (*R. arcticus* ssp. *stellatus*) and their hybrid (*R. arcticus* L. nothosubsp. *stellarcticus* G. Larsson). The virus in arctic bramble did not always induce foliar symptoms; however, yellowing of the leaves around central and lateral veins was observed.

*Raspberry bushy dwarf virus* has not been reported to occur in the field in eastern North American blackberry cultivars, but it sometimes occurs in Pacific Northwest blackberry cultivars. In the U.S., it is common in some blackberry-raspberry hybrids, such as the cultivar Boysen in California. This virus is the major cause of disease in New Zealand and is common in Europe (Converse, 1991).

*Raspberry bushy dwarf virus* is symptomless in many North American red raspberry cultivars (Converse, 1991). In some Pacific Northwest red raspberry cultivars, the virus may cause foliar ring and line patterns resembling those caused by several nepoviruses. In other cases it causes interveinal chlorosis, which produces a condition known as yellows (Converse, 1991). According to Converse (1987) mixed infections of RBDV with other viruses may cause more severe damage to red raspberry cultivars than single infections. The name of the virus is misleading, as plants are not dwarfed or bushy. The bushy dwarf name originally came from a plant that was infected with multiple viruses. In combination with *Black raspberry necrosis virus* (BRNV), RBDV causes dwarfing and shoot proliferation in red raspberry, a typical bushy dwarf condition. Although pollen-borne, RBDV does not cause pollen abortion but does cause drupelet abortion which leads to crumbly fruit in some red raspberry cultivars (Converse, 1991).

Most Pacific Northwest trailing blackberry cultivars and their hybrids that were studied in Scotland were symptomless when infected with RBDV, except the cultivar Marion, which developed yellows (Jones et al., 1982). Blackberry-raspberry hybrids, such as 'Boysen', appear to be symptomless when naturally infected with RBDV in the U.S. (Converse, 1984). A discrepancy occurs in the symptoms and damage caused by RBDV in the blackberry-raspberry hybrid 'Logan' in the United Kingdom and in the United States. In the United Kingdom, highly significant reductions occurred in fruit yield (36%) and cane weight (34%) in 'Logan' infected with RBDV. The disease is called Loganberry Degeneration (Converse, 1991). In a British Columbia field study, the red raspberry cultivars Canby and Meeker showed significant reductions in cane height (22%), cane diameter (14%)

and fruit yield (72%) compared to uninoculated controls (Converse, 1991). In a field study in Oregon on the black raspberry cultivar Munger, RBDV-infected plants had a significant loss of vigor (a 38% decrease in the number of primocanes and a 34% decrease in the weight of floricanes) compared to uninoculated controls, but there were no significant differences in fruit yield (Converse, 1991).

Finn and Martin (1996) studied the distribution of RBDV in clonal and seed propagules of *R. ursinus* and seeds of *R. leucodermis* throughout the Pacific Northwest (Oregon, Washington and British Columbia). None of these samples tested positive for RBDV. Martin (1998) found RBDV widespread and with a high incidence in raspberry in the Fraser Valley. However, fields that did not have RBDV in this region were less than two years old.

In the Czech Republic, two serologically indistinguishable strains of RBDV have been described, RB and S strains, that infect susceptible raspberry and blackberry and causes significant yield loss, cane vigor decline and druplet abortion (Spak and Kubelkova, 1999).

Symptoms of RBDV on raspberry are not diagnostic, as crumbly fruit can be caused by a variety of different stresses such as root rot, poor nutrition or poor pollination. Therefore reliable detection measures must be used to identify the disease. The easiest and quickest method for detecting RBDV is by using ELISA (Enzyme-Linked Immunosorbent Assay). Another detection method is to inoculate indicator plants such as *Chenopodium amaranticolor* Coste and Reynier and observing chlorotic leaf spot and mottle symptoms to verify RBDV infection. This test will differentiate RBDV infection in *Rubus* spp. from infection by other viruses (Kaufman, 2000).

*Raspberry bushy dwarf virus* is a seed- and pollen-borne virus that only spreads during bloom. The virus is found inside pollen grains. Raspberry is normally pollinated with insects so it is likely that insects spread the virus-infested pollen (Converse, 1987). It is not clear if the infection of bushes occurs via the pollination process or if there is a flower-visiting insect (other than bees) that transmits the virus (MacDonald et al., 1998). Pollen transmission can result in virus transmission through the seed (vertical transmission, one generation to the next). This could occur if the virus is present in the pollen tube and makes its way into the ovary. This means that when a raspberry flower is pollinated with RBDV infected pollen both embryo and mother plant can become infected. The exact mechanism by which RBDV infects the seed and mother plant is unknown. However, transmission to the maternal tissue and infection of the mother plant (horizontal transmission, transmission within a generation or through a field) is relatively rare with plant viruses. One proposed mechanism for horizontal transmission is that thrips fed on pollen, which had a very high titer of virus and then fed on maternal tissues and transmitted the virus (Sdoodee and Teakle, 1987). Pollen on the petals of a flower or on a leaf could serve as a source of virus for thrips transmission. This type of transmission has been shown for *Tobacco streak virus*. Another possibility is that as the pollen tube grows down the style, virus is transmitted to the maternal tissue of the flower and then spreads systemically through the plant. There are many possible factors that could affect transmission including temperature and humidity levels, insect vectors and spread from native vegetation. Studies have shown that native *Rubus* species such as thimbleberry (*R. parviflorus*) can be naturally infected with RBDV. This species is commonly found growing around raspberry fields in the Pacific Northwest

and could provide a source of RBDV inoculum. Mechanical transmission from plant to plant in the field is very unlikely, as it is very difficult to inoculate raspberry seedlings by this means in the greenhouse; also spread is radially from a point rather than down a row (Finn and Martin, 1996).

The RBDV particles are quasi-isometric, 33 nm in diameter. Total genome size 8.6 kb. The virus has three species of single-stranded RNA of 5.4 kb, 2.2 kb and 1 kb respectively. (Converse, 1991; Murphy et al., 1995).

### ***Tobacco streak virus***

*Tobacco streak virus* (TSV) is found in North America and has a broad host range including many species of *Rubus*, both in *Eubatus* and *Idaeobatus* (Converse, 1984). The *Rubus* strain of TSV (TSV-R) was first reported in California in 1966 in several blackberry and blackberry-raspberry cultivars (Converse, 1991). It is probably endemic and may be indigenous in wild Pacific Northwest trailing blackberry where it is latent (Brunt and Stacey-Smith, 1976). The major U.S. Pacific Northwest blackberry cultivars are complex hybrids having *R. ursinus* in their pedigrees and often are naturally infected, without symptoms, but with a decrease in plant vigor (Brunt and Stacey-Smith, 1976). *Rubus discolor* and *R. laciniatus* Willd. were not known to be naturally infected (Brunt and Stacey-Smith, 1976; Converse and Bartlett, 1979). Susceptibilities of wild and cultivated blackberries in the central and eastern U.S. have not yet been investigated; however, TSV-R has been found in red and black raspberries in the eastern U.S. (Converse, 1972; Peterson and Corbett, 1980). Its wide host range makes it likely that it will occur in blackberries in the central and eastern U.S.

No *Rubus* spp. had been found that are graft-immune to TSV-R (Converse, 1991). There are several strains of TSV. Strains of TSV have been transmitted in Brazil between herbaceous plants by thrips (*Frankliniella* spp.). Detection is by sap transmission using favorable buffers to suitable indicators like *Chenopodium quinoa* (Converse, 1984; Converse, 1991).

Natural infections of TSV-R in *Rubus* are usually symptomless. Some *Rubus* hosts, such as *R. henryi* Hemsl. & Kuntze, *R. phoenicolasius* Maxim., *R. procerus*, the blackberry-raspberry hybrid cultivar Logan and the Pacific Northwest trailing blackberry cultivars Olallie and Marion, exhibit hypersensitive necrotic reactions at the graft union when graft-inoculated with TSV-R (Converse, 1991). Other *Rubus* spp., such as red and black raspberry and the blackberry raspberry hybrid cultivar Boysen, remain symptomless when graft-inoculated with this virus (Converse, 1991).

Finn and Martin (1996) studied the distribution of TSV in clonal and seed propagules of *Rubus ursinus* and seeds of *R. leucodermis* throughout the Pacific Northwest (Oregon, Washington, and British Columbia). Using ELISA, none of *R. leucodermis* samples tested positive for TSV. However, samples of *R. ursinus* from 77% of the locations surveyed and 37% of the seedlings obtained tested positive for TSV. Martin (1998) did not find TSV in commercial red raspberry fields in the Fraser Valley and only eight of forty nine commercial fields were found sporadically infected with TSV in southern Washington and Oregon.

*Tobacco streak virus* (TSV-R) is pollen and seed-transmitted in *Rubus* and probably enters into breeders' crosses by these means. Some strains of TSV are known to be vectored by thrips (*Frankliniella occidentalis* (Pergande) and *Thrips tabaci* Lindeman). Although

TSV-R is strongly but not totally flower-associated in black raspberry fields, there are no published data on the ability of thrips to transmit TSV-R, with or without the involvement of viruliferous *Rubus* pollen. The insect vector involved in TSV-R spread in the field in *Rubus* is unknown (Converse, 1991).

*Tobacco streak virus* belongs to the family *Bromoviridae*, and the genus *Ilarvirus*, which is a group of ssRNA viruses with a tripartite genome. Particles of the genus have quasi-isometric shapes varying from roughly spherical to bacilliform. Total genome size 7.915 kb. The genome consists of three ssRNAs of 2.94 kb, 2.77 kb and 2.205 kb respectively, all ssRNAs are required for infection. (Converse, 1991; Hull, 2002; Murphy et al., 1995).

### ***Tomato spotted wilt virus***

*Tomato spotted wilt virus* (TSWV) belong to the family *Bunyaviridae* and the genus *Tospovirus*. Tospoviruses particles are quasispherical and enveloped by a membrane composed of lipid and protein (Daughtrey et al., 1995).

*Tomato spotted wilt virus* has a wide host range with more than 925 species infected in more than 70 families (Hull, 2002). The host range includes vegetable crops such as tomato, potato (*Solanum tuberosum* L.), pepper (*Capsicum* sp.), celery (*Apium graveolens* L.), eggplant (*Solanum melongenea* L.), many legumes, lettuce (*Lactuca sativa* L.), annual and perennial ornamentals and weeds (Jones et al., 1991). *Tomato spotted wilt virus* became a major problem in commercial vegetable and flower production in the U.S. and Europe in the late 1980s. Although the virus was known to occur in many plants, it was not widely

distributed. Thrips are the vectors of Tospoviruses. The Western flower thrips (*Frankliniella occidentalis* Pergande) is the most important TSWV vector (Daughtrey et al., 1997). In 1986-87, major outbreaks of Western flower thrips occurred in fields across the U.S. from the western to the eastern states. The thrips pest also became widespread in greenhouses, and the insect was identified in areas of eastern Canada where it was previously unknown. At the same time, the insect was first observed in Europe where it quickly became established. Transport of the virus and vector has apparently occurred on propagation stock as well as finished products. One reason for this rapid spread probably relates to the difficulty of controlling thrips that are often concealed in transported flowers (Lawson and Hsu, 1995).

Acquisition of the viruses by thrips occurs when the insects feed on infected plants in the larval stage (German et al., 1992). The infection cycle is initiated only when the female adult thrips lays eggs on *Tospovirus*-infected leaves that are suitable host for egg and larval development. *Tomato spotted wilt virus* is likely to be acquired after 30 min of larval feeding, but acquisition may occur throughout larval development. Tospoviruses are retained by thrips through molting and pupation and are transferred to the adult insect. Adult thrips may inoculate plants with TSWV soon after emergence, or several days may be required for the insects to initiate transmission (Lawson and Hsu, 1995).

In addition to *F. occidentalis*, TSWV is transmitted by *F. schultzei* (Trybom), blossom thrips; *Thrips tabaci* (Lindeman), onion thrips; and *F. fusca* (Hinds), the tobacco thrips (Lawson & Hsu, 1995); *F. intosa* (Trybom), the flower thrips; and *T. palmi* Karny, the melon thrips (Daughtrey et al., 1997).

*Tomato spotted wilt virus* differs in its protein composition from viruses of any other taxonomic group. There are four structural proteins contained in the viral particle: two glycoproteins (G1 and G2) located in the membrane, a nucleoprotein (N protein) which surrounds the RNA, and an enzyme which synthesizes new RNA. The genetic information is contained in three single-stranded RNA molecules (Daughtrey et al., 1995; Jones et al., 1991; Porter et al., 1984).

In 1989, a serologically distinct TSWV strain was reported from *Impatiens* and designated TSWV-I to distinguish it from the common or lettuce strain TSWV-L (Daughtrey et al., 1995). *Tomato spotted wilt virus*-I isolates have frequently been detected in a wide variety of flower crops throughout the U.S. *Tomato spotted wilt virus*-I shared many characteristics with TSWV, such as symptomatology and possession of three ssRNA species (L, M and S of 8.3 kb, 5.2 kb, 3.4kb, respectively) and three structural proteins (G1, G2 and N of 78K, 52K and 28K respectively (Law and Moyer, 1990). The TSWV-I G1 and G2 glycoproteins were serologically related to the respective proteins of TSWV, but the TSWV-I nucleocapsid or N protein was serologically unrelated to that of TSWV (Law and Moyer, 1990).

### ***Impatiens necrotic spot virus***

*Impatiens necrotic spot virus* (INSV) is a species of the *Tospovirus* genus (Law and Moyer, 1990) and was at one time classified as a strain of TSWV. Classification of INSV is further complicated by the occurrence of variants derived from the virus that are no longer serologically related to the original virus from which they were derived (Lawson et al.,

1993). The apparent plasticity of INSV may be reflected in the highly diverse host range of the virus that reportedly infects many different ornamental plants (Hausbeck et al., 1992; Lawson and Hsu, 1995).

*Impatiens necrotic spot virus* is a serious threat to a wide range of North American greenhouse flower crops; more than 300 plant species from 50 plant families are susceptible to INSV. Some ornamental plants susceptible to INSV include *Impatiens wallerana* Hook, *Cyclamen persicum* Mill, *Sinningia speciosa* (Lodd.) Hiern and *Exacum affine* Balf. Flowering annuals in the greenhouse are more often infected with INSV than TSWV (Daughtrey et al., 1995; Daughtrey et al., 1997; Windham et al., 1998).

According to Daughtrey et al. (1997), INSV is very labile. Temperature has significant effect on symptom expression of INSV in naturally infected seed-produced New Guinea impatiens (*Impatiens* hybrids). In general, high light above 300  $\mu\text{M m}^{-2}\text{s}^{-1}$  PPF and high temperatures above 27° C will suppress symptom development. In a greenhouse, symptoms are often more easily observed in plants growing during the winter months (Lawson and Hsu, 1995).

*Impatiens necrotic spot virus* and TSWV are characterized by RNA-containing isometric particles about 70-90 nm in diameter. The particles have a lipoprotein envelope, which can be removed by nonionic detergents, leaving a roughly spherical core of 60 nm in diameter. The two Tospoviruses, TSWV and INSV, although distinguished by serologically distinct N protein, cause similar symptoms and have overlapping host ranges (Daughtrey et al., 1995; Jones et al., 1991; Porter et al., 1984).

*Impatiens necrotic spot virus* and TSWV cause an extraordinarily broad range of symptoms on many plants, with symptoms ranging from subtle to severe. Possible symptoms include stunting, necrotic spotting, chlorotic spotting, areas of black or brown stem necrosis, ringspots, mosaic, line patterns and vein necrosis. Plants infected when young are the most severely affected. Complete necrosis and collapse of seedlings has been observed, especially in the case of young gloxinias (*Sinningia speciosa* (Lodd.) Hiern) infected with INSV. Extensive foliar necrosis can be also observed, which can be misattributed to chemical injury or bacterial or fungal pathogens. Plant hosts may exhibit several symptom types, showing variation from cultivar to cultivar and from plant to plant. Latent (symptomless) infections are common in certain cultivars of susceptible crops (Daughtrey et al., 1995; Daughtrey et al., 1997).

Mechanism of transmission of *Tomato ringspot virus*, *Tobacco ringspot virus*, *Tobacco streak virus*, *Raspberry bushy dwarf virus*, *Impatiens necrotic spot virus* and *Tomato spotted wilt virus*.

| <b>Virus</b>                         | <b>Acronym</b> | <b>Mechanism of transmission</b>                                                                                                                                                                                                                                                          |
|--------------------------------------|----------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Tomato ringspot virus</i>         | ToRSV          | Pollen<br>Seed<br>Nematodes<br>- <i>Xiphinema americanum</i>                                                                                                                                                                                                                              |
| <i>Tobacco ringspot virus</i>        | TRSV           | Pollen<br>Seed<br>Insects (species unknown)<br>Nematodes<br>- <i>Xiphinema americanum</i>                                                                                                                                                                                                 |
| <i>Tobacco streak virus</i>          | TSV            | Pollen<br>Seed                                                                                                                                                                                                                                                                            |
| <i>Raspberry bushy dwarf virus</i>   | RBDV           | Pollen<br>Seed                                                                                                                                                                                                                                                                            |
| <i>Impatiens necrotic spot virus</i> | INSV           | Insects<br>- <i>Frankliniella occidentalis</i> , the Western flower thrips)                                                                                                                                                                                                               |
| <i>Tomato spotted wilt virus</i>     | TSWV           | Insects<br>- <i>Frankliniella occidentalis</i> , the Western flower thrips<br>- <i>F. schultzei</i> , blossom thrips<br>- <i>Thrips tabaci</i> , onion thrips<br>- <i>F. fusca</i> , the tobacco thrips<br>- <i>F. intosa</i> , the flower thrips<br>- <i>T. palmi</i> , the melon thrips |

## Diagnosis

The early and accurate diagnosis of plant diseases is a crucial component of any crop-management system. Once the cause of a disease has been established as a virus, a series of tests is necessary to determine its identity.

Diagnosis of plant viruses can be difficult when one is dealing with unstable viruses, unusual strains, or viruses in woody plants. A diagnostic method useful for one virus in a given host may not be reliable for other viruses or for the same virus in a different host. Symptoms are of major importance because they are the main means by which a virus disease is diagnosed; although, positive identification of a virus or virus complex cannot be based on foliar symptoms alone. Greenhouse and laboratory tests using specific scientific techniques are required for positive identification of viruses.

Tests that may be utilized in diagnosing the viruses surveyed in this research are described as follows.

### **Biological tests**

Assay of infectivity is an essential aspect of plant virology. Since infectivity of a virus depends on the susceptibility of the host plant, the same basic procedure may simultaneously measure the infectivity of the virus and the susceptibility of the host (Gera et al., 1995).

Experimental host plants, under standardized conditions, will produce consistent and distinguishing disease symptoms when infected with a particular virus. Determining whether infection occurs and if it is local or systemic remains a simple and useful tool in plant virology. This procedure is usually essential for studying new viruses or virus strains. Sap transmission of many viruses by leaf rubbing is a valuable method for virus detection and disease diagnosis. In general, viruses transmitted in a persistent manner by vectors are transmitted poorly or not at all by mechanical inoculation (Gera et al., 1995).

Several herbaceous plants are susceptible to a large number of viruses. Some plant

species react to mechanical inoculation with several viruses by producing local lesions that are useful in quantitative virus assay. Indicator hosts react diagnostically with certain viruses, whereas plants that can distinguish between certain viruses are called differential hosts (Gera et al., 1995).

Biological techniques for virus diagnosis and detection are usually highly accurate but too slow and not amenable to large-scale application (Gera et al., 1995).

## **Serology**

Serological procedures are based on the interaction between a protein or proteins (termed the antigen) in the pathogen with antibodies raised against them in a vertebrate (Hull, 2002).

Antibodies are animal host proteins produced in response to the presence of foreign molecules in the body. They are synthesized primarily by  $\beta$ -lymphocytes. They belong to a large family of glycoproteins that share key structural and functional features. Structurally, antibodies are composed of one or more copies of a characteristic unit forming a Y-shape. Each unit contains four polypeptides: two identical copies of the heavy chain and two identical copies of the light chain joined by disulfide bonds. Functionally, they can be characterized by their ability to bind to specific antigens. Antibodies are divided into five classes of immunoglobulins known as IgG, IgM, IgA, IgE and IgD, based on the number of Y-like units and the type of heavy-chain polypeptide they contain. The first two classes are relevant to plant virology. IgG molecules have three protein domains. Two of the domains are identical (approx. 55 kD) and form the arms of the Y, and are termed the Fab domain; each arm contains an antigen-binding site at the end, making the IgG molecule bivalent. The

third domain, the Fc domain, composed of two identical light chains (approx. 25 kD), forms the base of the Y; IgM antibodies are pentamers of the Y-shaped units (Gera et al., 1995; Hull, 2002).

Most plant viruses are strong immunogens. When they are injected into an experimental animal, they stimulate the production of specific antibodies that can be used in various serological tests. Under suitable conditions the reactions between antibodies and the antigen can be demonstrated in vitro. Visible precipitates are formed as a result of the reaction between the bivalent antibodies and the multivalent viral-antigens. A prerequisite for the formation of precipitates is that both antibodies and antigen are present within a certain range of concentrations. Precipitation will not occur if one of the reactants is not present in the optimal concentration (Gera et al., 1995).

Any molecule that can bind to an antibody is known as an antigen. Antigens are usually fairly large molecules or particles consisting of protein or polysaccharides that are foreign to the vertebrate species into which they are introduced. Most have a molecular weight greater than 10 kDa, although smaller peptides can elicit antibody production (Hull, 2002).

There are two aspects to the activity of an antigen. First, the antigen can stimulate the animal to produce antibody proteins that will react specifically with the antigen; this aspect is known as the immunogenicity of the antigen. Second, the antigen must be able to combine with the specific antibody produced. This is generally referred to as the antigenicity of the molecule (Gera et al., 1995; Hull, 2002).

There are two basic types of antisera: polyclonal, which contain antibodies to all the available epitopes (sites on the surface of an antigen molecule to which a single antibody

molecule binds) on the antigen, and monoclonal, which contain antibodies to one epitope. There is much discussion as to which is the best for diagnosis, but this will depend on what question the diagnostician is addressing. Monoclonal antisera are much more specific than polyclonal antisera and can be used to differentiate strains of many pathogens. On the other hand, specificity can be a disadvantage and a variant of the pathogen may not be detected (Hull, 2002).

### **Enzyme-Linked Immunosorbent Assay (ELISA)**

The Enzyme-Linked Immunosorbent Assay (ELISA) is the most widely used method for virus diagnosis because it is simple and practical.

Since 1971, enzyme-amplified immunoassays have been developed to enhance the detectability of antigen-antibody reactions. In 1977, Clark and Adams showed that the microplate method of ELISA could be very effectively applied to the detection and assay of plant viruses. The most commonly applied immunoassay is the enzyme-linked immunosorbent assay (ELISA), in which the antigen-antibody complexes are adsorbed to wells in plastic microtitre plates. The most important advantages of ELISA over other serological tests for plant viruses are: very low concentrations of virus (1-10 ng/ml) can be detected; only small amounts of antibodies are required; the test can be applied to both crude virus preparations as well as purified virus suspensions; the test is suitable for large-scale testing of samples; the test can be standardized using kits and quantitative measurements are possible (Dijkstra & Jager, 1998; Hull, 2002).

In the last decade different variations of ELISA have become the preferred serological test for plant viruses because of their simplicity, adaptability, rapidity, sensitivity and accuracy. If the enzyme is linked covalently to the antiviral immunoglobulin, the test is classified as a type of direct ELISA. If the enzyme is linked to a molecule, which detects the antiviral immunoglobulin, the test is classified as a type of indirect ELISA. Many other serological tests rely on evaluation of a visible antigen-antibody precipitate. ELISA, on the other hand, registers the occurrence of antigen-antibody complexes by rapid enzymatic development of a distinctly colored product. This is accomplished by successive “layers” of reagents culminating in enzymatic hydrolysis of enzyme substrate, converting a colorless substance to a pigment (Converse and Martin, 1990).

### **1) Direct double-antibody sandwich method (DAS-ELISA)**

The method has been adapted for plant viruses by Clark and Adams (1977) and is carried out in wells of polystyrene microtitre plates. The wells are first coated with the antibody-containing immunoglobulin fraction of antiserum to the virus to be assayed (primary antibodies). After washing the wells, the virus samples are added, and after one more washing, primary antibodies labeled with an enzyme (conjugate) are added. Following another washing, enzyme substrate is added, yielding a colored product (Dijkstra & Jager, 1998). DAS-ELISA is especially useful for detecting antigens in complex mixtures. This is because the bound antibody specifically traps the antigens of interest, whereas non-specific antigens are removed in the washing step. This method suffers two limitations. First, it may be very strain specific, and for discrimination between virus strains, this can be a useful feature. However, for routine diagnostic tests it means that different viral serotypes may

escape detection. This high specificity is almost certainly due to the fact that the coupling of the enzyme to the antibody interferes with weaker combining reactions with strains that are not closely related. Second, this procedure requires a different antiviral enzyme-antibody complex to be prepared for each virus to be tested (Hull, 2002).

## **2) Indirect double-antibody sandwich method**

In the indirect procedure, the enzyme used in the final detection and assay step is conjugated to an anti-globulin antibody. For example, if the virus antibody were raised in a rabbit, a chicken anti-rabbit globulin might be used. Thus, one conjugated globulin preparation can be used to assay bound-rabbit antibody for a range of viruses. Furthermore, indirect methods detect a broader range of related viruses with a single antiserum (Hull, 2002). Although the indirect-ELISA is considered less strain-specific, the test is simple to perform (Gera et al., 1995).

## **Electron Microscopy**

The shape, size and any surface features of the virus particle are basic requirements for virus identification. Electron microscopy is commonly used to examine purified preparations of virus as well as crude extracts from infected plants. The development of the negative staining technique allowed simple, rapid and direct examination of crude extracts of infected plants to describe these features. Crude sap preparation may be made by grinding or chopping infected leaf, stem or root tissue in a buffer or directly in a negative stain. Alternatively, a fragment of epidermis can be stripped from the leaf and passed through a drop of water or stain on a specimen grid. Electron microscopy can provide information on

virus morphology within minutes. Approximate particle dimensions can also be determined. Measured particle size may depend on how the specimen is prepared and stained. Phosphotungstic acid, uranyl acetate, and ammonium molybdate are the most common stains used. These stains may also penetrate into the particles revealing the internal structure (Hull, 2002).

## **Molecular tests**

### **Polymerase chain reaction (PCR)**

Polymerase chain reaction (PCR) allows the amplification of very low concentration of specific target sequences of DNA and RNA. A prerequisite for the use of PCR for virus detection is the availability of sequence data on the viral genome. The technique involves the hybridization of synthetic complementary oligonucleotide primers to the target sequence and synthesis of multiple copies of cDNA of the sequence between the primers using heat-stable DNA polymerase. The process goes through a series of amplification cycles, each consisting of melting the double-stranded template DNA molecules in the presence of the oligonucleotide primers and the four deoxyribonucleotide triphosphates at high temperature (melting), hybridization of the primers with the complementary sequences in the template DNA's at lower temperature (annealing) and extension of the primers with DNA polymerase (DNA synthesis). During each cycle, the sequence between the primers is doubled so that after  $n$  cycles a  $2^n$  amplification should be obtained. Usually the reaction is of 30 to 50 cycles (Hull, 2002).

The selection of primers depends on the target sequence and it is absolutely essential that the 3' nucleotide is complementary to the desired nucleotide on the target sequence.

Since PCR is based on DNA, it is not directly applicable to most plant viruses that have RNA genomes. However, cDNA can be made to the desired region of the RNA genome using a primer and reverse transcriptase, and this used as the initial template. This procedure is now widely used and is called RT-PCR (Gera et al., 1995; Hull, 2002).

The specificity of the method depends on the choice of the synthetic oligonucleotide primers derived from the sequence of the target DNA. The use of highly conserved sequences for primer design is recommended for detecting all individuals of a particular virus. Alternatively, degenerate primers derived from a conserved amino acid sequence of viral coat protein may be used for the detection of all members of a virus group (Gera et al., 1995).

Polymerase chain reaction was used successfully to detect very low amount of poty-, nepo-, and luteoviruses and viroids. The method was also used for the detection of viruses in ornamental crops (Gera et al., 1995).

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## **Chapter 1**

# **Virus Identification, Geographical and Spatial Distribution and Symptom Expression in Blackberry (*Rubus* spp.) Cultivars in the Southeastern United States**

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**(In the format appropriate for submission to HortScience)**

Virus Identification, Geographical and Spatial Distribution and Symptom Expression in  
Blackberry (*Rubus* spp.) Cultivars in the Southeastern United States

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**Virus Identification, Geographical and Spatial Distribution and Symptom Expression in Blackberry (*Rubus* spp.) Cultivars in the Southeastern United States**

*Additional index words.* *Impatiens necrotic spot virus*, *Tobacco ringspot virus*, *Tomato ringspot virus*, *Tobacco streak virus*, *Tomato spotted wilt virus*, *Raspberry bushy dwarf virus*, Enzyme-linked Immunosorbent Assay, primocanes, floricanes, woody perennial, cultivars.

**Abstract.** Reports of virus diseases and virus-like symptoms have increased in blackberry cultivars in the southeastern U.S. over the past 5 years. However, the identity, distribution and symptom expression of these blackberry viruses were in most instances unknown. A survey was conducted at eight grower locations and at two research stations in North Carolina, South Carolina and Virginia in 2001 and 2002 to determine the presence and distribution of six viruses. Four viruses known to occur in *Rubus*: *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Tobacco streak virus* (TSV) and *Raspberry bushy dwarf virus* (RBDV). Two viruses not reported to occur in woody perennial plants but that were included in this study were *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) because of recent discoveries of these viruses in horticultural crops in this or other regions of the United States. Samples for testing collected from plants with virus-like symptoms included: 1) symptomatic plants (floricane leaves, primocane leaves, roots and seed), and 2) asymptomatic plants (primocane and floricane leaves). Blackberry cultivars sampled were Apache, Arapaho, Black Satin, Chester, Chickasaw, Choctaw, Kiowa,

Lochness, Navaho, Shawnee, Rosborough and Triple Crown. Samples were tested by enzyme-linked immunosorbent assay (ELISA) using kits obtained from Agdia, Inc., Elkhart, IN. *Tobacco ringspot virus*, ToRSV and INSV were the most prevalent viruses in the survey. *Tobacco ringspot virus* and ToRSV were detected in 8/10 locations, while INSV was detected in 8/8 locations. *Tobacco ringspot virus* was detected in 280/572 samples, ToRSV in 235/520 samples and INSV in 137/414 samples. *Raspberry bushy dwarf virus* was detected in only 4/362 samples in 3/10 locations, and all three locations were in North Carolina. *Tobacco streak virus* and TSWV were not detected in any of the samples tested. In general the ability to detect viruses in different plant parts varied. However, TRSV was readily detected at a high titer, than other viruses in most plant parts collected. *Tomato ringspot virus* and INSV were more easily detected in root samples. *Raspberry bushy dwarf virus* was detected in asymptomatic florican leaves and primocane leaves, as well as in roots and seed samples. Differences in virus incidence varied among cultivars. Cultivars with more than a 50% incidence for TRSV and ToRSV included Apache, Arapaho, Chester and Rosborough. Furthermore, ToRSV was detected in five additional cultivars with an incidence greater than 50% including Navaho, Shawnee, Choctaw, Kiowa and Triple Crown. *Impatiens necrotic spot virus* was detected in 'Apache', 'Arapaho', 'Chickasaw', 'Navaho' and 'Kiowa'. *Raspberry bushy dwarf virus* was found in 'Apache', 'Arapaho', and 'Kiowa'. Multiple infections were observed in most of the plants tested. Symptoms in multiple infections included mosaic, ringspots, necrosis, chlorotic line patterns, vein mosaic, shortened internodes, chlorosis of the mid ribs, yellow patterns, vein banding, oak leaf pattern and small and distorted leaves. Four of six viruses assayed during the survey were present in blackberries in the southeastern U.S. and one new *Crinivirus* detected by

Tzanetakis et al. (2003) lead us to suspect that more viruses might be present in the field as mix infections that are causing the fast declining in young plantings. Because many of these viruses were not seen in blackberry production areas in the region previously, their impact may be in the early stages and could increase in incidence, distribution and virulence rapidly.

Commercial nurseries were not included in this survey but it is likely that they were the major source of infected plants in the newer plantings. It is necessary to implement certification programs to ensure that the plants they propagate are free from known viruses and control of nematodes and insect vectors in the field is required.

Viruses have deleterious effects on many members of the *Rosaceae* family. In the genus *Rubus*, which contains blackberries, raspberries and related hybrids, 33 viruses and virus-like diseases have been reported worldwide (Ellis et al., 1991; Martin, 2002). Fifteen viruses are known to occur in wild and cultivated *Rubus* species in North America, and some of them are quite serious, causing severe plant decline and yield reduction (Brunt and Stace-Smith, 1976; Converse, 1987; Converse, 1991; Jennings, 1988).

Until recently virus characterization has been conducted mainly on raspberries in the western U.S. due to raspberries favored economic status and large concentrated production area (Converse, 1984). However, concurrent with a surge in blackberry production in the eastern U.S., reports of viruses or virus-like diseases in blackberry are increasing. Clark (2000) reported that virus and virus-like symptoms were observed in the blackberry cultivars Arapaho and Kiowa in Arkansas. In North Carolina, routine field visits by North Carolina State University (NCSU) Extension personnel in 2000 revealed an increase in the number of plants with virus-like symptoms and a concurrent decline in berry yield and quality in several blackberry cultivars (G. Fernández, personal communication).

According to Martin (2002) the most common viruses that currently cause problems in blackberry were *Raspberry bushy dwarf virus* (RBDV), *Tomato ringspot virus* (ToRSV) and *Tobacco ringspot virus* (TRSV). However, most of this knowledge was based on work conducted in the Pacific Northwest. Blackberry virology in the eastern U.S. is still in its early descriptive phase. For our present work, we identified a group of suspect viruses based on previous reports in the crop as well as the recent identification of viruses that are new or prevalent in other crops in the southeastern U.S. These included two nepoviruses: ToRSV

and TRSV; two seed and pollen-borne virus, RBDV and *Tobacco streak virus* (TSV), and two thrips transmitted tospoviruses not reported in *Rubus* previously: *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV).

Knowledge of viruses that infect blackberry, and their epidemiology and control, are important when considering establishment of blackberry plantings in distinct geographic production areas. Therefore we set out to identify, map out the distribution, and determine the symptom expression of six viruses of blackberry as a means to abate any potential viral epidemics. The specific objectives of this study were to: 1) identify viruses and their geographical distribution in blackberry cultivars commonly grown in the three southeastern states, 2) assess incidence in symptomatic and asymptomatic plants, 3) determine spatial distribution of virus in blackberry plant parts and 4) characterize symptom expression of single and multiple virus infection in selected blackberry cultivars.

## **Materials and Methods**

*Collection sites and sampling.* Eight grower locations and two research stations were selected in Virginia, North Carolina and South Carolina to determine the presence of TRSV, ToRSV, TSV, RBDV, INSV and TSWV on blackberry cultivars. These locations were distributed between the Mountain, Piedmont and Coastal Plain regions of the three states. The age of the plantings ranged from 2 to 18 years.

Blackberry cultivars predominant at the collection sites were ‘Apache’, ‘Arapaho’, ‘Black Satin’, ‘Chester’, ‘Chickasaw’, ‘Choctaw’, ‘Kiowa’, ‘Lochness’, ‘Navaho’, ‘Rosborough’, ‘Shawnee’ and ‘Triple Crown’. Plants showing virus-like symptoms were

selected and marked at each location using colored flags. Leaflets were collected from (1) primocanes and/or floricanes from symptomatic blackberry plants (2001 and 2002), and (2) primocanes and floricanes from asymptomatic blackberry plants (Collected in 2002 from symptomatic plants selected the previous year). Roots and fruit were also collected from symptomatic blackberry plants in 2002. Samples were placed in plastic storage bags, labeled, placed in a cooler and brought back to the virology lab at NCSU, where they were stored at 4°C until they were ready for testing, which was usually the next day.

Seed samples were obtained by mixing each fresh fruit sample in a blender at intervals of 30 sec several times. Next, the seed was collected on cheesecloth and washed several times with tap water until all the pulp was washed off of the surface of the seed; seed was then surface-sterilized using 0.52% w/v sodium hypochlorite (10% v/v Clorox bleach) for 5 min and rinsed three times with distilled water. Finally, seeds were dried for 24 hr at room temperature, placed in coin envelopes, labeled and stored in a zip lock bag at 4° C until the ELISA was conducted.

*Sample preparation.* Samples from symptomatic and asymptomatic plants consisted of leaflets from floricanes and primocanes and also root samples from these plants were weighed (0.5 to 1 g of tissue per sample) and homogenized. A roller grinder (Plant Disease and Insect Clinic, NCSU) was used to grind tissue samples using a blueberry extraction buffer (Agdia, Inc., Elkhart, IN) at a 1:10 ratio (tissue weight: blueberry extraction buffer volume). Seeds were ground using a mortar and a pestle at a ratio of six seeds per 1000 µl of buffer. Samples were dispensed in tubes and centrifuged for 10 min at 7,000 rpm, 100 µl of the supernatant solution was collected and used for detection.

*Detection.* Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) pathoscreen kits from Agdia, Inc., Elkhart, IN, were used to detect TRSV, ToRSV, TSV, RBDV, TSWV and INSV in samples. Coated polystyrene microtitre plates were used to detect the viruses. Detection was accomplished following the manufacture's protocol. All reagents were used at 100 µl per well in microtiter plates. Plates were washed five times after each step except for blocking. Positive and negative controls were included on each plate. Positive controls consisted of purified virus supplied in the kit by the manufacturer. Negative controls were of two types; wells that contained extraction buffer and wells that contained centrifuged plant sap from virus indexed and certified as virus free tissue culture plants produced in the NCSU Micropropagation Unit, Raleigh, N.C.

*Analysis.* Absorbance of each well at 405 nm ( $A_{405}$ ) was read in an ELISA plate reader (Molecular Devices, Sunnyvale, Calif.). Leaf, root and/or seed samples were considered positive for virus if  $A_{405}$  of the sample wells were greater than three times the mean of the values obtained for negative or healthy controls (Sutula et al., 1986).  $A_{405}$  values of negative controls ranged from 0.000 to 0.027; any value greater than 0.1 was considered positive.

## **Results**

*Virus identification and geographical distribution.* Tobacco ringspot virus, ToRSV, RBDV, and INSV were detected in North Carolina, South Carolina and Virginia (Fig. 1.1), while TSV and TSWV were not detected in any location in either year of the survey.

In North Carolina TRSV and ToRSV were detected in four of five locations, RBDV in three of five locations, and INSV in all five locations surveyed (Table 1.1). In South Carolina, TRSV and ToRSV were detected in all locations (4/4), while INSV was found in two locations. *Raspberry bushy dwarf virus* was not found at any location (Table 1.1). The only virus that was detected by ELISA in Virginia was INSV (Table 1.1).

*Overall virus incidence and incidence in symptomatic and asymptomatic cultivars.* Differences in virus incidence were detected among cultivars in each state. In North Carolina TRSV was detected with a higher incidence in ‘Arapaho’ 76.2% (212/278) and ‘Apache’ 65% (13/20) followed by ‘Chickasaw’ 33.3% (11/33), ‘Navaho’ 23.8% (5/21) and ‘Kiowa’ 20.4% (19/93); while in South Carolina it was detected in ‘Rosborough’ 100% (2/2), ‘Arapaho’ 52.4% (11/21), ‘Chester’ 50% (1/2), ‘Triple Crown’ 33.3% (1/3), ‘Chickasaw’ 25% (1/4), ‘Apache’ 18% (2/11) and ‘Kiowa’ 11% (1/9).

*Tomato ringspot virus* was detected in eight cultivars in North Carolina: ‘Apache’ 18.8% (4/22), ‘Arapaho’ 56.2% (127/226), ‘Chickasaw’ 44.8% (13/29), ‘Choctaw’ 14.3% (1/7), ‘Kiowa’ 26.5% (23/87), ‘Navaho’ 60% (12/20), ‘Shawnee’ 57% (4/7) and ‘Triple Crown’ 28.6% (4/14), and in ten cultivars in South Carolina: ‘Apache’ 58.3% (7/12), ‘Arapaho’ 69.6% (16/23), ‘Chester’ 100% (2/2), ‘Chickasaw’ 50% (2/4), ‘Choctaw’ 100% (1/1), ‘Kiowa’ 88.9% (8/9), ‘Navaho’ 66.7% (2/3), ‘Rosborough’ 100% (2/2), ‘Shawnee’ 33.3% (1/3) and ‘Triple Crown’ 100% (3/3).

*Raspberry bushy dwarf virus* was detected only in North Carolina in ‘Apache’ 5% (1/20), ‘Arapaho’ 0.9% (2/204), and ‘Kiowa’ 1.5% (1/69).

*Impatiens necrotic spot virus* was detected in seven cultivars in North Carolina: ‘Apache’ 31.8% (7/22), ‘Arapaho’ 45.3% (92/203), ‘Chickasaw’ 27.6% (8/29), ‘Choctaw’ 14.3% (1/7), ‘Kiowa’ 18.8% (13/69), ‘Navaho’ 31.3% (5/16) and ‘Triple Crown’ 10% (1/10), in two cultivars in Virginia, ‘Black Satin’ 16.6% (1/6) and ‘Navaho’ 25% (1/4), and in two cultivars (Arapaho and Kiowa) in South Carolina.

There were differences in virus detection in symptomatic and asymptomatic plants (primocanes and floricanes) (Table 1.2). *Tobacco ringspot virus*, ToRSV and INSV were detected in symptomatic and asymptomatic primocane and floricane leaves, roots and seed in the North Carolina and South Carolina locations (Table 1.2) while RBDV was detected only in asymptomatic floricane and primocane leaves, roots and seeds (Table 1.2).

*Spatial distribution of viruses in plant parts.* The presence of viruses varied in plant parts tested in this study (Table 1.2). Incidence of TRSV, INSV and ToRSV was higher in roots and seeds than in either primocane or floricane leaves in North Carolina. In several locations incidence was 100% in roots and only approximately 4 to 82% in leaf tissues collected at the same time. Despite smaller sample sizes from South Carolina, virus incidence was again higher in roots than in floricane or primocane leaves. In general, primocane and floricane leaves had similar incidence of viruses in symptomatic or asymptomatic plants. In North Carolina, all four of the viruses were detected in the seeds sampled, with TRSV, ToRSV and INSV being most prevalent.

*Multiple infections and symptom expression.* Multiple virus infections were detected in blackberry samples from North Carolina and South Carolina. In both states 82 samples tested

positive for TRSV, ToRSV and INSV; 56 samples tested positive for TRSV and ToRSV; 18 samples tested positive for TRSV and INSV, 34 samples tested positive for ToRSV and INSV, 1 sample tested positive for TRSV, ToRSV and RBDV, 1 sample tested positive for TRSV, INSV and RBDV, and two samples tested positive for TRSV, ToRSV, RBDV and INSV (Table 1.3).

Symptoms observed in plants that tested positive for one or more viruses included mosaic, chlorotic line patterns, ringspots, chlorosis, vein chlorosis, leaf distortion, crumbly fruit, necrosis and oak leaf pattern. However, there were no clear patterns of virus incidence and symptom expression. For example, single infections of TRSV were associated with vein chlorosis and yellow blotches in ‘Arapaho’ (Fig. 1.2) and ringspots in ‘Kiowa’ (Fig. 1.3). Multiple infections of INSV and TRSV had yellow blotches, necrosis, vein chlorosis and oak leaf patterns in ‘Kiowa’ (Fig. 1.4). Other non-foliar symptoms included cane splitting and a lack of primocane emergence in some plants.

Plants that tested positive for three viruses (TRSV, INSV and ToRSV) displayed a wide range of symptoms in just one cultivar ‘Arapaho’. We observed crumbly fruit, oak leaf pattern, and small and distorted leaves (Fig. 1.5), vein chlorosis and ringspots (Fig. 1.6), ringspots, vein chlorosis, yellow blotches, necrotic spots and crumbly fruit (Fig. 1.7) and yellow blotches, ringspots, vein chlorosis, ringspots and crumbly fruit (Fig. 1.8).

*Tomato ringspot virus*, TRSV, TSV, RBDV, INSV and TSWV were not detected in some samples with virus and virus-like symptoms using ELISA. However, these samples that presented chlorotic line patterns were analyzed using RT-PCR (Tzanetakis et al., 2003). Ds-RNA extracted had multiple bands with the largest approximately of 9000 bp. Cloning with random primers, sequencing and a BLAST search revealed sequence similarity to the minor

coat protein of criniviruses (Tzanetakis et al., 2003). In addition to this new *Crinivirus*, another new virus that presents similarity to a *Potyvirus* was also detected in samples from the three states in our survey (R.R. Martin and R. Gergerich, personal communication).

## Discussion

This survey demonstrates the current widespread incidence of TRSV, ToRSV and INSV in blackberry cultivars at commercial grower locations and at research stations in the southeastern U.S. These viruses occurred most commonly in mixed infections, while RBDV was less prevalent. The symptoms of the mixed infections varied widely with virus(es) present and cultivar(s). This latter situation has made the identification of viruses in plants by visual means nearly impossible.

One factor that could play an important role in the incidence and detection of ToRSV and TRSV is the growth habit of blackberry plants, which have perennial roots and crowns and biennial canes. In general TRSV and ToRSV were more readily detected and in a higher titer in roots than in floricanes and primocane leaves (data not shown). Roots could be considered as a virus reservoir in infected plants and could allow for a higher titer due to this perennial nature. Further testing is needed to determine if there is a seasonal shift in the ability to detect viruses in these various plant parts.

Additionally, we experienced a low concentration of virus at times during our testing. This could be due to uneven spatial or seasonal distribution of the virus in the plant. *Rubus* species are known to have dramatic shifts in carbon and nutrient allocation to their various plant parts over a growing season which is associated with climatic conditions (Fernandez

and Pritts, 1994; Prive et al., 1997; Mohadjer et al., 1998). Virus titer could therefore vary with climactic conditions prevalent in the locations surveyed. For example, Daughtrey et al. (1997) and Lawson and Hsu (1995), found that INSV was very labile and temperature has significant effect on symptom expression of INSV in naturally infected seed-produced and New Guinea impatiens. In general, high light above  $300 \mu\text{M m}^{-2}\text{s}^{-1}$  PPF and high temperatures above  $27^{\circ}\text{C}$  will suppress symptom development. Therefore our ability to detect virus may parallel this situation. *Tomato ringspot virus* and TRSV both belong to the *Nepovirus* genus and are transmitted by dagger nematodes (*Xiphinema americanum* Cobb and *X. rivesi* Dalmasso) (Converse, 1991). The spread of these viruses and their effects on the host will depend with a variety of factors including climate, nematode population density and cultural practices such as weed control. Both viruses ToRSV and TRSV were detected in old (more than 12 years) and new plantings (less than 5 years) and populations of *X. americanum* were found to be present in soil samples collected from two locations during the survey (data not shown). It was not determined whether the infection occurred in the field or through the introduction of infected material from nurseries in other states. However, detection of TRSV and ToRSV in old and young plantings led us to suspect that the infections of these plantings had two origins: presence of viruliferous nematodes in the soil where the plantings were established and/or the introduction of infected material from regions where dagger nematodes are present and infect mother plants and propagated material. According to Converse (1987; 1991) ToRSV is the most important and widespread of the nematode-transmitted viruses affecting cultivated *Rubus* in North America. Where dagger nematodes are present in the field, ToRSV spreads along rows at a rate of approximately 2 m per year (Converse, 1991). New infections occurred next to previously

infected plants, resulting in expanding oval patches of infected plants in a field (Converse, 1991). Thus, ToRSV incidence could continue to increase as the age of the planting increases and may add to the decline of the plantings.

Prior to the present study, TRSV was not thought to be common in *Rubus* (Jennings, 1988) and knowledge of its occurrence was limited to occasional reports on blackberries close to tobacco fields in North Carolina. In 1987, Converse stated that TRSV had no economic importance because there are essentially no commercial raspberry or blackberry plantings in those areas of North America where TRSV and its nematode vector are endemic. However, this may be the case of single virus infections, which can result in little to no observable symptoms in blackberries. Multiple virus infections often result in a faster decline in infected plants, producing great losses. Several symptomless primocanes and floricanes were found positive for both TRSV and ToRSV; these results indicate that it is possible to detect ToRSV and TRSV before symptom development. Seeds tested from infected plants were 100% positive for TRSV and 68.1% for ToRSV. This confirms previous studies that stated that these two viruses are seed-transmitted (Converse, 1991). ‘Arapaho’ and ‘Apache’ seem to be the most affected cultivars by ToRSV and TRSV. This is in accordance with Clark (2000) who mentions that viral symptoms were more common in ‘Arapaho’ in Arkansas.

*Raspberry bushy dwarf virus* was detected with a low incidence (4/362) and only in North Carolina. Converse (1991) stated that RBDV has not been reported to occur in the field in eastern North American blackberry cultivars, but occurred in Pacific Northwest blackberry cultivars. Positive samples for RBDV came from asymptomatic primocanes and floricanes, roots and seed collected during the second growing season (2002) from symptomatic plants

selected the previous year. *Raspberry bushy dwarf virus* is symptomless in many Pacific Northwest cultivars (Converse, 1991), the virus may cause yellows on the cultivar Marion, the only Pacific Coast trailing blackberry known to produce symptoms (Jones et al., 1982). However, Spak and Kubelkova (1999), found no correlation between yellowing symptoms and the presence of the virus presence proven by ELISA. This low incidence could be due to RBDV was present as a latent infection and it does not produce symptoms in the cultivars that are being utilized in the southeastern region.

*Raspberry bushy dwarf virus* was detected in ‘Apache’, ‘Arapaho’ and ‘Kiowa’. The epidemiological significance of pollen-borne viruses stems from their potential for being transmitted horizontally from an infected host to a healthy host (Bulger et al., 1990). The detection of RBDV in seeds agrees with Jones et al. (1996) who indicated that in nature RBDV is transmitted to the progeny seedlings, through the ovule and among flowering plants through infected pollen; no other means of natural spread is known.

*Impatiens necrotic spot virus* was found in all locations surveyed. This virus has not been reported in woody perennial plants previously, but has been reported in a broad range of many different ornamental crops (Hausbeck et al., 1992; Lawson and Hsu, 1995). The Western flower thrips *Frankliniella occidentalis* (Pergande) is the only thrips species currently known to vector INSV (German et al., 1992; Lawson and Hsu, 1995; Daughtrey et al., 1997). The high incidence found in our results seems to be associated to the presence of populations of thrips observed within the blackberry plantings and other surrounding crops in the locations surveyed. Even though the thrips species present in blackberry fields has not been identified, the incidence of this virus could be associated to the presence of thrips in these locations and the resistance to pesticides that the vector has developed. This is the first

time to report the presence of INSV in a woody perennial plant, these results might be related to the virus-vector specificity and the persistent transmission of the virus by the thrips rather than the type of host infected. Variations in INSV detection was observed during this research. Plant that tested positive for INSV at one sampling date, tested negative in subsequent dates and visa versa. The detection of the virus may have been strongly influenced by environmental conditions as mentioned before. This variation in INSV detection would lead us to assume that the virus may be hard to detect in this and other semi-woody or woody host plants.

*Tomato spotted wilt virus* and TSV were not detected in any of the locations surveyed. *Tomato spotted wilt virus* has not been found in woody perennial plants (Hull, 2002). *Tomato spotted wilt virus* is more problematic on outdoor vegetable and field crops in areas where the virus is endemic: tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* L.), pepper (*Capsicum* sp.) and peanut (*Arachis hypogaea* L.) in particular, were dramatically affected by TSWV (Daughtrey et al., 1997). We did not detect the presence of TSV and its *Rubus* strain (TSV-R) in the locations surveyed in any of the cultivars tested. Previous reports by Brunt and Stace-Smith (1976) and Converse and Bartlett (1979) mention that this virus is probably endemic and may be indigenous in wild Pacific Northwest trailing blackberry (*R. ursinus* Cham. & Schlecht.) where it is latent. Susceptibilities of wild and cultivated blackberries in the central and eastern U.S. have not yet been investigated; however, TSV-R has been found in red and black raspberries in the eastern U.S. (Converse, 1972; Peterson and Corbett, 1980). Its wide host range makes it likely that it will occur in blackberries in the central and eastern U.S. Natural infections of TSV-R in *Rubus* are usually symptomless (Converse, 1991). However, in a survey in the Pacific Northwest, none of the

*R. leucodermis* samples tested positive for TSV and samples of *R. ursinus* from 77% of the sites where cuttings were taken and 37% of the seedling populations obtained tested positive for TSV (Finn and Martin, 1996).

Thus far no clear patterns have emerged regarding symptom expression based on virus or cultivar. Our studies have shown that at least four viruses were present in blackberry cultivars commonly planted in the region. However, our ability to visually identify symptom expression and presence of a virus remains unclear. There is speculation that one or more viruses yet to be determined may be interacting with the viruses we detected and therefore be responsible for some of the variation in symptoms we have observed (R. Gergerich, personal communication).

This survey demonstrated that four of six viruses assayed were present in blackberries in the southeastern U.S. Some have been known to occur before, and at least one (INSV) is the first time report in blackberry. Detection of one new *Crinivirus* by Tzanetakis et al. (2003) and the presence of its vector lead us to suspect that more viruses might be present in the field and mix infections of these viruses are causing the fast declining observed in young plantings. Although disconcerting, viruses cannot be solely attributed with a widespread decline in plant health or yield. However, because many of these viruses were not seen in blackberry production areas in the region previously, their impact may be in the early stages and could increase in incidence, distribution and virulence rapidly. In general, the majority of newer plantings in this survey had a higher incidence of virus than older plantings. Although commercial nurseries were not included in this survey, it is likely that they were the major source of infected plants in the newer plantings. Nursery producers should implement certification programs to ensure that the plants they propagate are free from known viruses.

Growers should use cultural practices that reduce chances of infection from nematodes and viruliferous insects. Research that will help to develop controls of these viruses will aid in the continued growth of the blackberry industry.

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Table 1.1. Incidence of *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Tobacco streak virus* (TSV), *Raspberry bushy dwarf virus* (RBDV), *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) in blackberry in ten locations in North Carolina, South Carolina and Virginia in 2001 and 2002.

| State                    | County                       | Virus tested  |                      |               |           |               |         |               |         |                 |           |
|--------------------------|------------------------------|---------------|----------------------|---------------|-----------|---------------|---------|---------------|---------|-----------------|-----------|
|                          |                              | TRSV          |                      | ToRSV         |           | TSV           |         | RBDV          |         | INSV            |           |
|                          |                              | Incidence (%) |                      | Incidence (%) |           | Incidence (%) |         | Incidence (%) |         | Incidence (%)   |           |
| North Carolina           | Pender                       | 90.7          | (78/86) <sup>z</sup> | 41.1          | (37/89)   | 0.0           | (0/85)  | 1.2           | (1/85)  | 41.1            | (37/90)   |
| North Carolina           | Cleveland                    | 2.0           | (1/50)               | 23.2          | (13/55)   | 0.0           | (0/49)  | 0.0           | (0/49)  | 2.3             | (1/43)    |
| North Carolina           | Lenoir                       | 47.6          | (89/187)             | 65.8          | (106/160) | 0.0           | (0/140) | 0.7           | (1/140) | 38.3            | (54/141)  |
| North Carolina           | New Hanover                  | 66.2          | (92/138)             | 31.1          | (32/102)  | 0.0           | (0/82)  | 2.4           | (2/82)  | 41.0            | (34/83)   |
| North Carolina           | Sampson                      | 0.0           | (0/6)                | 0.0           | (0/6)     | 0.0           | (0/6)   | 0.0           | (0/6)   | 16.7            | (1/6)     |
| North Carolina           | <i>Sub-total<sup>y</sup></i> | 55.7          | (260/467)            | 45.6          | (188/412) | 0.0           | (0/362) | 1.1           | (4/362) | 35.0            | (127/363) |
| South Carolina           | Spartanburg                  | 30.0          | (3/10)               | 100.0         | (10/10)   | 0.0           | (0/10)  | 0.0           | (0/10)  | nt <sup>x</sup> | nt        |
| South Carolina           | Charleston                   | 100.0         | (6/6)                | 100.0         | (6/6)     | 0.0           | (0/6)   | 0.0           | (0/6)   | 16.7            | (1/6)     |
| South Carolina           | Greenville                   | 33.3          | (1/3)                | 100.0         | (3/3)     | 0.0           | (0/3)   | 0.0           | (0/3)   | nt              | nt        |
| South Carolina           | Pickens                      | 20.5          | (10/44)              | 59.6          | (28/47)   | 0.0           | (0/44)  | 0.0           | (0/44)  | 28.0            | (7/25)    |
| South Carolina           | <i>Sub-total<sup>y</sup></i> | 31.7          | (20/63)              | 59.6          | (47/66)   | 0.0           | (0/63)  | 0.0           | (0/63)  | 25.8            | 8/31)     |
| Virginia                 | Nottoway                     | 0.0           | (0/42)               | 0.0           | (0/42)    | 0.0           | (0/12)  | 0.0           | (0/12)  | 10.0            | (2/20)    |
| Virginia                 | <i>Sub-total<sup>y</sup></i> | 0.0           | (0/42)               | 0.0           | (0/42)    | 0.0           | (0/12)  | 0.0           | (0/12)  | 10.0            | (2/20)    |
| <i>Total<sup>w</sup></i> |                              | 49.0          | (280/572)            | 45.2          | (235/520) | 0.0           | (0/437) | 0.9           | (4/437) | 33.1            | (137/414) |

<sup>z</sup> Positive samples/total samples tested.

<sup>y</sup> Total positive samples/total plants tested/state (2001-2002).

<sup>x</sup> nt = no sample was tested in this location for this virus.

<sup>w</sup> Total incidence and total positive samples/total plants tested in all three states.

Table 1.2. Incidence of *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Tobacco streak virus* (TSV), *Raspberry bushy dwarf virus* (RBDV), *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) on different plant parts of blackberry collected from North Carolina, South Carolina and Virginia in 2001 and 2002.

| State | Virus | Plant part tested for virus       |                       |                                    |         |                                   |         |                                    |         |                 |         |               |         |
|-------|-------|-----------------------------------|-----------------------|------------------------------------|---------|-----------------------------------|---------|------------------------------------|---------|-----------------|---------|---------------|---------|
|       |       | Symptomatic<br>Floricane leaflets |                       | Asymptomatic<br>Floricane leaflets |         | Symptomatic<br>Primocane leaflets |         | Asymptomatic<br>Primocane leaflets |         | Roots           |         | Seeds         |         |
|       |       | Incidence (%)                     |                       | Incidence (%)                      |         | Incidence (%)                     |         | Incidence (%)                      |         | Incidence (%)   |         | Incidence (%) |         |
| NC    | TRSV  | 43.3                              | (78/179) <sup>z</sup> | 34.4                               | (11/32) | 63.3                              | (50/79) | 39.1                               | (36/96) | 100.0           | (38/38) | 100.0         | (47/47) |
| NC    | ToRSV | 40.6                              | (67/164)              | 41.7                               | (10/23) | 45.8                              | 27/58   | 23.3                               | (20/85) | 91.4            | (32/35) | 68.1          | (32/47) |
| NC    | TSV   | 0.0                               | (0/114)               | 0.0                                | (0/23)  | 0.0                               | (0/58)  | 0.0                                | (0/85)  | 0.0             | (0/35)  | 0.0           | (0/47)  |
| NC    | RBDV  | 0.0                               | (0/114)               | 4.2                                | (1/23)  | 0.0                               | (0/58)  | 1.2                                | (1/85)  | 2.9             | (1/35)  | 2.1           | (1/47)  |
| NC    | INSV  | 31.3                              | (35/111)              | 25.0                               | (6/23)  | 23.7                              | (14/58) | 19.8                               | (17/85) | 80.0            | (28/35) | 57.4          | (27/47) |
| NC    | TSWV  | 0.0                               | (0/101)               | 0.0                                | (0/23)  | 0.0                               | (0/58)  | 0.0                                | (0/85)  | 0.0             | (0/35)  | 0.0           | (0/47)  |
| VA    | TRSV  | 0.0                               | (0/15)                | 0.0                                | (0/1)   | 0.0                               | (0/16)  | 0.0                                | (0/10)  | nt <sup>y</sup> |         | nt            |         |
| VA    | ToRSV | 0.0                               | 0/15                  | 0.0                                | (0/1)   | 0.0                               | (0/16)  | 0.0                                | (0/10)  | nt              |         | nt            |         |
| VA    | TSV   | 0.0                               | (0/4)                 | nt                                 |         | 0.0                               | (0/5)   | 0.0                                | (0/3)   | nt              |         | nt            |         |
| VA    | RBDV  | 0.0                               | (0/4)                 | nt                                 |         | 0.0                               | (0/5)   | 0.0                                | (0/3)   | nt              |         | nt            |         |
| VA    | INSV  | 25.0                              | (1/4)                 | 0.0                                | (0/1)   | 20.0                              | (1/5)   | 0.0                                | (0/10)  | nt              |         | nt            |         |
| VA    | TSWV  | 0.0                               | (0/4)                 | 0.0                                | (0/1)   | 0.0                               | (0/5)   | 0.0                                | (0/10)  | nt              |         | nt            |         |
| SC    | TRSV  | 25.0                              | (11/44)               | nt                                 |         | 37.5                              | (6/16)  | nt                                 |         | 100.0           | (3/3)   | nt            |         |
| SC    | ToRSV | 81.8                              | (36/44)               | nt                                 |         | 31.3                              | (5/16)  | nt                                 |         | 100.0           | (6/6)   | nt            |         |
| SC    | TSV   | 0.0                               | (0/44)                | nt                                 |         | 0.0                               | (0/16)  | nt                                 |         | 0.0             | (0/3)   | nt            |         |
| SC    | RBDV  | 0.0                               | (0/44)                | nt                                 |         | 0.0                               | (0/16)  | nt                                 |         | 0.0             | (0/3)   | nt            |         |
| SC    | INSV  | 11.1                              | (1/9)                 | nt                                 |         | 6.3                               | (1/16)  | nt                                 |         | 100.0           | (6/6)   | nt            |         |
| SC    | TSWV  | 0.0                               | (0/21)                | nt                                 |         | 0.0                               | (0/16)  | nt                                 |         | 0.0             | (0/3)   | nt            |         |

<sup>z</sup> Number of positive samples for virus/number of total samples tested for each plant part.

<sup>y</sup> nt = no sample was tested for this virus in this location.

Table 1.3. Single and multiple infections of *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Raspberry bushy dwarf virus* (RBDV) and *Impatiens necrotic spot virus* (INSV) in blackberry cultivars in North Carolina, South Carolina and Virginia in 2001 and 2002.

| Virus                      | Number of positive samples/state |                |          | Total |
|----------------------------|----------------------------------|----------------|----------|-------|
|                            | North Carolina                   | South Carolina | Virginia |       |
| Single infection           |                                  |                |          |       |
| TRSV                       | 99                               | 5              | 0        | 104   |
| ToRSV                      | 32                               | 9              | 0        | 41    |
| INSV                       | 3                                | 0              | 2        | 5     |
| RBDV                       | 4                                | 0              | 0        | 4     |
| Multiple infections        |                                  |                |          |       |
| TRSV + ToRSV               | 48                               | 8              | 0        | 56    |
| ToRSV + INSV               | 29                               | 5              | 0        | 34    |
| TRSV + INSV                | 18                               | 0              | 0        | 18    |
| TRSV + ToRSV + INSV        | 79                               | 3              | 0        | 82    |
| TRSV + ToRSV + RBDV        | 1                                | 0              | 0        | 1     |
| TRSV + INSV + RBDV         | 1                                | 0              | 0        | 1     |
| TRSV + ToRSV + INSV + RBDV | 2                                | 0              | 0        | 2     |

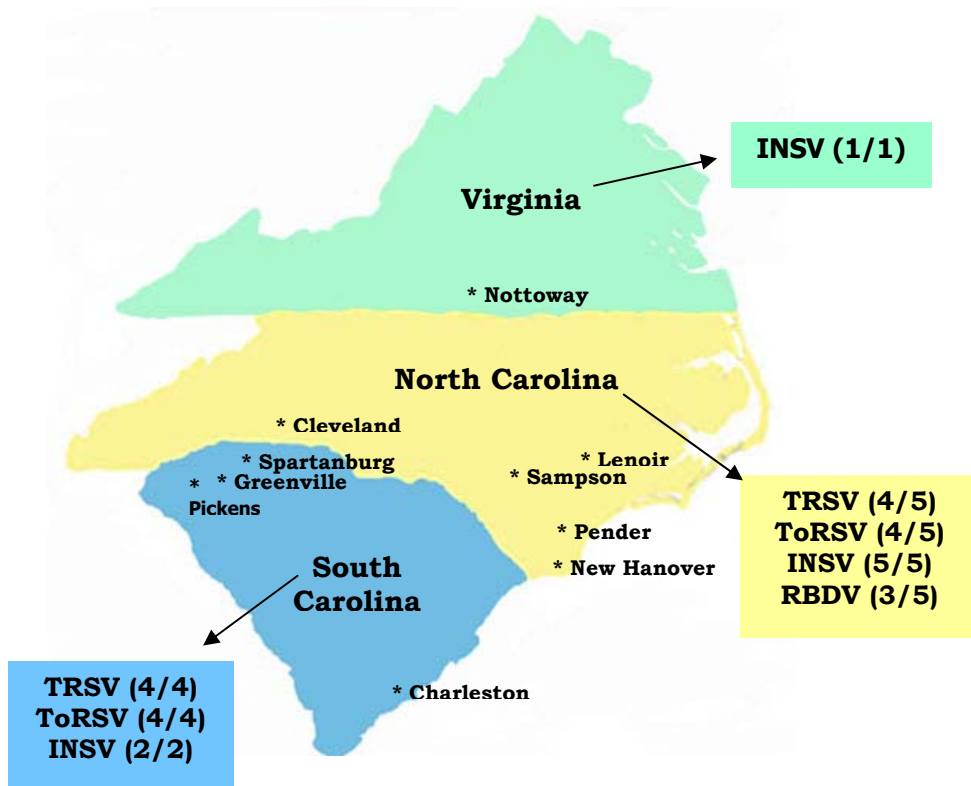


Figure 1.1. Location of the collection sites and the distribution (number of sites with virus/number of sites surveyed) of *Tobacco ringspot virus* (TRSV), *Tomato ringspot virus* (ToRSV), *Tobacco streak virus* (TSV), *Raspberry bushy dwarf virus* (RBDV), *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV) on blackberry samples collected from North Carolina, South Carolina and Virginia in 2001 and 2002.



Figure 1.2. 'Arapaho' blackberry leaves infected with *Tobacco ringspot virus*, showing yellow blotches and vein chlorosis.



Figure 1.3. Infected 'Kiowa' blackberry plant showing ringspots caused by *Tobacco ringspot virus*.



Figure 1.4. Yellow blotches, necrosis, vein chlorosis and oak leaf pattern on leaves of 'Kiowa' blackberry plant infected with *Tobacco ringspot virus* and *Impatiens necrotic spot virus*.



Figure 1.5. Multiple infections of *Tomato ringspot virus*, *Impatiens necrotic spot virus* and *Tobacco ringspot virus* on ‘Arapaho’ blackberry plant showing crumbly fruit, oak leaf pattern and small-distorted leaves.



Figure 1.6. Multiple infections of *Tomato ringspot virus*, *Impatiens necrotic spot virus* and *Tobacco ringspot virus* on 'Arapaho' blackberry plant showing vein chlorosis and ringspots.



Figure 1.7. Multiple infections of *Tomato ringspot virus*, *Impatiens necrotic spot virus* and *Tobacco ringspot virus* on 'Arapaho' blackberry plant showing ringspots, vein chlorosis, yellow blotches, necrotic spots and crumbly fruit.



Figure 1.8. Yellow blotches, vein chlorosis, ringspots and crumbly fruit caused by multiple infections of *Tomato ringspot virus*, *Impatiens necrotic spot virus* and *Tobacco ringspot virus* on ‘Arapaho’ blackberry.

## **Chapter 2**

### ***Impatiens necrotic spot virus* in Blackberry (*Rubus* spp.) Cultivars; a New Host for the Virus**

**Tania L. Guzmán, Gina Fernández, and Zvezdana Pesic-VanEsbroeck**

**(Abstract in the format appropriate for submission as a Disease Note in Plant Disease)**

***Impatiens necrotic spot virus* in Blackberry (*Rubus* spp.) Cultivars; a New Host for the Virus.**

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***Impatiens necrotic spot virus* in Blackberry (*Rubus* spp.) Cultivars; a New Host for the Virus.**

**ABSTRACT**

In 2000 blackberry plants with multiple visual symptoms consistent with the presence of viral diseases were observed in several locations in North Carolina. As a result of these observations, a field survey was conducted in eight locations in North Carolina, South Carolina and Virginia in 2001 and 2002. Leaflets from primocanes, floricanes, roots and seeds were collected and tested using Agdia kits (Elkhart, IN, USA). *Impatiens necrotic spot virus* was detected in all locations surveyed with an incidence from 2 to 41%. To our knowledge this is the first report of INSV on blackberry, a woody perennial plant. The high incidence found in our results seems to be associated to the presence of populations of thrips in these locations and the introduction on infected vegetatively propagated plant material from other states that may not satisfy the certification standards required for distribution of virus-free material. There were differences in INSV detection during the growing season. This may have been due to temperature extremes we experienced and that play an important role in detection of this virus in other plant species. The titer of INSV was higher in roots than in other plant part tested. Blackberry roots and crowns may be a virus reservoir and therefore should be included for diagnosis. In addition, INSV was also found in multiple infections associated with *Tobacco ringspot virus*, *Tomato ringspot virus* and *Raspberry bushy dwarf virus*. Symptoms observed for INSV varied from cultivar to cultivar and included vein chlorosis, vein necrosis, necrotic spotting, line patterns, leaf distortion and oak

leaf pattern. Certification programs should be implemented to insure that plants propagated are free of INSV. Also growers should use certified material and cultural practices such as weed control, rouging and control of populations of thrips. In order to prove that INSV is in blackberry the remainder of Koch's postulates is necessary to verify that blackberries are indeed a new host for INSV. Mechanical inoculation of infected plant sap onto *Nicotiana benthamiana* Domin., electron microscopy and/or PCR are necessary in order to fully prove pathogenicity. The identification of the thrips species present in the field is also pending.

*Additional keywords:* Enzyme-Linked Immunosorbent Assay (ELISA), thrips, woody perennial, ornamental, vector, *Nicotiana benthamiana*.

*Impatiens necrotic spot virus* (INSV) is a species in the *Tospovirus* genus, family *Bunyaviridae* (Law and Moyer, 1990). *Impatiens necrotic spot virus* was first isolated from New Guinea Impatiens (*Impatiens* Hybrids) exhibiting symptoms similar to *Tomato spotted wilt virus* (TSWV) and was termed TSWV-I (Law et al., 1992). However serological comparison of TSWV-I and TSWV showed that while the two envelope proteins (G1 and G2) were conserved, the nucleoproteins (N) did not cross-react in heterologous assays (Law and Moyer, 1990). Differences in the serological relationship of the structural proteins and nucleotide sequence of the S RNA N ORFs indicate that the two viruses represent different serogroups, and TSWV-I was renamed INSV (Law et al., 1992). The two tospoviruses, TSWV and INSV, although distinguished by serologically distinct N protein, cause similar symptoms and have overlapping host ranges (Daughtrey et al., 1995; Jones et al., 1991; Porter et al., 1984).

*Impatiens necrotic spot virus* is currently a serious threat to a wide range of North American greenhouse flower crops (Daughtrey et al., 1995); according to Widham et al. (1998) more than 300 plant species from 50 plant families are susceptible to INSV. A partial listing of ornamental plants susceptible to INSV includes cineraria (*Senecio cruentus* Masson ex L'Hér.), ranunculus (*Ranunculus* sp.), impatiens (*Impatiens wallerana* Hook.), New Guinea impatiens (*Impatiens*), cyclamen (*Cyclamen persicum* Mill.), exacum (*Exacum affine* Balf.), begonia (*Begonia* sp.), primula (*Primula* sp.), African violet (*Saintpaulia ionantha* H. Wendl.), ageratum (*Ageratum houstonianum* Mill.), amaranthus (*Amaranthus* sp.), anemone (*Anemone* sp.), aster (*Aster* sp.), calceolaria (*Calceolaria mexicana* Benth.), calendula (*Calendula officinalis* L.), chrysanthemum (*Chrysanthemum* sp.), coleus (*Plectranthus* L'Hér.), fuchsia (*Fuchsia triplylla* L.), marigold (*Tagetes erecta* L.), petunia (*Petunia*

*hybrida* Vilm.), snapdragon (*Antirrhinum majus* L.), verbena (*Verbena* sp.), zinnia (*Zinnia violacea* Cau.) and gloxinia (*Sinningia speciosa* (Lodd.) Hiern) (Daughtrey et al., 1995; Daughtrey et al., 1997; Windham et al. (1998). Daughtrey et al. (1997) and Ullman et al. (1998) mention that roses (*Rosa* hybrids), poinsettias (*Euphorbia pulcherrima* Willd. Ex Klotzsch) and zonal geraniums (*Pelargonium hortorum* L.H. Bailey) are the only major flower crops not susceptible to INSV and TSWV; these are woody plants; tospoviruses are usually associated with herbaceous hosts.

According to Daughtrey et al. (1997) and Lawson and Hsu (1995), INSV is very labile and temperature has significant effect on symptom expression of INSV in naturally infected seed-produced and New Guinea impatiens. In general, high light above 300  $\mu\text{M m}^{-2}\text{s}^{-1}$  PPF and high temperatures above 27° C will suppress symptom development. In a greenhouse symptoms are often more easily observed in plants growing during the winter months.

*Impatiens necrotic spot virus* can cause an extraordinarily broad range of symptoms on many plants, with symptoms ranging from subtle to severe. The possible symptoms include stunting, necrotic spotting, chlorotic spotting, areas of black or brown stem necrosis, ringspots, mosaic, line patterns, vein necrosis, flower breaking and death (Daughtrey et al., 1995; Daughtrey et al., 1997; Windham et al., 1998). Plants infected when young are the most severely affected (Daughtrey et al., 1995). Complete necrosis and collapse of seedlings has been observed, especially in the case of young gloxinias infected with INSV (Daughtrey et al., 1995). Foliar necrosis is likely to be misattributed to chemical injury or bacterial or fungal pathogens (Daughtrey et al., 1997). Any of the plant hosts may exhibit several of the

symptom types, showing variation from cultivar to cultivar and from plant to plant. Latent (symptomless) infections are common in certain cultivars of susceptible crops (Daughtrey et al., 1995; Daughtrey et al., 1997).

*Impatiens necrotic spot virus* is transmitted by the Western flower thrips, *Frankliniella occidentalis* (Pergande) (Daughtrey et al., 1997). These thrips have become the most common and difficult-to-control pest in the greenhouse industry nationwide (Daughtrey et al., 1997) due to their ability to develop resistance to insecticides (Windham et al., 1998).

The purpose of this study was to detect INSV in blackberry cultivars in the southeastern U.S. In addition we would like to determine the incidence and distribution of the virus in the locations surveyed.

## **Materials and Methods**

*Collection sites and sampling.* A survey was conducted at six grower locations and at two research stations during 2002 to detect the presence of INSV in blackberry cultivars in three states of the southeastern U.S. New Hanover, Sampson, Cleveland, Lenoir and Pender Counties were surveyed in North Carolina; Pickens and Charleston in South Carolina and Nottoway in Virginia. These locations were distributed in the Mountain, Piedmont and Coastal Plain regions at these states. Samples were collected throughout the growing season in 2002. Blackberry cultivars predominant at the collection sites were ‘Navaho’, ‘Arapaho’, ‘Triple Crown’, ‘Shawnee’, ‘Kiowa’, ‘Choctaw’, ‘Chickasaw’, ‘Rosborough’, ‘Lochness’, ‘Black Satin’, ‘Chester’ and ‘Apache’. Plants showing virus-like symptoms were selected and marked at each location using colored flags. Leaflets were collected from (1) primocanes

and floricanes from symptomatic blackberry plants in 2001 and 2002, and (2) primocanes and floricanes from asymptomatic blackberry plants in 2002 from plants selected the previous year. Roots and fruit were collected from symptomatic blackberry plants. Samples were placed in plastic storage bags, labeled, placed in a cooler and brought back to the virology lab at NCSU, where they were stored at 4°C until they were ready for testing.

Seed samples were obtained by mixing each fresh fruit sample from each plant in a blender at several intervals of 30 sec. Next, the seed was collected on cheesecloth and washed several times with tap water until all the pulp was washed out from the surface of the seed; then, it was surface sterilized using 0.52% w/v sodium hypochlorite (10% v/v Clorox bleach) for 5 min and rinsed three times with distilled water. Finally, seeds were dried for 24 hr at room temperature, placed in coin envelopes, labeled and stored in a zip lock bag at 4° C.

*Sample processing.* Leaflets and roots collected from symptomatic and asymptomatic floricanes and primocanes were homogenized and weighed (0.5 to 1 g of tissue per sample). Using a roller grinder (Plant Disease and Insect Clinic, NCSU), tissue samples were ground using a blueberry extraction buffer (Agdia, Inc., Elkhart, IN, USA) at a 1:10 ratio (tissue weight: blueberry extraction buffer volume). Seeds were ground using a mortar and a pestle at a ratio of six seeds per 1000 µl of buffer. Samples were dispensed in tubes and centrifuge for 10 min at 7,000 rpm, 100 µl of the supernatant solution was collected and used for detection.

*Detection.* Double Antibody Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA) pathoscreen kits from Agdia, Inc., Elkhart, IN, USA, were used to detect INSV in blackberry samples. Coated polystyrene microtitre plates were used to detect the virus. Detection was accomplished following the manufacturer protocol. Positive and negative controls were included in each plate. Positive controls consisted of purified virus supplied in the kit by the manufacturer. Negative controls were of two types; wells that contained extraction buffer and wells that contained centrifuged plant sap from indexed virus-free tissue culture plants produced in the Micropropagation Unit, NCSU, Raleigh, NC.

*Analysis.* Absorbance of each well at 405 nm ( $A_{405}$ ) was read in an ELISA plate reader (Molecular Devices, Sunnyvale, CA, U.S.). Tissue and/or root samples were considered positive for virus if  $A_{405}$  of the sample wells were greater than 3 times the mean of the values obtained for negative or healthy controls (Sutula et al., 1986).  $A_{405}$  values of negative controls ranged from 0.000 to  $\pm 0.027$ ; any value greater than 0.1 was considered positive.

## **Results**

*Impatiens necrotic spot virus* was detected in all locations surveyed in North Carolina, South Carolina and Virginia as shown in Table 2.1. In North Carolina 127 of 363 tested positive for INSV, while in Virginia 2 of 20 and in South Carolina 8 of 31 tested positive for this virus (Table 2.1).

Differences in virus incidence in various plant parts tested (primocanes, floricanes, seeds and roots) were also observed. The titer of INSV was higher and therefore easier to

detect in roots than in other plant parts tested. *Impatiens necrotic spot virus* was detected in the field throughout mid June to mid July in all three states.

Symptoms of INSV varied from cultivar to cultivar. Leaf distortion, necrosis and oak leaf pattern (Fig. 2.1.), yellow blotches and necrosis of the mid ribs (Fig. 2.2.), and interveinal chlorosis and necrotic spots (Fig. 2.3.) were observed on ‘Triple Crown’. Vein chlorosis and necrosis at the margins and in the mid ribs (Fig. 2.4.) and leaf distortion, yellow blotches and necrosis (Fig. 2.5.) were observed on ‘Apache’.

## Discussion

*Impatiens necrotic spot virus* was found in 100% of the locations surveyed. This virus has not been reported in woody perennial plants previously, but has been reported in a broad range of ornamental crops (Hawsbeck et al., 1992; Lawson and Hsu, 1995). The Western flower thrips *F. occidentalis* (Pergande) is the only thrips currently known to vector INSV (German et al., 1992; Lawson and Hsu, 1995; Daughtrey et al., 1997). The high incidence found in our results seems to be associated to the presence of populations of thrips observed within the blackberry plantings and other surrounding crops in the locations surveyed. Even though, the species of thrips present in blackberry fields has not been identified, the incidence of this virus could be associated with the presence of thrips in these locations. According to Ullman et al. (1998) one insect can infect several plants and low levels of infective thrips can reflect a high level of virus infection; a higher number of lesions on petunias in an area where the Western flower thrips populations were relatively low were

also observed. Another factor that could play an important role in INSV presence and dissemination might be the introduction on infected vegetatively propagated plant material from nurseries in other states without certification programs.

There were differences in our detection of INSV during the growing season. We detected INSV in plants tested from mid June to mid July in 2002. However, the same infected plants tested negative in previous and later sampling dates. *Impatiens necrotic spot virus* is considered very labile (Daughtrey et al., 1997) and temperature plays an important role in the detection of this virus. Light levels of  $300\mu\text{m m}^{-2}\text{s}^{-1}$  and high temperatures above  $27^{\circ}\text{C}$  will suppress symptom development (Lawson and Hsu, 1995). In the states where this survey was conducted, temperature and light conditions often exceed those that have been found to suppress expression. Additionally, INSV was also found in multiple infections associated with *Tobacco ringspot virus*, *Tomato ringspot virus* and *Raspberry bushy dwarf virus* (Guzmán-Baeny, 2003).

The titer of INSV was higher in roots than in other plant part tested (data not shown). Blackberries present perennial roots and crowns, and biennial canes that are trimmed after fruit production, roots and crowns could be virus reservoir and therefore should be sampled and tested. Roggero et al. (1999) mentioned that high temperatures did not alter INSV replication but only impeded systemic movement of the virus in infected peppers. This might be also valid for blackberries where inhibition of long distance transport of INSV at high temperature may explain the variation of INSV detection in upper parts of the plant. In addition, because soil temperatures are lower than air temperatures, the titer of the virus may be higher in the roots where temperatures are more favorable for this virus.

Symptoms observed for INSV varied from cultivar to cultivar and from plant to plant as observed in ‘Triple Crown’ and ‘Apache’ blackberries. Symptoms of INSV observed in blackberry cultivars include vein chlorosis, vein necrosis, necrotic spotting, line patterns and oak leaf pattern. According to Daughtrey et al. (1995), Daughtrey et al. (1997), and Windham et al. (1998), INSV can cause an extraordinarily broad range of symptoms on many plants.

Based on the results obtained in this research we found that blackberries are a new host for INSV. We have concluded that the incidence and distribution of this new virus would have two origins: introduction of vegetatively propagated infected plant material and the presence of populations of thrips which tend to build up during warmer temperatures (spring to early summer).

In the future, blackberry root samples should be included for INSV diagnosis. Nurseries should implement certification programs to insure that the plants they propagate are free of INSV. Growers should use certified material and cultural practices such as weed control, rouging, control of populations of thrips with pesticides when necessary, avoid insect resistance to pesticides by rotating pesticides used in control.

Note to readers: The reminder of Koch’s Postulates and the identification of the thrips species present in the field are pending.

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Table 2.1. Incidence of *Impatiens necrotic spot virus* (INSV) in blackberry (*Rubus* spp.) cultivars in three states in the southeastern United States in 2002.

| State          | County      | INSV<br>Incidence (%)     |
|----------------|-------------|---------------------------|
| North Carolina | Pender      | 41.1 (37/90) <sup>z</sup> |
| North Carolina | Cleveland   | 2.3 (1/43)                |
| North Carolina | Lenoir      | 38.3 (54/141)             |
| North Carolina | New Hanover | 41.0 (34/83)              |
| North Carolina | Sampson     | 16.7 (1/6)                |
| South Carolina | Charleston  | 16.7 (1/6)                |
| South Carolina | Pickens     | 28.0 (7/25)               |
| Virginia       | Nottoway    | 10.0 (2/20)               |

<sup>z</sup> Number of positive samples for INSV/number of total samples tested.



Figure 2.1. Leaf distortion, necrosis and oak leaf pattern in ‘Triple Crown’ blackberry infected with *Impatiens necrotic spot virus*.



Figure 2.2. 'Triple Crown' blackberry showing yellow blotches and necrosis of the mid ribs caused by *Impatiens necrotic spot virus*.



Figure 2.3. Symptoms of *Impatiens necrotic spot virus* in ‘Triple Crown’ blackberry showing interveinal chlorosis and necrotic spots.



Figure 2.4. ‘Apache’ blackberry showing vein chlorosis and necrosis at the margins and in the mid ribs caused by *Impatiens necrotic spot virus*.



Figure 2.5. Symptoms of *Impatiens necrotic spot virus* in ‘Apache’ blackberry showing leaf distortion, yellow blotches and necrosis.

## **Chapter 3**

### ***Tobacco ringspot virus* Infection in Blackberry (*Rubus* spp.) Seeds**

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**(This chapter fulfills part of the requirements for Special Topics Project in Plant Pathology and is in the format for submission to HortScience)**

*Tobacco ringspot virus* Infection on Blackberry (*Rubus* spp) Seeds

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***Tobacco ringspot virus* Infection in Blackberry (*Rubus* spp.) Seeds**

*Additional index words.* Protein A Enzyme-Linked Immunosorbent Assay (ELISA), seed coat, embryo, maternal tissue, seedlings.

**Abstract.** The extent to which viruses are present in seed lots of commercial crop species is seldom reported. *Tobacco ringspot virus* is known to be transmitted through seed but the incidence of the virus in seed lots and seedlings in blackberries has not been studied. Fruit was collected from blackberry (*Rubus* spp.) plants infected with TRSV in 2002 from three locations in North Carolina. A total of twelve seed lots were tested. Six seed lots were tested from New Hanover, one lot from Lenoir and five lots from Pender Counties. An Indirect ELISA was used to detect TRSV in embryo and seed coat of blackberry seeds. *Tobacco ringspot virus* was detected in 107 of the 130 embryos tested, and 10 of the 130 seed coats were also positive for the virus. Seven of twelve seed lots had 100% incidence of TRSV and the overall incidence of TRSV on the twelve seed lots for the three locations ranged from 30 to 100%. Incidence of TRSV in seedlings from infected plants was not studied but the most striking feature of seedlings from infected seeds in other crops is that they rarely appear diseased, this unfortunately would facilitate virus dissemination. We found that *Tobacco ringspot virus* infection in seeds came from the ovule of infected seeds. Therefore virus infected seeds could have major impacts on breeding programs by unknowingly introducing

and transmitting viruses through breeding stock that will facilitate the spread of the virus in nature.

About one-fifth of the known plant viruses are transmitted through the seed of infected host plants (Matthews, 1991). At least half of the members of the *Comovirus*, *Hodeivirus*, *Ilarvirus*, *Nepovirus*, *Potyvirus* and *Tobravirus* groups are seed transmitted (Lawson et al., 1995). Seed transmission provides a very effective means of introducing virus into a crop at an early stage, given randomized foci of infection throughout the planting. Viruses may persist in seed for long periods so that commercial distribution of a seed-borne virus to new areas may occur (Matthews, 1991; Taylor and Brown, 1997). Transmission of viruses through seed can be of considerable ecological significance for virus perpetuation and dissemination, as well as economic consequence for the plant grower (Johansen et al., 1994). According to Lawson et al. (1995), Matthews (1991), and Agrios (1997) the virus in most seed-transmitted seems to come primarily from the ovule of infected plants, but several cases are known in which the virus in the seed is derived from the pollen that fertilized the flower; with the exception of *Tobacco mosaic virus* (TMV) which is a contaminant on the surface of

tomato (*Lycopersicon esculentum* Mill.), pepper (*Capsicum* sp.) and tobacco (*Nicotiana tabacum* L.).

The extent to which viruses are present in seed lots of commercial crop species has seldom been reported (Taylor and Brown, 1997). Some viruses, including several transmitted by nematodes, infect a high percentage of seed from diseased mother plants (Lawson et al., 1995). The proportion of infected seeds set by diseased plants may depend on the virus, the host plant, the age at which plants first become infected and high temperatures (Cadman, 1963; Lawson et al., 1995). The absence of reports of seed infection is probably due to the fact that progeny seedlings show no symptoms and almost certainly nepoviruses occur much more widely in commercial seed lots than is currently apparent (Taylor and Brown, 1997). *Tobacco ringspot virus* been demonstrated to be transmitted through seeds of petunia (*Petunia hybrida*) (Henderson, 1931) and 'Lincoln' soybean (*Glycine max* (L.) Merr.) (Desjardins et al., 1953). According to Lawson et al. (1995), TRSV seed transmission may occur only through the female gametophytic tissue (ovule). If seed transmission is to occur, the plant usually must be infected before the ovules are fertilized. The percentage of infected seed also depends on the time of flowering in relation to the time of infection. Experiments with TRSV have shown that few seeds become infected if inoculation is delayed until the plants are in flower (Athow and Bancroft, 1959; Crowley, 1959). Seed transmission probably plays an important part in the survival and spread of some kinds of nepo (nematode-transmitted viruses with polyedral particles) viruses (Cadman, 1963). The most striking feature of seedlings from infected seeds is that they rarely appear diseased. They are protected from infection by the viruses they contain and at no stage do they display the

symptoms typical of newly infected plants (Lister, 1960). Virus infected seeds could have major impacts on breeding programs by unknowingly transmitting viruses through breeding stock. Therefore, it is important to determine if TRSV, a virus that is known to occur in blackberry, is present in blackberry seeds.

Furthermore, it is important to know if the virus is present in the seed coat, embryo or both as it could impact breeding programs and propagation of seedlings. Therefore the purpose of this study was to determine: 1) if TRSV is present in blackberry seeds and 2) the incidence of TRSV in embryos and seeds coats.

## **Materials and Methods**

*Collection sites and sampling.* Fruit from infected 'Arapaho' blackberry plants that tested positive for TRSV in previous experiments (Guzmán-Baeny, 2003) were collected from three locations in North Carolina: Lenoir, Pender and New Hanover Counties in 2002. Fruit samples were placed in plastic storage bags, labeled, placed in a cooler and brought back to the virology lab at NCSU, where they were stored at 4°C until they were ready for testing. Seeds were separated into 12 different lots based on infected plant number and location.

Seed samples were prepared by mixing each fresh fruit sample in a blender at several intervals of 30 sec. Mixed seed was collected on cheesecloth and washed several times with tap water until all the pulp was washed out from the surface of the seed. Then, the seed was surface sterilized using 0.52% w/v sodium hypochlorite (10% v/v Clorox bleach) for 5 min and rinsed three times with distilled water. Finally, seeds were dried for 24 hr at room temperature, placed in coin envelopes, labeled and stored in a zip lock bag at 4° C.

Sample processing. Approximately one third of each seed lot was allowed to re-hydrate in disposable Petri dishes with wet filter paper for 48 hrs. After this period seed was dissected under a stereoscope using forceps, thin needles and razor blades, and this material was disinfected with 70% v/v of ethanol for each seed. The endocarp (outer and harder layer) was removed and the seed coat and the embryo were collected and separated. Each embryo and its corresponding seed coat were ground separately using a mortar and a pestle at a ratio of 1 embryo or 1 seed coat per 300 µl of blueberry extraction buffer (Agdia, Inc., Elkhart, IN, USA). Samples were dispensed in tubes and centrifuged for 10 min at 7,000 rpm. 100 µl of the supernatant solution was collected and used for detection. Six seed lots were tested from New Hanover, one seed lot from Lenoir and five seed lots from Pender.

*Detection.* An indirect ELISA test, protein A ELISA, was used to detect TRSV in embryos and seed coats (Barbara and Clark, 1972). This test utilizes enzyme-conjugated *Staphylococcus aureus* Protein A that binds specifically with the Fc portion of immunoglobulin molecules (Barbara and Clark, 1982).

1. Microtiter plates were coated with 1 µg/ml of protein A (Sigma Chemical Co., St. Louis, MO, USA) in coating buffer (1.59g Na<sub>2</sub>CO<sub>3</sub> plus 2.93 g Na HCO<sub>3</sub> plus 0.2 g NaN<sub>3</sub> - adjusted to pH 9.6). All reagents were used at 100 µl per well in microtiter plates.
2. Microtiter plates were incubated for 2 hr in a humid box at room temperature. Plates were washed three times with PBS-Tween (Agdia, Inc., Elkhart, IN, USA) allowing plates to stand at least 3 min between washings.

3. Plates were coated with antiserum (Courtesy of Dr. Rose Gergerich) diluted 1:1000 in PBS-Tween. They were then incubated for 2 hr in a humid box at room temperature (27-30 °C), then washed as above.
4. Plates were coated with test samples diluted at 1:10 in virus extraction buffer (PBS-Tween) and incubated overnight at 4°C and washed as above.
5. Plates were coated with antiserum diluted 1:1000 in PBS-Tween, incubated 2 hr in a humid box at room temperature, and washed as above.
6. Plates were coated with 1 µg/ml of protein-A-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA) diluted in PBS-Tween, incubated for 2 hr at room temperature, and washed as above.
7. Freshly prepared substrate (p-nitrophenyl phosphate, Agdia, Inc., Elkhart, IN, USA) was added to each well, and plates were incubated at room temperature or as long as necessary to observe a reaction.

Positive and negative controls were included in each plate. Positive controls consisted of purified virus supplied in the kit by the manufacturer. Negative controls were of two types; wells that contained extraction buffer and wells that contained centrifuged embryos from seeds obtained from the field and tested negative for TRSV using ELISA in previous experiments (Guzmán-Baeny, 2003).

*Analysis.* Absorbance of each well at 405 nm ( $A_{405}$ ) was read in an ELISA plate reader (Molecular Devices, Sunnyvale, CA, U.S.). Embryo and seed coat samples were considered positive for TRSV if  $A_{405}$  of the sample wells were greater than 3 times the mean of the values obtained for negative or healthy controls (Sutula et al., 1986).  $A_{405}$  values of negative controls ranged from  $\pm 0.003$  to 0.049.

## Results

*Detection of TRSV in embryos and seed coats.* Tobacco ringspot virus was detected in the embryos of the blackberry seed tested (Table 3.1). The incidence of the virus in the embryo from the twelve seed lots tested ranged from 30 to 100%. Embryos from seed collected from Lenoir County had the highest incidence of 100% (10/10), in New Hanover County the incidence ranged from 50 to 100%, and from Pender from 30 to 100%. Out of the six seed lots tested from New Hanover County; four of five embryos were found positive in the first lot; 16/18 in the second; 17/17 in the third; 5/10 in the forth; 10/10 in the fifth and 62/70 in the sixth (Table 3.1). Results obtained from the five seed lots from Pender County were: 10/10 in the first seed lot; 10/10 in the second; 5/10 in the third; 3/10 in the forth and 35/50 in the fifth (Table 3.1.). Ten of ten embryos were found positive for TRSV from the seed lot tested from Lenoir County (Table 3.1.)

One of the 18 seed coats from seed lot 2 and 4/17 from seed lot 3 from New Hanover County were found positive for TRSV. Five seed coats were also found positive for TRSV in the seed lots from Pender County (2/10 seed lot 1; 2/10 seed lot 2; and 1/10 seed lot 3).

## Discussion

*Tobacco ringspot virus* was detected in 82.3% (107/130) of the embryos tested. This high incidence might be because the virus may infect only the ovule (Lawson et al., 1995) and transmission through the gametophyte is probably more efficient for most host-viruses combinations than transmission through the pollen (Matthews, 1991). Cross-pollination experiments showed that the principle route for seed transmission of TRSV in soybean was by infection of the megagametophytes (Matthews, 1991). Infected pollen may be able to compete only poorly with normal pollen during fertilization (Matthews, 1991). Yan and Hamilton (1974) observed TRSV in the megagametophyte as well as in pollen of soybean. The high rate of seed transmission of TRSV in soybean was seemingly related to the capacity of TRSV to invade meristematic tissue and infect the megaspore mother cells.

The high rate of transmission observed in this study also corresponded with the findings of Lister (1978) who found that the rate of seed transmission of TRSV was close to 100% in soybean, but the virus tends toward self-elimination due to the severe effects on flower development and seed set.

The low incidence of infection (7.7%) on the seed coats in this experiment can be attributed to contamination. Small pieces of embryos likely remained attached to the seed coat during the early process of the development of the seed separation protocol.

Incidence of TRSV in seedlings from infected plants was not studied. Blackberry seeds vary tremendously in germination (J. Ballington pers. comm.) This could therefore influence in the spread in the population of infected seedlings. In many cases, germination does not take place, therefore spread of the virus is averted. However, when germination

does occur, the virus could be passed on to seedlings. According to Cadman (1963) one of the most striking features of seedlings from infected seeds is that they rarely appear diseased which will facilitate virus dissemination.

Based on our results we concluded that *Tobacco ringspot virus* infection in seeds came from the ovule of infected plants. The presence of virus infected seeds and seedlings could have a major impact on breeding programs by unknowingly introducing and transmitting viruses through breeding stock. Therefore, screening of new varieties that are destined to be released from breeding programs is essential to ensure that these material is free from any known seed transmitted viruses such as TRSV.

Previous studies have found seed transmission is low in comparison to our findings. This high incidence could have a major impact on cultivar development programs, where there is the likelihood of virus infection via seed transmission.

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Table 3.1. *Tobacco ringspot virus* incidence in embryos and seed coats of blackberry (*Rubus* spp.) seeds collected in 2002 from Lenoir, New Hanover and Pender Counties in North Carolina.

| County          | Seed lot No | Plant No <sup>z</sup> | Total seed tested | No positive Embryo <sup>y</sup> | No positive Seed Coat <sup>x</sup> | Incidence (%) Embryo <sup>w</sup> | Incidence (%) Seed Coat <sup>v</sup> |
|-----------------|-------------|-----------------------|-------------------|---------------------------------|------------------------------------|-----------------------------------|--------------------------------------|
| New Hanover     | 1           | 36                    | 5                 | 4                               | 0                                  | 80.0                              | 0.0                                  |
| New Hanover     | 2           | 49                    | 18                | 16                              | 1                                  | 88.9                              | 5.6                                  |
| New Hanover     | 3           | 120                   | 17                | 17                              | 4                                  | 100.0                             | 23.5                                 |
| New Hanover     | 4           | 116                   | 10                | 5                               | 0                                  | 50.0                              | 0.0                                  |
| New Hanover     | 5           | 30                    | 10                | 10                              | 0                                  | 100.0                             | 0.0                                  |
| New Hanover     | 6           | 45                    | 10                | 10                              | 0                                  | 100.0                             | 0.0                                  |
| <i>Subtotal</i> |             |                       | 70                | 62                              | 5                                  | 88.6                              | 7.1                                  |
| Lenoir          | 1           | 6                     | 10                | 10                              | 0                                  | 100.0                             | 0.0                                  |
| <i>Subtotal</i> |             |                       | 10                | 10                              | 0                                  | 100.0                             | 0.0                                  |
| Pender          | 1           | 6                     | 10                | 10                              | 2                                  | 100.0                             | 20.0                                 |
| Pender          | 2           | 36                    | 10                | 10                              | 2                                  | 100.0                             | 20.0                                 |
| Pender          | 3           | 22                    | 10                | 5                               | 1                                  | 50.0                              | 10.0                                 |
| Pender          | 4           | 27                    | 10                | 3                               | 0                                  | 30.0                              | 0.0                                  |
| Pender          | 5           | 31                    | 10                | 7                               | 0                                  | 70.0                              | 0.0                                  |
| <i>Subtotal</i> |             |                       | 50                | 35                              | 5                                  | 70.0                              | 10.0                                 |
| <i>Total</i>    |             |                       | 130               | 107                             | 10                                 | 82.3                              | 7.7                                  |

<sup>z</sup> Infected blackberry plant in the field from where fruit lot was collected.

<sup>y</sup> Number of positive embryos.

<sup>x</sup> Number of positive seed coats.

<sup>w</sup> Incidence of TRSV in the embryo/seed lot/County.

<sup>v</sup> Incidence of TRSV in the embryo per seed lot/County.