ISOLATION AND DETECTION OF BEAN YELLOW MOSAIC, CLOVER YELLOW VEIN AND PEANUT STUNT VIRUSES FROM TRIFOLIUM L. SPECIES

by

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(ABSTRACT)

Trifolium L. (clover) are annual or perennial species established in pasturelands to improve forage productivity and quality. In the southeastern United States, *Trifolium repens* L. (white clover) and *Trifolium pratense* L. (red clover) are important species, susceptible to virus infection. Objectives of this research were to isolate bean yellow mosaic (BYMV), clover yellow vein (CYVV) and peanut stunt (PSV) viruses from naturally infected white and red clovers from different locations in Virginia; and, to compare Indirect Enzyme-Linked ImmunoSorbent Assay (i-ELISA) and tissue immunoblot assay (TIBA) as methods for virus detection. A total of five white clover samples from Augusta, Richmond and Washington Counties were positive against CYVV antiserum and four white clover samples from Augusta County were positive against PSV antiserum. Single red clover samples from Frederick and Montgomery Counties were positive against BYMV antiserum. There were notable differences in host range with samples that tested positive for CYVV and BYMV, indicating they may be different strains. PSV was evenly distributed in the plant, whereas CYVV was higher in older plant parts. Viruses were successfully detected by blotting leaf samples directly onto

membranes, thereby simplifying the sample preparation step. A number of membranes, such as nitrocellulose, nylon, chromatography paper, filter paper and writing pad could be used to detect viruses. In terms of specificity, immunoblots were equal or superior to i-ELISA. The TIBA should be useful in support of breeding and plant pathology studies as it is simple and rapid, and is less laborious and less expensive than i-ELISA.

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CHAPTER 1

INTRODUCTION

Trifolium (clovers) are important annual or perennial forage species established in pasturelands to improve forage productivity and quality. There are a number of *Trifolium* species that exist, but, in the southeastern United States including Virginia, *T. repens* L. (white clover) and *T. pratense* L. (red clover) are among the commonly found species.

Annual or perennial clovers serve as sources of natural virus inoculum and during spring or summer months aphid vectors transmit the viruses to other legumes (Anonymous, 1982-1987). The virus/es infect field plants and as a result the yield and stand persistence decreases (Barnett and Diachun, 1986).

In 1977, scientists in 11 southeastern states initiated Southern Regional Project S-127, Forage Legume Viruses, now known as S-228 to identify important forage legume viruses. Surveys conducted earlier and during the project have identified alfalfa mosaic tricornavirus (AMV), bean yellow mosaic potyvirus (BYMV), clover yellow vein potyvirus (CYVV), peanut stunt cucumovirus (PSV), clover yellow mosaic potexvirus (CYMV), white clover mosaic potexvirus (WCMV), tobacco ringspot nepovirus (TRSV) and tomato ringspot nepovirus (TmRSV) in Virginia (Anonymous, 1982-1987; Hunst and Tolin, 1982; McLaughlin and Boykin, 1988; Milbrath and Tolin, 1977; Tolin et al., 1970; Tolin and Miller, 1975). Surveys conducted during the past 2-3 yrs have also identified

luteoviruses (Anonymous, 1990). Among the above mentioned viruses peanut stunt (PSV), bean yellow mosaic (BYMV) and clover yellow vein (CYVV) are the most important and widely present viruses in Virginia (McLaughlin et al., 1984; McLaughlin and Boykin, 1988). All three are mechanically transmissible (Bos, 1970; Pratt, 1969; Tolin et al., 1970) and can also be transmitted by aphids in a non-persistent manner (Hobbs and McLaughlin, 1990; Tolin et al., 1970).

Accurate identification of viruses is important to control the virus-induced diseases (McLaughlin and Scott, 1986). In the past scientists have used a number of reliable methods to detect and identify the viruses. These are Enzyme-Linked ImmunoSorbent Assay (ELISA), radioimmunosorbent assay (RIA), the latex agglutination test (LAT), Ouchterlony gel diffusion, electron microscopy, viral protein analysis, dsRNA analysis and indicator plants. The above methods have some drawbacks, making them less desirable to use. Labeled antibody tests such as ELISA are time-consuming and need specific equipment and supplies (Graddon and Randles, 1986). Radioimmunosorbent assay is not preferable in the present days due to potential health hazards from radioactive waste and its disposal. Ouchterlony requires large quantities of antisera (Ball, 1974) and electron microscopy involves sizable investments of money, labor and time. Viral proteins and dsRNA analysis are laborious, time consuming and require special equipment, supplies and experience to perform. Symptom expression using indicator plants can be confusing and misleading (McLaughlin

and Scott 1986).

A rapid viral detection method in which results can be obtained in a shorter period of time using fewer items of equipment and less labor is required. Such a detection method could prove useful in the laboratory or greenhouse, or even to growers or breeders who have large field plots of high economic value.

The purpose of this research is to establish cultures of BYMV, CYVV and PSV from naturally infected white and red clovers present in Virginia and compare them to known isolates of these viruses. Indirect enzyme-linked immunosorbent assay (i-ELISA) and tissue immunoblot assay (TIBA) will be compared as methods for their detection. TIBA will be compared to i-ELISA and analyzed to show the cost or effective benefits of this new and promising technique.

CHAPTER 2

LITERATURE REVIEW

GENUS *TRIFOLIUM* L.: TAXONOMY, MORPHOLOGY AND DISTRIBUTION

The genus *Trifolium* L. belongs to the tribe Trifolieae of the subfamily Papilinoideae, family Leguminosae (alternate name, Fabaceae). The genus contains approximately 240 species and Zohary further divided it into eight sections. Clover or trefoil is a plant which belongs to this genus (Gillett, 1985).

The word *Trifolium* comes from Latin, in which, 'tres' means "three" and 'folium', means "leaf" (Allen and Allen, 1981). According to Shaw (1906), "the term *Trefoil* was given because the leaves are divided into three leaflets". The main characteristics of the genus as given in Gillett (1985) are:

> "Clovers are annual, biennial, or perennial herbs.
> Leaves are palmately trifoliate or rarely, pinnately trifoliate; occasionally digitate with five to nine usually denticulate leaves; stipules mostly ovate, entire, fused to the petiole for about half their length.
> Flowers incapitate or dense subcapitate spikes, the petal white, purple, red, rarely yellow, sometimes bicolored; stamens diadelphous.
> Fruit a small, 1-4 seeded pod, enclosed in the persistent calyx and corolla at maturity.
> Basic chromosome number, as determined from counts on approximately 40 species, x = 6,7,8,9".

The genus *Trifolium* is widely distributed throughout the northern hemisphere and to some extent in the southern hemisphere. The largest concentration of the species is in the eastern Mediterranean region, while other species spread northwards as far as Scandinavia or northeastwards across Asia including Japan.

Trifolium occurs in North Africa, Tanzania, Uganda, Ethiopia, Egypt, southern Arabia and the Atlas Mountains. In America, it extends from central coastal British Columbia to Baja, California. A few species are found in the U.S. Rocky Mountains, and a few others are found in the Appalachians Mountains of the East. There are a few that extend from Arizona to Mexico and through central America as far as Peru, Chile, Argentina, Brazil and Uruguay. Although it was introduced in great numbers into Australia, no species is native to that region (Gillett, 1985). "The principal agricultural value of clover is in its use as forage, hay, and pasture crops" (Turner, 1959). Speaking from an American farmer's point of view, "the genus *Trifolium* may be defined in the collective sense as a family of plants leguminous in character, which are unexcelled in furnishing forage and fodder to domestic animals, and unequaled in the renovating influences which they exert upon land" (Shaw, 1906).

Trifolium repens L.

History, distribution and characteristics. The origin of many forage legumes is generally regarded as the Near East, and it may have included *Trifolium repens* L. (white clover) (Harlan, 1971). Although the history of white clover in the U.S. is obscure, according to Carrier and Borth, it was brought to America by the early European settlers. By 1750 white clover was well established in North America and preceded English settlers to Ohio and Kentucky. Since it simultaneously appeared with the early European settlers, American Indians referred to it as "white man's

foot grass" (Leffel and Gibson, 1973). It is also commonly known as Dutch, White Dutch, White Trefoil, Creeping Trifolium and Honeysuckle clover (Shaw, 1906).

The first official report of white clover in the United States was made in 1891 by the North Carolina Station (Leffel and Gibson, 1973). Due to the lack of high quality seeds, white clover could not be grown successfully. Later white clover seeds were successfully produced in the western parts of United States, followed by the northeastern, north central and southeastern parts. White clover became popular during the 1940s and 1950s, and its hectarage has been increased since then (Leffel and Gibson, 1973). The general geographical distribution of white clover within and beyond the temperate regions of the world is presented by Daday (1958). White clover is found in the humid eastern half of the country, in the Pacific northwest, and along river valleys and in irrigated pastures of the inter-mountain region (Leffel and Gibson, 1973).

The main characteristics of white clover as given in Leffel and Gibson (1973) are:

> "White clover is a glabrous pasture plant with prostrate habit of growth, polymorphic, perennial in the temperate regions or winter annual in subtropical regions. Grows up to 50 cm in length. Germinates as a typical clover by producing a pair of cotyledons; a simple leaf bearing a single leaflet; and many compound leaves, each normally composed of three leaflets.
>
> Short primary stem contains a number of internodes. Axillary buds of the primary stem gives rise to stolons 6-8 wks after germination. Elongation of the primary stem stops or is limited after stolon growth begins. Stolons develop

radially from the primary stem. The stolon, contrasted to the primary stem, has elongated internodes and an apical bud that remains vegetative. It is solid, varies greatly in size and may develop one or more adventitious roots at each of its nodes. Erect petiole is borne from each node on the stolon, 14 cm or more in length.

Leaflets inversely cordate or shallowly notched, about 10-30 mm in size, elliptical to heart-shaped. Presence or absence of a V-shaped white mark in the middle of the leaflet is a simply inherited genetic trait. Stipules are membranous, veiny, white, and lanceolate with a short filiform tip.

Primary taproot grows to a depth of 1 m or more but dies before or during the second year of growth of the plant.

Flowering heads are produced on peduncles somewhat longer than leaves. The almost round head is composed of individual flowers, each of which is borne on a short pedicel. Number of flowers ranges from 20 to 150 per head.

Flowers are white or pink in color".

Trifolium pratense L.

History, distribution and characteristics. *Trifolium pratense* L. (red clover) may have originated within countries that border the Mediterranean and Red Sea, particularly in Asia Minor and southeastern Europe. By 1500 it was known as an early flowering type of clover in Spain. By 1550 it had spread to Holland and Lombardy and then to the German Rhineland. Around 1650 it moved from Germany to England. The earliest definitive reference to finding clover in the English colonies was around the year 1663. Several people thought that the English colonists must have carried the red clover to the United States, but the exact date is not clear (Taylor, 1973).

Red clover is grown throughout the world. In Europe, it is grown in Scandinavia, England, Scotland, Wales and Ireland. It is

also grown extensively in Poland, Yugoslavia, Hungary, USSR and Japan. It is grown less extensively in South Africa, Chile, New Zealand and Australia (Smith et al., 1985). In the United States, clover is extensively grown in the humid regions of the eastern North and South Dakota and throughout Nebraska, and Kansas. It is also grown in the South in states such as Tennessee and North Carolina (Taylor, 1973).

Red clover is grouped into 3 divisions: early flowering, late flowering and wild red. Most of the red clovers found in the United States belong to the early flowering group, otherwise called medium red clover (Taylor, 1973). Red clover is also known as Common red clover, Broad-leaved clover and Meadow trefoil (Shaw, 1906).

The main characteristics of red clover as given in Smith et al. (1985) are:

> "Red clover is ordinarily biennial in its habit of growth, but under some conditions it is perennial. The plant is herbaceous, grows rapidly, and spreads and stands upright.
>
> Several branches rise up from the crown of each plant, and these in turn frequently become branched more or less in their upward growth.
>
> The stems of the plant are slightly hairy, and ordinarily they stand at least fairly erect and reach the height of about 1 ft or more.
>
> Lower leaves are long-petioled, the petioles become shorter upwards, the upper leaves are nearly sessile. Stipules fused to the petiole much of their length, ovate-lanceolate, pale with dark venation and setaceous tip.
>
> Leaflets ovate, elliptic to cuneate-obovate, 1.5 to 4.0 cm long, to 1.5 cm wide, subentire.
>
> Flowers are purple, red, or white in color".

Red clover is the most important legume hay crop in the northeastern U.S. It is also used for pasture, soil improvement, and fits well into 3 and 4 year rotations (Taylor, 1973).

Trifolium subterraneum L.

History, distribution and characteristics. More than 100 years ago *Trifolium subterraneum* L. (subterranean clover) was introduced into Australia, probably from England, Portugal, Spain, Madeira, or Canary Islands. It has been successfully introduced now to other countries including New Zealand, South Africa, Argentina, Chile, Uruguay, Japan, Kenya and Venezuela. In the U.S. first introductions were probably made by the Department of Agriculture around 1921 (McGuire, 1985).

The main characteristics of subterranean clover as given in McGuire (1985) are:

> "Subterranean clover is a glabrous, prostrate, annual or winter annual, about 1 to 8 m long. The stems are soft appressed or spreading, short pilose or glabrate.
>
> Stipules are ovate, obtuse, acute to acuminate. Leaves long petioled, growing about 1 to 20 cm tall. Leaflets are broadly obcordate, 0.8 to 1.2 cm long. Peduncles axillary, elongating and reflexing and appressed to the soil or burying the inflorescence following anthesis. Flowers are white or pink in color".

IMPORTANT CLOVER VIRUSES FOUND IN VIRGINIA

Peanut stunt virus

Classification and characteristics. Peanut stunt virus belongs to the Cucumovirus group which was compiled by Harrison et al. (1971). Kaper and Waterworth (1981) revised the properties of the viruses within the group. Cucumber mosaic virus (CMV) strain

S, as described by van Regenmortel (1967), is the type member of the group. Two other viruses, peanut stunt virus (PSV) and tomato aspermy (TAV), are also included as members of the Cucumovirus group. Other possible members include: soybean stunt virus, cowpea ringspot virus, and chrysanthemum mild mottle virus. The group name 'cucumo' is derived from its type member cucumber mosaic (Edwardson and Christie, 1991).

The main characteristics of the Cucumovirus group as summarized in Matthews (1979) are:

> "A: Properties of the virus particle. Nucleic acid: Three molecules of linear positive sense ss-RNA with MWs (approximately equal to) 1.27, 1.13 and 0.82 x 10^6; 0.35 x 10^6 coat protein mRNA is also encapsidated. Protein: One coat polypeptide, MW = 24,000. Lipid: None reported. Carbohydrate: None reported. Physiochemical properties: MW (approximately equal to) 5.8 X 10^6; density in CsCl (approximately equal to) 1.37 g/cm^3; density of fixed virus in cesium sulfate = 1.304 g/cm^3 $S_{20,w}$ (approximately equal to) 99; particles readily disrupted in neutral chloride salts and by sodium dodycl sulfate; particles sensitive to ribonuclease. Morphology: Polyhedral particles, (about) 28 nm diameter, with icosahedral T = 3 surface lattice symmetry. Although all particles have approximately the same $S_{20,w}$, three different particles exist, one containing 1 molecule of RNA of MW (approximately equal to) 1.27 x 10^6, one containing 1 molecule of MW (approximately equal to) 1.13 x 10^6, and one containing 2 molecules with MWs (approximately equal to) 0.82 X 10^6 and 0.35 X 10^6. Antigenic properties: Poor immunogens.
>
> B: Replication: Sometimes virus crystals seen in vacuoles but usually no inclusions. Virus particles scattered in cytoplasm.
>
> C: Biological aspects: Host Range: Wide host range among plants. Transmission: Readily transmissible experimentally by mechanical inoculation. Seed transmission in several host plants. Transmitted by aphids in non-persistent manner."

Strains of PSV can be divided into three serotypes: one typified by strains E and V; one typified by strains W and B; and one typified by strain Tp (Beczner and Devergne, 1979). Most strains are closely related serologically to the Eastern or type strain, PSV-E (Echandi and Hebert, 1971). The Virginia strain (V) from peanut (Tolin and Boatman, 1972) has been extensively studied by serology (Devergne and Cardin, 1975; Xu et al., 1986).

Distribution and symptoms. The stunt disease of peanuts (*Arachis hypogaea* L.) was first observed in the U.S. on August 3, 1964 on a farm near Manry in Southampton County, Virginia. The disease was also observed on September 17, 1964 on a farm near Littleton in Sussex County, and in Virginia in 1965 in a 12-acre field on a farm near Moonlight in Isle of Wight County (Miller and Troutman, 1966). The first description of the virus was made by Troutman (1966) and Silbernagel et al. (1966). The disease has also been reported from various other locations in the U.S. including North Carolina (Echandi and Hebert, 1970; Cooper, 1966), South Carolina (Choopanya and Halpin, 1968) and Washington (Silbernagel et al., 1966). It has also been reported from other countries including Hungary (Beczner and Devergne, 1979) and Morocco (Fischer and Lockhart, 1978). Miller and Troutman (1966) described the symptoms on diseased peanut plants to resemble rosette, another destructive disease of peanuts found in Africa (Storey and Bottomley, 1928). They described the foliar symptoms on peanuts as reduced petiole and leaflets, upward curled or malformed leaves, necrotic leaves, yellow coloration of new leaves

when they unfolded that turned normal green, entirely chlorotic or chlorotic only on the veins. The plant exhibited severe stunting, which was seen along the entire vine, branch, or portion of the branch or leaves. Diseased plants were slightly less green in color than the healthy. The fruit production was reduced and the peanuts were small, malformed and had split pericarp walls (Miller and Troutman, 1966).

Host range and crop losses. Peanut stunt virus has been isolated from naturally infected white clover (*Trifolium repens* L.) (Hebert, 1967; Tolin et al., 1970), beans (*Phaseolus vulgaris* L.) (Echandi and Hebert, 1970), crown vetch (*Coronilla varia* L.) (Tolin and Miller, 1975) and soybean (*Glycine max* (L.) Merr.) (Milbrath and Tolin, 1977). In white clover the symptoms range from a light to dark green mosaic, reduced primary and secondary stolon, nodes, leaves, petiole and root lengths. Primary leaves of 'Henderson' lima bean (*Phaseolus limensis* MacF.) and 'Bountiful' bean (*P. vulgaris* L.) showed chlorotic spots and occasional rings (Miller and Troutman, 1966). *T. incarnatum* L., *T. repens* L., *T. pratense* L., *T. micranthum* L., *T. resupinatum* L., *T. subterraneum* L., T. *dubium* Sibth. and *T. alexandrinum* L. showed stunting, mosaic symptoms on the leaflets and reduction in number of leaves and stems. In *T. repens*, PSV also reduced flowering (Choopanya and Halpin, 1968). Strap-like malformed leaflets resembling 2,4-D injury were seen on *Lycopersicon esculentum* Mill., *Sesbania exaltata* (Raf.) Cory and certain bean varieties (Silbernagel et al., 1966). In tobacco, the whole

leaves or part of the leaves become yellow (a symptom known as 'white top') and stunted. Leaves of infected plants showed a mosaic pattern of green and yellow areas. The virus is also transmitted through seed (Troutman et al., 1967), dodder (Miller and Troutman, 1966), and by aphids, such as *Myzus persicae* and *Aphis craccivora* (Isakson, 1970) and *Aphis spiraecola* (Hebert, 1967). Studies have shown that PSV and aphids, especially *A. craccivora*, overwinter in white clover and aphids may transmit PSV to neighboring peanut plants (Tolin et al., 1970).

PSV plays a significant role in reducing yield of all crops and stand persistence of many forage legumes, including clover. In 1964, the stunt disease caused a reduction of the marketable nuts in 2 separate fields by 10% and 50% respectively and also an estimated 95% loss to VA 56-R peanuts in 1966 (Miller and Troutman, 1966). Considerable effort was made to prevent the spread of the virus in white clover in the field (Gibson et al., 1982). In the experiment conducted for PSV, yield losses in white clover averaged 28% over a 12 month period. In growth chamber-related studies, PSV caused significant reductions in leaf dry weight yields and nodulations at 25 and 30C (Gibson et al., 1981).

Bean yellow mosaic virus and clover yellow vein virus

Classification and characteristics. Bean yellow mosaic virus (BYMV) and clover yellow vein virus (CYVV) belong to the potyvirus group, the type member of which is potato virus Y (Edwardson and Christie, 1991) which is the largest and "economically most

important" group of plant viruses (Harrison et al., 1971). The group contains, as of 1992, 47 members (Edwardson and Christie, 1991), 153 possible members (Martelli, 1992) and 29 viruses (Edwardson and Christie, 1991) which have yet to be assigned to the group by the Plant Virus Subcommittee of the International Committee on Taxonomy of Viruses. The term "possible members" applies to viruses exhibiting characteristics of potyviruses which may be distinct members of the group, or they may be strains or synonyms of group members. The term does not imply that the "possible members" might be members of some other plant virus group. The identification, classification and nomenclature of potyviruses has been in a very unsatisfactory state due to the large size of the group, the apparent wide variation among the viruses in the group, serological cross-reactions between members and the inability of workers to understand the taxonomic parameters that will distinguish distinct viruses from strains (Barnett et al., 1985; Moghal and Francki, 1976; Shukla and Ward, 1988; Shukla and Ward, 1989; Tracy et al., 1992).

The main characteristics of the potyvirus group as given in Edwardson and Christie (1991) are:

> "A: Properties of the virus particle. Nucleic acid: One molecule of linear positive-sense ss RNA. MW = 3.0-3.5 X 10^6; 5% by weight of particle. RNA molecules of some viruses have poly (A) tracts at their 3' ends. Protein: One coat polypeptide, MW = 32-36 X 10^3. Lipid: None reported. Carbohydrate: None reported. Physiochemical properties: $S_{20,w}$ = 150-160; density in CsCl = 1.31g/cm^3. Morphology: Flexuous filaments 680-900 nm long and 11 nm wide, with helical symmetry and pitch = 3.4 nm. Antigenic properties: Moderately immunogenic;

serological relationships between some members.

B: Replication: Characteristic cylindrical or conical inclusions, appearing as pinwheels when seen in transverse section, are induced in the cytoplasm; protein of inclusions (MW = 70 X 10^3) serologically unrelated to virus coat protein but specified by viral genome. Some members also induce nuclear inclusions. RNA from some members has been translated in vitro into proteins of MW corresponding to more than 90% of the genome coding potential.

C: Biological aspects: Host range: Narrow for individual viruses. Transmission: Transmissible experimentally by mechanical inoculation; transmitted by aphids in a non persistent manner. Others very tentatively included as possible members of the group are transmitted by whiteflies, mites or fungi."

The potyvirus group has been separated into four subdivisions on the basis of the cylindrical inclusion structures (Edwardson, 1966; Edwardson, 1974). It was recognized as a main characteristic of the group by the ICTV in 1976 (Fenner, 1976). The BYMV, CYVV and PMV (Pea mosaic virus strain of BYMV) have been placed in the subdivision II of Edwardson's classification of potyviruses (Edwardson, 1974; Moghal and Francki, 1976). These three viruses have many properties in common, such as overlapping host ranges and pathogenicity (Barnett et al., 1987; Bos et al., 1974; Bos et al., 1977), pinwheel inclusions that are similar morphologically and serologically (Edwardson and Christie, 1991) and form nuclear inclusions (Chang et al., 1988). In the U.S. BYMV-204-1, BYMV-Scott and CYVV-Pratt are well-characterized isolates. BYMV-204-1 was originally isolated from red clover (Jones and Diachun, 1977), BYMV-Scott was isolated from USDA-type

isolate (Barnett et al., 1987) and CYVV-Pratt from white clover (Pratt, 1969). A distant serological relationship between CYVV and BYMV (Barnett and Gibson, 1975; Pratt, 1969), and between BYMV and PMV (Jones and Diachun, 1977) has been reported.

Description, distribution and symptoms

Bean yellow mosaic virus. Bean yellow mosaic virus (BYMV) was the first legume virus to be well described and identified (Pierce, 1934). Since then many isolates with widely varying host ranges have been reported (Bos, 1970; Bos et al., 1977; Zaumeyer and Goth, 1963). Since the work of Doolittle and Jones (1925) and Pierce (1935) it was generally accepted that PMV is a distinct entity (Bos et al., 1974), but Bos (1970) classified PMV as a strain of BYMV. Goodchild (1956) proposed that BYMV and PMV were strains of the same virus based on symptom expression produced in red clover (Bos 1970; Diachun and Henson, 1956; Leath and Barnett, 1981). However PMV has some host range differences from BYMV. For example, PMV induces a bright yellow mosaic on *Pisum sativum*, whereas BYMV induces only a mild mosaic. *Phaseolus vulgaris* is susceptible to BYMV, but not to PMV (Edwardson and Christie, 1991). However, occasionally it infected beans (Zaumeyer and Wade, 1935) but was much less pathogenic than was normal BYMV (Schroeder and Provvidenti, 1966).

BYMV is worldwide in distribution, and is most commonly occurring virus in red clover (Bos, 1970; Diachun and Henson, 1956; Leath and Barnett, 1981). The virus has also been isolated from Australia (Abu-Samah and Randles, 1981), India (Singh et al.,

1988); and the Netherlands (Bos et al., 1974). The virus has been reported in natural infections of 188 spp in 54 genera of 14 families, including 50 spp in 26 genera of the family Leguminosae (Table 19, Edwardson and Christie, 1991). Red clovers infected by BYMV exhibit systemic mottling or interveinal yellowing. There is a greater prevalence of BYMV in red or crimson perennial clovers than of CYVV (Barnett and Diachun, 1986). The virus is disseminated from red clover by aphids, especially the *Acrythosiphon pisum, Macrosiphum euphorbiae, Myzus persicae* and *Aphis fabae*, in a non-persistent manner (Barnett and Diachun, 1986). A number of strains have been described which show only differences in host range, severity of symptoms, and different types of symptoms. The strains designated B-25, E-198 (Bos, 1970), BYMV-204-1 and BYMV-Scott (Jones and Diachun, 1977) from bean, peas, and red clover are well-characterized legume isolates.

Clover yellow vein virus. In 1965, Hollings and Nariani isolated clover yellow vein virus (CYVV) from white clover in England. Subsequently the virus was isolated from white clover in Canada (Pratt, 1969) and the U.S. (Pratt, 1969; Barnett & Gibson, 1975). In 1969, Bos described pea necrosis virus (PNV) which is now considered a strain of CYVV. However it has a host range different from CYVV in *N. clevelandii* and broad bean 'Compacta'. Since the differences fell within the range of variation of a single virus, PNV was considered to be a strain of CYVV (Bos et al., 1977).

CYVV is worldwide in distribution, and is the most commonly

distributed virus in natural white clover. The virus has been reported in natural infections in Canada (Hollings and Stone, 1974); Colombia (Lawson et al., 1985); England (Hollings and Nariani, 1965); Italy (Lisa and Dellavalle, 1987), and the Netherlands (Bos et al., 1977). The virus induces symptoms of mosaic, chlorotic or occasional necrotic sectioning, or no symptoms at all (Barnett and Diachun, 1986). CYVV has been reported in natural infections of 25 spp in 14 genera of 4 families, including 21 spp in 10 genera of the family Leguminosae (Table 27, Edwardson and Christie, 1991). It is known to be transmitted by *Myzus persicae* and *Acyrthosiphon pisum* (not by *Aphis fabae*) in a non-persistent manner, but not through seed (Edwardson and Christie, 1991).

SEROLOGICAL TESTING METHODS

Classical methods

In the past scientists have developed or adapted a number of serological procedures for detection and identification of plant viruses. Details of these procedures are presented in several reviews or text books (Ball, 1974; Gibbs and Harrison, 1976; Tolin, 1977; van Regenmortel, 1982; and Voller et al., 1976). The main advantage of serological diagnosis is its specificity, rapidity, and reliability. From the earliest serological studies on plant viruses it became apparent that the antigenic specificity of individual viruses would be extremely useful for the diagnosis of virus diseases. The classical serological methods that were used for the detection of forage legume viruses were

microprecipitin, the latex agglutination, and Ouchterlony gel diffusion. The classical methods are no longer used for extensive sampling, because they use large amounts of antisera and are less sensitive.

Modern methods

Modern serology is also based on antigen-antibody reactions, but the sensitivity of detection can be increased by attaching an enzyme label to either of the two (van Regenmortel, 1982). Radioimmunoassay was the first to develop based on this principle, but, gave birth to Enzyme-Linked ImmunoSorbent Assay (ELISA). Virus detection based on ELISA was first reported by Avrameas (1969). In 1974, Voller et al. (1976) adapted ELISA to a microtiter plate method and in 1976, they first described the "double antibody sandwich" (DAS) form of ELISA. This form of detection system uses a multi-step method where first the pathogen is selectively trapped using a specific antibody that is previously attached to the plate, and using an enzyme of high sensitivity the pathogen is detected. The basic methods of DAS-ELISA as described by Clark and Adams (1977) have undergone a number of changes, with respect to the solid phase, sample preparation and basic assay methodology. Although the principle remains the same, these small variations in procedure have made major breakthroughs in the various serological techniques (Hampton et al., 1990).

The traditional "double antibody sandwich" (DAS) method was modified to an "indirect ELISA" (i-ELISA) by Keonig (1981). In

DAS-ELISA, the antibody is applied first and is attached to the plate, followed by the addition of the antigen. But in i-ELISA the antigen is added first, followed by the antibody. This change has helped in moving the virus detection from a strain level to a more broader range of detection. Ideally DAS method is more strain specific than indirect method. This is because enzyme conjugates prepared with antibodies against one virus strain cannot react with closely related strains as the binding capabilities of the antibody is reduced due to its conjugation with the enzyme (Koenig, 1978). DAS-ELISA may be advantageous when one has to differentiate between different strains of the same virus. But if a broader range of viruses have to be detected, i-ELISA would be better to use because the narrow specificity of the reaction found with direct method can be overcome. In this method the enzyme is conjugated with immunoglobulins obtained from a goat immunized with rabbit IgG, and is capable of detecting the presence of any rabbit antibody (van Regenmortel, 1982). Therefore, with the indirect method, there is no need for an antibody conjugation step. Moreover this enzyme conjugate is available commercially.

Another modification of the technique is in the solid phase. Generally with ELISA, microtiter plates are used as the solid-phase onto which the virus particles adhere. An alternative to plates has been the use of nitrocellulose membranes (Banttari and Goodwin, 1985) or even filter paper (Haber and Knapen, 1989), for the detection of viruses. The technique known as

dot-immunoassay (DIA) is also an ELISA methodology, but the difference is that a membrane is used as a solid phase, instead of the microtiter plate. Sample preparation for DIA is done by grinding the tissue in a suitable buffer as done with ELISA (Banttari and Goodwin, 1985). This is the most tedious, laborious and time consuming step. Sample preparation could be simplified by simply blotting the tissue directly onto membranes (Lin et al., 1990) to detect viruses. The blotting technique is known as tissue immunoblot assay (TIBA) and is based on the same principle as ELISA. Blotting samples directly onto membranes for virus detection has given diagnostic work a facelift, and thus it plays an important part in the future of daily diagnosis of viruses.

CHAPTER 3

MATERIALS AND METHODS

COLLECTION OF PLANTS FROM THE FIELD

During the months of June and August of 1990 and 1991, and October 1991 a number of white and red clover plants were collected from various locations in Virginia. The locations were Steeles Tavern Experimental Farm, Augusta County; Glade Spring Experimental Station, Washington County; Warsaw Experimental Station, Richmond County; Winchester, Frederick County and Blacksburg, Montgomery County. All these locations were long-term established pastures or meadowlands. A complete description of the collected plants is given in Table 1.

The white clovers from Steeles Tavern, Glade Spring and Warsaw were collected on a schematic basis as shown in Fig. 1. This design came through the S-228 Regional project. Starting from the right hand corner of the field, 3-5 entire plants, including roots, were dug from a small area, 25 cm or less in diameter, and were labelled as sample 1. After taking 10 additional steps towards the center of the field, another site was selected and the plants dug from that site were labelled as sample 2. At site 3, the direction was changed toward the left hand corner of the field, and in this way plants from five different sites were dug and collected. The collected plants were brought to the greenhouse and grown in pots during the entire study. The plants were trimmed and weeded as required.

Table 1. Original host, collection site and designation of clover plants and virus isolates

Original Host	Site	Designation
White Clover	Steeles Tavern Augusta Co.	WC\ST1 WC\ST2 WC\ST3 WC\ST4 WC\ST5
White Clover	Glade Spring Washington Co.	WC\GS1 WC\GS2 WC\GS3 WC\GS4 WC\GS5
White Clover	Warsaw Station Richmond Co.	WC\WS1 WC\WS2 WC\WS3 WC\WS4 WC\WS5
Red Clover	Winchester Frederick Co.	RC\W
Red Clover	Blacksburg Montgomery Co.	RC\B

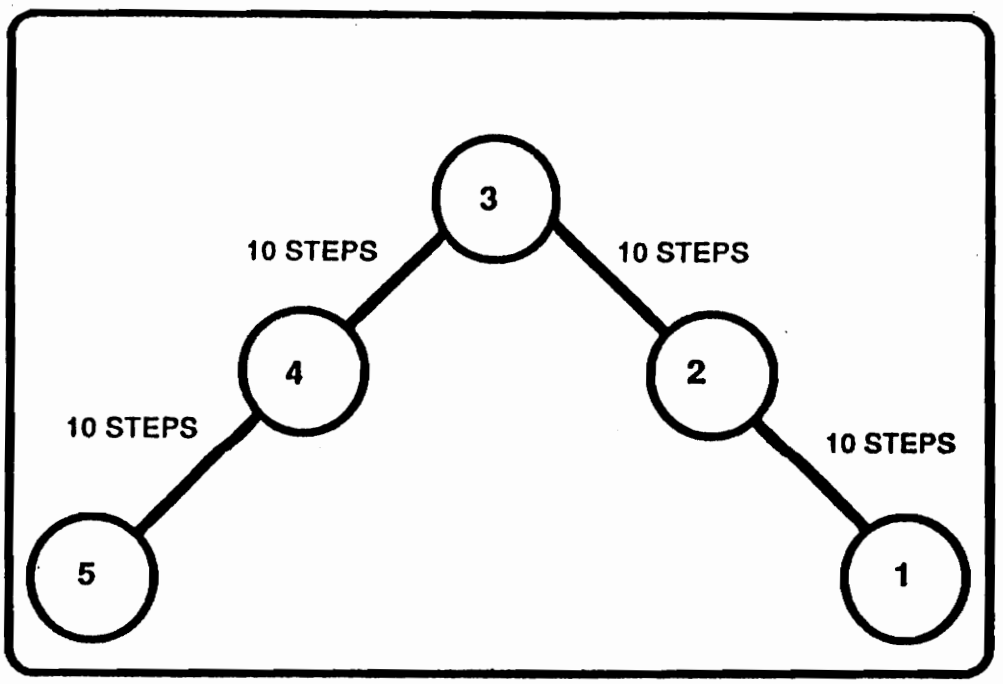


Fig. 1. Schematic diagram showing the method of field plant collection. Numbers 1-5 represent collection site for 3-5 plants.

Additional white clover plants were tested from Blacksburg, Montgomery County. These plants were grown from seeds and transplanted into a test plot in the field for use as bait plants to trap viruses transmitted by aphids.

The red clover plants were single samples collected from two different locations. One was near Winchester and other one from Blacksburg at the Glade Road Research Center.

BYMV, CYVV AND PSV: KNOWN TYPE ISOLATES AND ANTISERA

Well characterized isolates and their corresponding whole polyclonal antisera made to three viruses BYMV-204-1, CYVV-Pratt and PSV, were obtained from the following sources. Type isolates BYMV-204-1 and CYVV-Pratt and their antisera were received from O. W. Barnett (Clemson University), and a type isolate of PSV and its antiserum was received from S. A. Tolin (Virginia Polytechnic Institute & State University). Stock cultures of the three virus isolates were stored in small glass vials as diced tissue and kept at 4C over calcium chloride. At the time of inoculation the vials were brought to room temperature, and the required plants were inoculated according to the general mechanical inoculation procedures, which is described under mechanical inoculation in this chapter. BYMV-204-1 was propagated in *Pisum sativum* L. cv. Dwarf Gray Sugar, CYVV-Pratt was propagated in *Nicotiana benthamiana* Domin. or in *Pisum sativum* L. cv. Dwarf Gray Sugar, and PSV was propagated in *Vigna unguiculata* (L.) Walp. cv. California Blackeye. Working volumes of antisera were stored at 4C.

MECHANICAL INOCULATION

The virus inoculum was prepared from infected plants by triturating approximately 1-2 gms of symptomatic leaves in about 8-10 mls of 0.01 M sodium phosphate buffer, pH 7.0. Cotyledons and primary leaves of each host plant were lightly dusted with 600-mesh carborundum and then rubbed with expressed sap.

Each pot contained about 3-4 plants, and all plants were inoculated by the same method using the same expressed sap. One pot was left uninoculated to serve as a control. While inoculating *Nicotiana* and *Chenopodium* species there was only one plant in the pot to inoculate. The excess sap and remaining carborundum were rinsed from the plant with tap water.

ISOLATION OF VIRUSES FROM THE COLLECTED FIELD CLOVERS

BYMV, CYVV and PSV are known to be mechanically transmissible (Bos, 1970; Bos et al., 1974; Barnett and Gibson, 1975; Hollings and Stone, 1974; Pratt, 1969; Tolin et al., 1970; Miller and Troutman, 1966). Hence an experimental host range study was conducted under greenhouse conditions using various host species such as *Chenopodium amaranticolor* Willd., *C. quinoa* Cost & Reyn., *Cucurbita pepo* L. cv. Small Sugar (pumpkin), *Nicotiana benthamiana* Domin. (tobacco), *Phaseolus vulgaris* L. cvs. Bush Blue Lake 274 and Tennessee Green Pod (bean), *Pisum sativum* L. cvs. Alaska, Nugget, Progress and Dwarf Gray Sugar (peas), *Trifolium repens* L. cv. Tillman (white clover), *Trifolium subterraneum* L. cv. Mt. Barker (sub clover), *Vicia faba* L. cv. faba bean (broad bean) and *Vigna unguiculata* (L.) cv. California Blackeye (cowpea). Initial

virus isolation from all white clovers were made to cowpea and broad bean. Based on the results obtained (see Chapter 4) and initial indirect ELISA (Table 2, Chapter 4) additional hosts (as mentioned above) were incorporated, for samples WC\ST2, WC\ST5, WC\GS3, WC\GS4, WC\WS5, RC\W and RC\B, by selecting hosts as required for the virus infecting each sample.

Since *Trifolium pratense* (red clover) was not transplanted into pots in the greenhouse, the virus had to be mechanically transmitted to other suitable hosts for maintenance. Symptoms were observed over a period of 3-4 wks.

Seeds of *Chenopodium* were provided by O. W. Barnett, Clemson, South Carolina; *Nicotiana benthamiana* was from R. K. Jones, Raleigh, North Carolina 27695; *Phaseolus vulgaris* cv. Tennessee Green Pod was from Wetsel's Seed Co., Harrisonburg, Virginia; *Cucurbita pepo* cv. Small Sugar, *Pisum sativum* cv. Alaska and some initial seeds of Dwarf Gray Sugar, *Vicia faba* cv. faba bean, *Vigna unguiculata* cv. California Blackeye, came from Burpee, Warminster, Pennsylvania, 18974; *Phaseolus vulgaris* cv. Bush Blue Lake 274 and some later seeds of *Pisum sativum* cv. Dwarf Gray Sugar came from Southern States, Virginia; *Trifolium* cultivars came through the regional project S-127/228; and *Pisum sativum* cvs. Nugget & Progress was provided by Dr. Ruth Alscher, Virginia Polytechnic Institute & State University.

CHECKING FOR VIRUS PARTICLES IN ELECTRON MICROSCOPE

Some of the virus isolates (not from white clover) were examined to determine the morphology of the viruses present in

tissue. With forceps, thin epidermal peels were made from the leaf. The torn surface was allowed to touch a water droplet on a carbon-formvar coated grid, and adhering particles were negatively stained using 1% uranyl acetate. The stained grids were viewed using a Zeiss EM-10 C transmission electron microscope.

ANALYZING FOR VIRAL PROTEINS IN THE CLOVER PLANTS

All the white clover isolates and one red clover isolate (RC\W) were prepared for a total protein analysis. The protein isolation was done following the Alper et al. (1984) method. Extracted proteins were stored at -20C until further use.

Viral proteins were analyzed by SDS-PAGE gel electrophoresis, following the Laemmli system (Laemmli, 1970). To each protein sample one-third volume of disruption buffer containing 0.062M tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue was added. The mixture was boiled for 3 min and kept immediately on ice. The samples were microcentrifuged for 1 min prior to applying them on the gels. A 12% "resolving gel" and 4% "stacking gel" with 0.1% SDS was used. Electrophoresis was done using a Hoeffer Scientific SE 200, "mighty small" gel apparatus. All the gels were run at a constant 100 volts for 1.5 hrs. The gels were stained using 0.2% Coomassie brilliant blue R 250 in 50% methanol and 30% glacial acetic acid, and destained using 7.5% glacial acetic acid and 5% methanol.

The following marker proteins were used: Ovalbumin (43,000) and Bovine serum albumin (66,000).

DETECTION OF BYMV, CYVV AND PSV USING INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY (i-ELISA)

Indirect ELISA was done on polystyrene microtiter plates (Immulon 1, Fisher Scientific) with the following modification of the various methods (Barbara and Clark, 1982; McLaughlin et al., 1984). The procedures for BYMV and CYVV were similar and are as follows. Samples were ground at 1:50 (w/v) in carbonate buffer (0.015M Na_2CO_3, 0.034M $NaHCO_3$, pH 9.6) with 1% PVP using a Tissuemizer (Tekmar, Cincinnati, OH). Whenever the antigen cross-reacted with both BYMV and CYVV antisera, a two fold dilution of the sap extract was done as follows. From the 1:50 ground sap, a 2-fold serial dilution was made up to 1:102,400 in the same carbonate buffer. Any change in the dilution series is given in the results section. All the tests included a known positive, healthy negative and buffer controls.

The assay was initiated with three successive, 3 min rinses of the plate in Phosphate buffered Saline - Tween (PBS, 0.137M NaCl, 0.0147M KH_2PO_4, 0.01514M Na_2HPO_4, 0.00268M KCl, pH 7.4 with 0.05% Tween 20), followed by adding 200ul of the ground sample to respective wells. The plate was then incubated for at least 1 hr at 37C. After three successive washes in PBS-Tween as done earlier, 100ul of the viral-specific whole antisera diluted 10^{-4} to 10^{-5} in a buffer containing PBS-Tween, 2% PVP and 0.2% BSA was added and the plate was incubated at 4C overnight.

The following day the plate was rinsed in PBS-Tween as before, then 100ul of secondary antibody, goat antirabbit IgG (GAR

F(ab'$_2$), Sigma Chemical Co., St. Louis, MO), diluted 1:2000 in PBS-Tween, 2% PVP, 0.2% BSA were added, and incubated at 37C in a water bath for 4 hrs. Finally, following a similar rinse in PBS-Tween, the color was developed by adding 200ul of substrate consisting of p-nitrophenyl phosphate in 9.7% Diethanolamine, pH 9.8. Results were read as absorbance at 405 nm, using an EL 307 ELISA reader (Bio-Tek), and analyzed using LOTUS 123. Results were positive when the mean value of the sample exceeded the mean value of the healthy plus 3 times its standard deviation.

For PSV the ELISA procedure was similar to BYMV and CYVV except that incubations were for 1 hr in a water bath at 37C and samples (antigen) were ground in only carbonate buffer, pH 9.6. Following the addition of antigen, a blocking step of PBS and 0.5% BSA for 1 hr was used. The primary antibody and secondary antibody dilutions were used as mentioned for BYMV and CYVV, but the diluent was PBS and 0.5% BSA.

DETECTION OF BYMV, CYVV AND PSV USING TISSUE IMMUNOBLOT ASSAY (TIBA)

The assay was performed by modifying a method described by Lin et al., (1990) and by Hsu and Lawson (1991). The method is based on the principle of ELISA performed on microtiter plates, but utilizes a membrane as the solid phase. Blots were made by gently pressing freshly torn surfaces of leaves, petioles and stolons of 2 naturally infected white clover samples, and only leaves of other samples, onto a membrane. Several different types of membranes were used such as nitrocellulose, 0.45-um pore size,

(Micron Separation Inc.), nytran, 0.2-um pore size (Schleicher & Schuell Inc., Keene, NH 03431), chromatography paper (3 Mm), filter paper Whatman # 1, and notepad (white) ruled paper (Advantage No. 5156, SCM Office Supplies Group, Marion, Indiana 46952). The tissue blots were developed immediately, or stored at room temperature up to one month before developing.

The entire procedure was conducted at room temperature. The blotted membranes were first rinsed in 5% Triton X-100 to remove the residual green color caused by attachment of chlorophyll-containing cell contents. This was followed by a brief rinse in PBS-Tween (PBS containing 0.05% Tween 20) followed by a 30 min rinse in PBS (0.02M K_2HPO_4, 0.15M NaCl, pH 7.4) containing 1% non-fat dry milk (Carnation) and 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The blocking solution was made fresh each time just prior to performing the assay. After blocking, the membranes were incubated with antigen-specific primary antibodies diluted 10^{-4} or 10^{-5} in PBS for 1 hr.

Following three successive washings of 10, 5 and 5 min each in a PBS-Tween solution, the membrane was incubated with alkaline phosphatase-labeled secondary antibodies (goat antirabbit F(ab'$_2$), Sigma Chemical Co., St. Louis, MO), for 1 hr. The membranes were again rinsed in PBS-Tween as before, and then incubated in the substrate solution for color development. The substrate was freshly prepared each time and consisted of 14 mg of nitroblue tetrazolium (NBT) dissolved in 300 ul methanol, and 7 mg of

5-bromo-4-chloro-3-indoyl-phosphate (BCIP) dissolved in 50 ul dimethyl sulfoxide (DMSO) in 40 ml of freshly prepared substrate buffer (0.1 M Tris, 0.1M NaCl, 5mM $MgCl_2$, pH 9.5). A positive result was indicated by the development of a purple color on the membrane. No color on the membrane indicated a negative result. Results were read with the naked eye or by using a dissecting microscope.

COST AND FEASIBILITY OF APPLYING ELISA AND TIBA

The two serological methods used in this study, namely ELISA on microtiter plates and on membranes (TIBA), were analyzed to see which method is beneficial or promising. Analysis was done based on the experience gained through performing these assays, and by consulting the literature (Lin et al., 1990; Powell, 1987) and catalogs (Fisher Scientific; Sigma Chemical Co.) that were generally used for purchasing most of the items for the assays. For analysis the cost was divided into 2 sections: capital equipment and expendable supplies. Capital equipment included items that were purchased only once during the initial establishment stage. Expendable supplies were items that were needed on a regular basis and had to be replaced when finished. Time and labor involved in performing these assays will also be compared.

CHAPTER 4

RESULTS

ISOLATION OF VIRUSES FROM FIELD CLOVERS

All white clover samples collected from Steeles Tavern (ST), Glade Spring (GS) and Warsaw (WS) were initially inoculated from white clover (WC) to cowpea and broad bean. Depending on the symptoms or initial indirect ELISA results additional host range tests were conducted with WC\ST2, WC\ST4, WC\ST5, WC\GS3, WC\GS4 and WC\WS5 samples (Table 1). The red clover (RC) sample RC\W was initially inoculated to *Nicotiana benthamiana* and sample RC\B was initially inoculated to broad bean.

White clovers collected from Steeles Tavern. When WC\ST1 and WC\ST3 were inoculated from white clover to broad bean, symptoms were seen as mild local necrotic spots 1 mo after inoculation. On cowpea, local necrotic spots, mild vein banding and mild mottling were seen by day 12 and up to 1 mo after inoculation.

WC\ST2 inoculated from white clover to cowpea did not show any symptoms. Inoculations made from WC\ST2 in white clover to broad bean showed necrotic local lesions within a week, followed by systemic chlorotic spots, mosaic, upward curl and necrotic spots on the leaves. Severe necrosis was also seen on the stem. The plant died within 3-4 wks after inoculation. Moderate local and systemic chlorosis, systemic vein clearing, vein banding, mottling and puckered leaf appearance were seen when WC\ST2 cultured in broad bean was inoculated to *Nicotiana benthamiana.*

Similar symptoms were also seen when the culture from *N. benthamiana* was inoculated back to broad bean.

Mild local chlorosis and systemic vein clearing followed by severe mosaic were seen 1 mo after inoculating Dwarf Gray Sugar pea with WC\ST2 cultured in broad bean and in *N. benthamiana*. When subterranean clover (sub clover) was separately inoculated with WC\ST2 cultured in Dwarf Gray Sugar pea and in *N. benthamiana*, symptoms seen were vein clearing, severe stunting of the entire plant, severe systemic mosaic and ruffled or unopened leaves. The plant showed complete necrosis 1 mo after inoculation, and eventually died.

Inoculations made from WC\ST2 in white clover to *Chenopodium quinoa* showed numerous local pin point chlorotic lesions and a few necrotic lesions 5 days after inoculation. After 9 days a number of local and systemic necrotic lesions were seen. The leaves with lesions abscised easily. Systemic veinal chlorosis was seen between 2-4 wks after inoculation. On *C. amaranticolor* 5 days after inoculation symptoms were similar to *C. quinoa*, but after 9 days local chlorosis and severe necrosis and lesions were seen. Systemic necrosis and spots, stunting and tip necrosis were evident on *C. quinoa* about 2 wks after inoculation. The plant died following the severe systemic necrosis.

Bush Blue Lake bean inoculated with WC\ST2 cultured in broad bean developed a mild local chlorosis, systemic mosaic, stunting and epinasty of newly developing leaves about a month after inoculation. On Alaska pea inoculated with the WC\ST2 cultured in

broad bean, chlorosis, vein banding and vein clearing were seen. Local and systemic chlorotic spots were seen on pumpkin when inoculated with WC\ST2 culture in white clover. The spots disappeared 10 days after inoculation. Little Marvel pea, inoculated with WC\ST2 cultured in Dwarf Gray Sugar pea and also with WC\ST2 culture in *N. benthamiana*, showed complete collapse of the inoculated leaves within a week after inoculation. It was difficult to say if there was systemic necrosis as the plant collapsed within a week.

At the end of the study (Jan 1992), symptoms on Dwarf Gray Sugar pea inoculated with the maintenance culture of WC\ST2 in Dwarf Gray Sugar pea showed different symptoms. The symptoms seen 1 mo after inoculation were mild local chlorosis, systemic mosaic and local and systemic necrotic spots. This WC\ST2 culture in Dwarf Gray Sugar pea was inoculated to broad bean, *N. benthamiana*, sub clover, *C. amaranticolor* and *C. quinoa*. On Dwarf Gray Sugar pea a few necrotic spots, systemic ringspots, hardly any mosaic and systemic necrosis on the leaves and systemic necrosis and spots on the stem were seen up to 1-1.5 mo after inoculation. *N. benthamiana* showed severe systemic mosaic, strap-like leaf, downward curling of leaf and a mild stunting of the newly developing leaves. Sub clover showed mosaic and moderate stunting of the plant. On *C. quinoa* and *C. amaranticolor* 5 days after inoculation, numerous tiny local chlorotic lesions were seen. About 9 days after inoculation *C. quinoa* showed local chlorosis and necrotic patches; and *C. amaranticolor* showed systemic veinal

chlorosis. About 2 wk after inoculation *C. amaranticolor* showed fawn-colored spots on the local and systemic leaves. Most of these symptoms were similar to what was obtained with WC\ST4 (given later in this section). The symptomatically different WC\ST2 cultured in Dwarf Gray Sugar pea and *N. benthamiana* were positive by i-ELISA to PSV (data not shown), whereas previous tests were negative to PSV (Table 2).

The virus transferred from WC\ST4 induced chlorotic spots on the inoculated leaves of cowpea 3 days after inoculation, followed by necrotic spots and veinal necrosis. Within 1 wk systemic infection was seen as vein banding, puckering of leaves and mosaic. When WC\ST4 in white clover was inoculated to broad bean, mild mosaic and necrotic spots and patches were seen 1-4 wks after inoculation.

Sub clover, white clover, *N. benthamiana* and broad bean were inoculated with WC\ST4 cultured in cowpea. Sub clover showed moderate to severe stunting of the plant and systemic mosaic. White clover showed mild to moderate systemic mosaic and no stunting. *N. benthamiana* showed mosaic, stunted leaf and petiole, strap-like leaf and downward curling. The plant appeared to be normal in longitudinal growth. No symptoms were seen on broad bean. Tennessee Green Pod bean inoculated with WC\ST4 cultured in cowpea showed diffuse necrotic lesions 3-5 days after inoculation. Local veinal necrosis was also seen. Single lesions of WC\ST4 cultured in from Tennessee Green Pod bean were inoculated to cowpea and symptoms exactly resembled those produced when WC\ST4

in white clover was inoculated to cowpea. From the first single lesion inoculation, 4 additional subcultures (P1, P2-1, P2-2, P3) were obtained by performing similar single lesion on Tennessee Green Pod bean. Numerous pin point chlorotic spots were seen 5 days after inoculating *C. amaranticolor* with WC\ST4-P2-2 cultured in cowpea. Systemic veinal chlorosis was seen about 9 days later. Between 3-4 wks after inoculation a few fawn-colored lesions were seen on the inoculated leaves of cowpea.

Inoculations from WC\ST5 induced symptoms which overlapped between WC\ST2 and WC\ST4. When WC\ST5 was inoculated from white clover to cowpea and from cowpea to Tennessee Green Pod bean and sub clover, symptoms exactly resembled those produced by WC\ST4 in those hosts. Similarly when WC\ST5 was inoculated from white clover to broad bean symptoms resembled those produced by WC\ST2. The broad bean culture was inoculated to *N. benthamiana*, Bush Blue Lake bean, Dwarf Gray Sugar pea and Alaska pea. Symptoms on *N. benthamiana* resembled WC\ST2 in all respects, but differed in that there was no vein banding symptoms. On Dwarf Gray Sugar pea systemic necrotic spots were seen, which was not seen with WC\ST2. On Bush Blue Lake bean, the symptoms were similar to WC\ST2, and on Alaska pea the symptoms were similar but milder than that caused by WC\ST2. Inoculations made from white clover to Small Sugar pumpkin showed only mild local chlorotic patches. When virus in sub clover was inoculated to *C. quinoa* and *C. amaranticolor*, local chlorosis was seen *on C. quinoa* 5 days after inoculation and local pin point chlorotic lesions were seen on *C.*

amaranticolor. After 9 days systemic chlorotic lesions were evident on *C. quinoa*, and on *C. amaranticolor* necrosis appeared. About 2 wks later necrotic areas were seen on *C. quinoa* and mild veinal chlorosis and fawn-colored spots were seen on *C. amaranticolor*.

White clovers collected from Glade Spring. When white clovers were inoculated to broad bean, WC\GS1 induced local necrosis within 8 days post inoculation, WC\GS2 induced moderate local necrosis, necrotic ringspots and systemic mosaic, WC\GS3 did not show any symptoms in the beginning, but about 25 days after inoculation systemic mosaic was seen, WC\GS4 showed mild local chlorosis and necrotic spots, systemic mosaic and tiny necrotic spots which turned severe 20 days later and WC\GS5 did not show any symptoms.

All the GS cultures were inoculated from white clover to cowpea and observations were made 5 days after inoculation. WC\GS1 showed very mild chlorosis, WC\GS2 did not show any symptoms, WC\GS3 showed mild local chlorosis and veinal necrosis, WC\GS4 showed mild local chlorosis and veinal necrosis and WC\GS5 showed very mild chlorosis and local necrosis. No other symptoms were seen later on any of these isolates.

When WC\GS3 cultured in broad bean was inoculated to Dwarf Gray Sugar pea symptoms were evident as local chlorosis, systemic mosaic and necrotic spots 10 days after inoculation. Similar symptoms, with the exception of necrotic spots, were evident 10 days later on Dwarf Gray Sugar pea inoculated with WC\GS4 cultured

in broad bean. Inoculations made from WC\GS4 cultured in Dwarf Gray Sugar pea to Bush Blue Lake bean resulted in moderate local chlorosis, systemic mild mosaic, epinasty and reduction in size of the new leaves about 20 days later. WC\GS4 cultured in Dwarf Gray Sugar pea was also inoculated to sub clover and symptoms were evident as systemic vein clearing, mosaic, severe stunting and ruffling or unopened leaves. *N. benthamiana* inoculated with WC\GS4 cultured in Dwarf Gray Sugar pea, developed mild chlorosis and puckered leaves. Similar inoculation of WC\GS3 to *N. benthamiana*, gave local chlorosis, systemic necrotic spots and leaf tip curl. The Glade Spring clovers were not tested on *Chenopodium* species.

White clovers collected from Warsaw Station. When Warsaw white clovers were inoculated to broad bean, WC\WS1 showed local necrosis and systemic ringspots, WC\WS2 showed systemic ringspots, WC\WS3 showed local necrotic spots and systemic ringspots, WC\WS4 showed systemic mild flecking on leaves and WC\WS5 showed few necrotic spots, systemic mosaic and mild necrotic spots. About 20 days later the entire plant became severely necrotic and died.

Similarly when the white clovers were inoculated to cowpea, WC\WS1 showed mild local veinal necrosis, WC\WS2 showed only one necrotic spot, WC\WS3 showed mild veinal necrosis, WC\WS4 did not show any local symptoms but only systemic mild chlorosis and darkening of leaves and WC\WS5 showed mild veinal necrosis and white systemic flecking. The Warsaw clovers were not tested on *Chenopodium* species.

Red clover collected from Winchester. Inoculations made from RC\W to Small Sugar pumpkin did not show any clear symptoms, but on *N. benthamiana* mild mosaic followed by severe systemic chlorosis were seen. In the initial transfers the plant died within 1 wk after inoculation, but later during the study, symptoms weakened on *N. benthamiana*. When RC\W cultured in *N. benthamiana* was inoculated to sub clover, mild yellow mosaic symptoms with no stunting were evident, and when inoculated to white clover and Small Sugar pumpkin, no symptoms were seen.

Inoculations made from RC\W cultured in sub clover to Dwarf Gray Sugar pea showed mild systemic mosaic with some vein banding and yellowing symptoms. When Nugget and Progress pea were inoculated with RC\B cultured in sub clover, symptoms on Progress pea were more severe and were seen earlier than on Nugget pea. Symptoms were seen as mild vein clearing, downward leaf curl and green mottle, which turned bright yellow within 25 days after inoculation.

Red clover collected from Blacksburg. When RC\B was inoculated from red clover to broad bean very mild mosaic was seen. Inoculations made with RC\B cultured in broad bean to sub clover showed moderate mosaic, few necrotic spots and severe stunting of the entire plant. When RC\B cultured in sub clover was inoculated to Dwarf Gray Sugar pea, systemic bright mottle was seen. On *C. quinoa* and *C. amaranticolor* large local chlorotic spots were seen 5 days after inoculation. On *C. quinoa* 9 days later mild necrotic spots were seen. About 2 wks later systemic

chlorotic spots and necrotic areas were seen on *C. quinoa* and on *C. amaranticolor* systemic veinal chlorosis, fawn colored areas at leaf edges were seen. Cowpea was not tested.

ELECTRON MICROSCOPY

Long flexuous rods were seen from WC\ST2 and WC\ST5 cultured in Dwarf Gray Sugar pea. The size of the particles were not determined. Similarly flexuous rods were also seen from RC\W cultured in Dwarf Gray Sugar pea. The virus particles ranged from 500-800 nm. The most frequently found length was about 650 nm. Spherical particles were seen with sample WC\ST4 cultured in sub clover.

VIRAL PROTEIN EXTRACTION

The Steeles Tavern white clovers WC\ST2 and WC\ST5 showed a protein band of approximately 33 kDa. WC\ST5 showed an additional minor band of 28-29 kDa. Though WC\ST4 did not show any band from the white clover sample, a protein band measuring 24 kDa was seen when extractions from cowpea were used. WC\ST1 and WC\ST3 did not show any distinct band. A 34 kDa band was seen from WC\GS1 and a 32 kDa and a weak 29-30 kDa from WC\GS4. No band was seen from WC\GS2 or WC\GS3. From WC\WS1 and WC\GS4 a 29 kDa was seen and from WC\GS5 a 30 kDa. No band was seen from WC\WS2 or WC\WS3. The absence of protein band with certain samples confirms that no virus was present, and for those samples that gave a protein band it indicates the presence of virus/es. Ideally PSV should have a protein band equal to 24 kDa and potyviruses should have a band approximately equal to 30 kDa.

DETECTION OF BYMV, CYVV AND PSV BY i-ELISA

The white clover (WC) samples from Steeles Tavern (ST) were tested initially against PSV (whole) in Feb 1991, and against CYVV-Pratt (IgG) antisera during Mar 1991. The samples were tested against CYVV-Pratt (whole) and BYMV-204-1 (whole) antisera for the first time during the month of Sept 1991. Samples from Glade Spring (GS) and Warsaw (WS) were tested initially during the months of Oct 1991 for PSV and Dec 1991 for BYMV and CYVV. Red clover (RC) from Winchester was tested initially in Oct 1991 against BYMV, CYVV and PSV and similarly Blacksburg red clover was tested in Nov 1991. All the samples were ground at 1:50 dilution.

Testing against BYMV-204-1 antiserum at 10^{-4} dilution. All white clover samples from Steeles Tavern when tested in Sept 1991 against BYMV-204-1 antiserum gave negative results except for WC\ST2 which gave mild positive reactions (Table 2). Tests done from other hosts inoculated with WC\ST2 also gave positive reactions. For example the culture in *N. benthamiana* gave A_{405} value of 0.821 in Oct 1991 test and the culture in broad bean gave a value of 0.758 in Nov 1991 test.

When WC\ST2 and WC\ST5 cultured in Small Sugar pumpkin were tested in July 1991, WC\ST2 was negative, whereas WC\ST5 was mildly positive. WC\ST5 cultured in *N. benthamiana* was tested in Dec 1991 and gave a A_{405} value of 1.111. The samples from Glade Spring gave negative results except for WC\GS4 when tested in Dec 1991 (Table 2). An A_{405} value of 1.150 was also obtained from the same culture in broad bean when tested in Dec 1991.

Table 2. Initial serological detection of viruses from clover using indirect enzyme-linked immunosorbent assay[a]

	Antisera[b]					
Antigen	BYMV-204-1		CYVV-Pratt		PSV	
WC\ST1	0.105[c]	-[d]	0.163	-	0.323	++
WC\ST2	0.162	+	0.524	+++	0.098	-
WC\ST3	0.121	-	0.167	-	0.617	+++
WC\ST4	0.127	-	0.186	+/-	0.824	++++
WC\ST5	0.076	-	0.098	-	0.500	+++
WC\GS1	0.052	-	0.073	-	0.192	+/-
WC\GS2	0.060	-	0.122	-	0.188	+/-
WC\GS3	0.098	-	0.112	-	0.189	+/-
WC\GS4	0.479	++	0.632	+++	0.152	-
WC\GS5	0.053	-	0.156	-	0.213	+
WC\WS1	0.089	-	0.117	-	0.153	-
WC\WS2	0.052	-	0.093	-	0.150	-
WC\WS3	0.050	-	0.105	-	0.120	-
WC\WS4	0.140	+/-	0.206	+	0.125	-
WC\WS5	0.278	+	0.577	+++	0.126	-
RC\B-SC	0.703	+++	0.685	+++	0.122	-
RC\W-SC	0.238	+	0.471	++	0.099	-

[a]200 microliters of antigen and substrate buffer were added, 100 microliters of whole polyclonal antibody 1:10,000 and goat antirabbit 1:2000 were added to each well.
[b]BYMV-204-1 = bean yellow mosaic virus. CYVV-Pratt = clover yellow vein virus. PSV = peanut stunt virus. All are North American type isolates.
[c]Mean absorbance values at 405nm from duplicate wells after 30 min substrate incubation.
[d]Response given as + or -, based on + being greater than the mean of healthy reading plus 3 standard deviations.
+/- = weak, + = mild, ++ = moderate, +++ = moderately strong, ++++ = strong

Among the Warsaw samples, all were negative except for a mild positive reaction from WC\WS4 and WC\WS5 (Table 2).

Sample RC\W cultured in sub clover (RC\W-SC) gave a low A_{405} value at 1:50 sap dilution during Oct 1991 test (Table 2), but on repeating the test it was found that the A_{405} value increased as the sap dilution decreased (Fig 3, will be discussed later).

Similarly RC\W in *N. benthamiana* also reacted with a low A_{405} value at 1:50 sap dilution when tested during Oct 1991. RC\B cultured in sub clover (RC\B-SC) gave high value when tested during Nov 1991 (Table 2). Sample RC\B in Dwarf Gray Sugar pea also gave high value against the antiserum when tested in April 1992 (data given elsewhere pg. 48).

Testing against CYVV-Pratt at 10^{-4} antiserum dilution. The white clover samples from Steeles Tavern when tested against CYVV-Pratt (IgG) at 1:5000 antiserum dilution gave A_{405} value of 0.482 for WC\ST2 and 0.466 for WC\ST5. The rest of the ST samples were negative (data not shown). But when the same set of white clovers were tested against CYVV-Pratt (whole) at 10^{-4} dilution, WC\ST2 gave moderately strong reaction, whereas WC\ST5 was negative. In addition WC\ST4 gave very mild positive reaction (Table 2).

The WC\ST2 and WC\ST5 cultures in *N. benthamiana*, Alaska pea and broad bean tested during Feb 1991 gave strong positives against CYVV-Pratt (IgG) antiserum, but Bush Blue Lake when tested during the same time and also later gave negative results. None of them were tested against BYMV antiserum. When WC\ST2 and

WC\ST5 cultured in Small Sugar pumpkin were tested against CYVV during July 1991, WC\ST2 tested negative but WC\ST5 gave very mild positive reaction. Sample WC\ST4 cultured in broad bean and cowpea tested negative against CYVV (ELISA results for different hosts mentioned above are not shown).

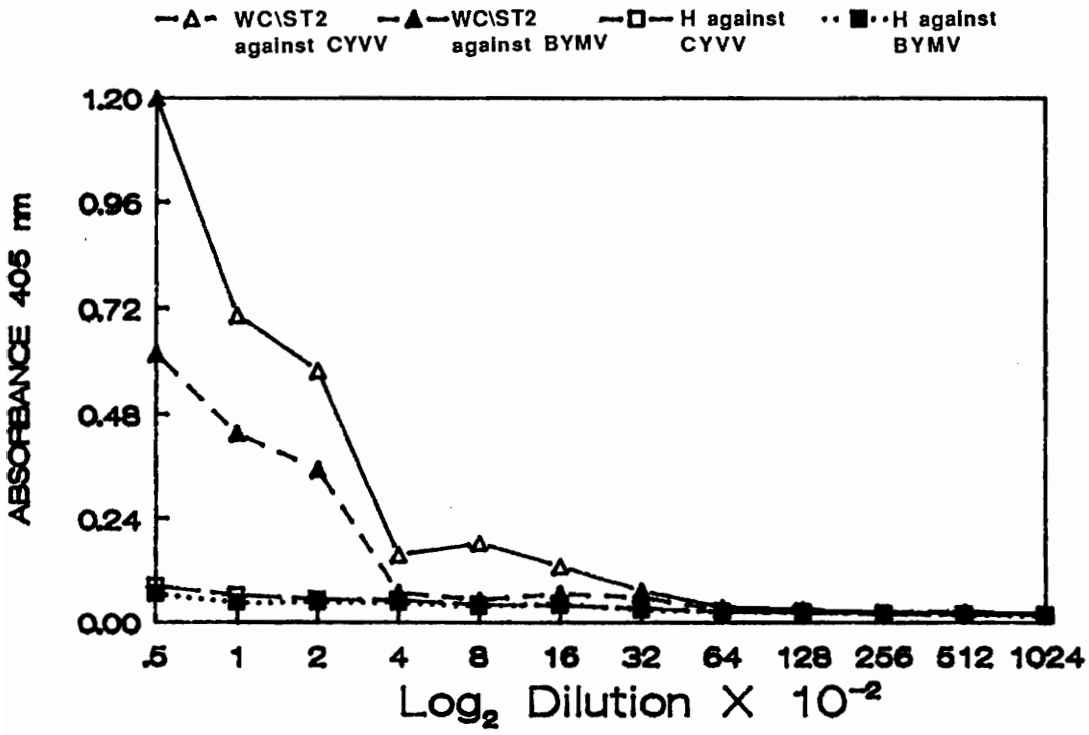
The white clover samples from Glade Spring gave negative reactions except for WC\GS4. Although WC\GS3 was negative, the same culture in broad bean gave A_{405} value of 0.662, indicating it is positive. Similarly WC\GS4 cultured in broad bean also gave a high A_{405} value of 1.201. The white clover from Warsaw, WC\WS4 and WC\WS5 gave mild and moderately strong positive reactions respectively (Table 2).

The red clover sample RC\B cultured in sub clover (RC\B-SC) gave lower value against CYVV antiserum than against BYMV antiserum, whereas RC\W gave low values against BYMV antiserum and a slightly higher value against CYVV antiserum (Table 2).

Effect of antigen dilution showing the ability to distinguish between BYMV and CYVV. The Steeles Tavern samples WC\ST2 and WC\ST5 showed a high level of cross-reaction between BYMV and CYVV antisera as indicated in Table 2 and in later tests, hence were tested further by diluting the antigen. The Warsaw and Glade Spring white clover samples were not tested. The red clover samples RC\W and RC\B were also tested.

At 1:50 sap dilution the absorbance value of WC\ST2 against CYVV-Pratt (Fig. 2) was higher than against BYMV-204-1 antiserum (Fig. 2). As the sap dilution increased the absorbance decreased.

Fig. 2. Reactivity of WC\ST2 culture in white clover in indirect enzyme-linked immunosorbent assay (i-ELISA) showing the effect of antigen dilution. The culture and a corresponding healthy white clover (H) were ground in coating buffer pH 9.6 with 1% PVP at 1:50 (w/v) and then 2-fold serial dilutions were made until 1:102,400. They were tested with antisera against CYVV-Pratt and BYMV-204-1 diluted to 1:10,000.

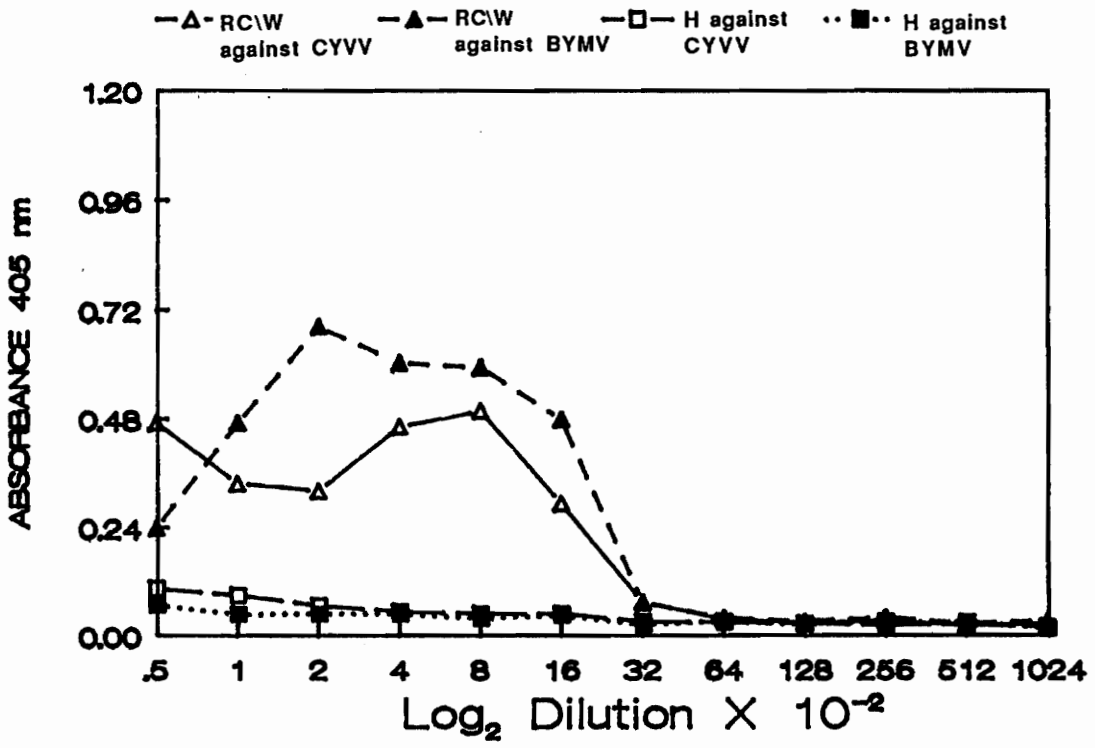

—Δ— WC\ST2 against CYVV
—▲—WC\ST2 against BYMV
—□— H against CYVV
··■·· H against BYMV
ABSORBANCE 405 nm
1.20
0.96
0.72
0.48
0.24
0.00
.5
1
2
4
8
16
32
64
128
256
512
1024
Log2 Dilution X 10-2

At 1:400 sap dilution the A_{405} was almost equal to a healthy when tested against BYMV antiserum, and at 1:3200 sap dilution against CYVV antiserum. However in a similar test done during Sep 1991 and Dec 1991, there were no cross-reaction of WC\ST2 with BYMV antiserum. In that test the A_{405} value against CYVV-Pratt antiserum was 0.522 at 1:50 and 0.080 at 1:50 against BYMV antiserum. The type isolate CYVV-Pratt in sap was tested in a separate test. The CYVV-Pratt cultured in *N. benthamiana* gave an A_{405} value of 1.288 at 1:50, 1.256 at 1:100, 1.093 at 1:200, 0.790 at 1:400, 0.248 at 1:800 and decreased thereafter.

Sample RC\W cultured in sub clover (RC\W-SC) at 1:50 gave a lower A_{405} value against BYMV and a higher A_{405} value against CYVV (Fig. 3). But as the sap dilution increased to 1:100 the value was higher against BYMV and lower against CYVV. RC\B cultured in sub clover also showed an increase in absorbance value against BYMV at 1:100 (A_{405} value 0.937) and a decrease against CYVV antiserum at 1:100 (A_{405} value 0.431). However the A_{405} value at 1:50 was higher against BYMV (0.850) and lower against CYVV (0.477). Similarly in Dwarf Gray Sugar pea the same sample gave A_{405} value of 1.297 at 1:50 against BYMV and 1.099 against CYVV. But at 1:1600 against CYVV the A_{405} value was 0.243 and against BYMV it was 0.464.

With WC\ST5 the absorbance at 1:50 against CYVV was 1.046 and against BYMV was 0.470. But at 1:400 against CYVV A_{405} was 0.358 but against BYMV it was 0.100.

Fig. 3. Reactivity of RC\W cultured in subterranean clover in indirect enzyme-linked immunosorbent assay (i-ELISA) showing the effect of antigen dilution. The culture and a corresponding healthy subterranean clover (H) were ground in coating buffer pH 9.6 with 1% PVP at 1:50 (w/v) and then 2-fold serial dilutions were made until 1:102,400. They were tested with antisera against CYVV-Pratt and BYMV-204-1 diluted to 1:10,000.


RC\W against CYVV
RC\W against BYMV
H against CYVV
H against BYMV
ABSORBANCE 405 nm
1.20
0.96
0.72
0.48
0.24
0.00
.5
1
2
4
8
16
32
64
128
256
512
1024
Log2 Dilution X 10-2

Testing against PSV at 10^{-4} antiserum dilution. When the white clover samples were tested against antiserum to PSV, WC\ST1, WC\ST3, WC\ST4 and WC\ST5 gave positive reactions (Table 2). WC\ST4 and WC\ST5 cultures in cowpea (single lesions and linear passages) and the cultures in sub clover tested positive. Values for sub clover were higher than the original white clovers. The WC\ST4 cultured in *N. benthamiana* was not tested against PSV and the same culture in broad bean tested negative against PSV (data not shown). All the samples from Glade Spring, Warsaw and the red clovers tested negative (Table 2). But WC\WS4 gave mild positive reactions from the culture tested in cowpea (data not shown).

White clover samples from the test plot used as bait for viruses gave mild positives to PSV from three of the samples. Randomly selected white clover samples from the field close to field grown beans also gave positive results with PSV antiserum (data not shown).

INFLUENCE OF SEASON ON RETESTING CLOVERS FOR VIRUSES USING i-ELISA

The clover plants from Steeles Tavern which had been maintained in the greenhouse since 1990/1991 were tested again for the 3 viruses during Apr 1992. The samples from Glade Spring and Warsaw were tested during Nov 1991 for PSV and Apr 1992 for BYMV and CYVV. The red clover sample RC\W cultured in subterranean clover (RC\W-SC) was tested during Apr 1992 for all the 3 viruses, and similarly sample RC\B cultured in subterranean clover (RC\B-SC) was tested for BYMV and CYVV during Apr 1992 and for PSV during Nov 1991. Results are shown in Table 3. Summer tests were

Table 3. Influence of season on retesting the clovers for viruses by indirect enzyme-linked immunosorbent assay[a]

	Antisera[b]		
Antigen	BYMV-204-1	CYVV-Pratt	PSV
WC\ST1	0.190[c] +[d]	0.401 ++	0.253 +
WC\ST2	0.530 +++	1.073 ++++	0.096 -
WC\ST3	0.138 -	0.201 +	0.632 +++
WC\ST4	0.194 +	0.140 +/-	0.690 +++
WC\ST5	0.539 +++	1.073 ++++	0.426 ++
WC\GS1	0.140 -	0.188 +/-	0.080 -
WC\GS2	0.235 +	0.757 +++	0.076 -
WC\GS3	0.154 +/-	0.174 +/-	0.075 -
WC\GS4	0.249 +	0.713 +++	0.072 -
WC\GS5	0.179 +/-	0.170 +/-	0.073 -
WC\WS1	0.067 -	0.124 -	0.074 -
WC\WS2	0.062 -	0.096 -	0.078 -
WC\WS3	0.078 -	0.054 -	0.071 -
WC\WS4	0.068 -	0.145 +/-	0.068 -
WC\WS5	0.633 +++	0.913 ++++	0.080 -
RC\B-SC	1.254 ++++	0.711 +++	0.125 -
RC\W-SC	1.057 ++++	0.569 +++	0.100 -

[a] 200 microliters of antigen and substrate buffer were added, 100 microliters of whole polyclonal antibody 1:10,000 and goat antirabbit 1:2000 were added to each well.

[b] BYMV-204-1 = bean yellow mosaic virus. CYVV-Pratt = clover yellow vein virus. PSV = peanut stunt virus. All are North American type isolates.

[c] Mean absorbance values at 405nm from duplicate wells after 30 min substrate incubation.

[d] Response given as + or -, based on + being greater than the mean of healthy reading plus 3 standard deviations.

+/- = weak, + = mild, ++ = moderate, +++ = moderately strong, ++++ = strong

done during the months of June - Aug 1991.

It was generally found that the A_{405} value for the 3 viruses decreased during the winter months, and increased during spring or summer (results of summer tests not shown). The cross-reactivity between BYMV and CYVV antisera was easily demonstrated during the spring testing (compare Table 2 and 3 with respect to samples WC\ST2, WC\ST5, WC\WS5, RC\B-SC and RC\W-SC). The absorbance value of PSV appears to drop more than BYMV or CYVV during winter (Eg. compare Table 2 & 3 for Warsaw (WS) and Glade Spring (GS) samples).

IDENTIFICATION AND DESIGNATION FOR VIRGINIA ISOLATES

Only 1 or 2 samples were selected to represent each of the virus. Based on host range and i-ELISA results the Virginia isolates were given the following designations. They are: CYVV-WC\ST2, PSV-WC\ST4, CYVV-WC\GS3, CYVV-WC\GS4, CYVV-WC\WS5, BYMV-RC\B and BYMV-RC\W.

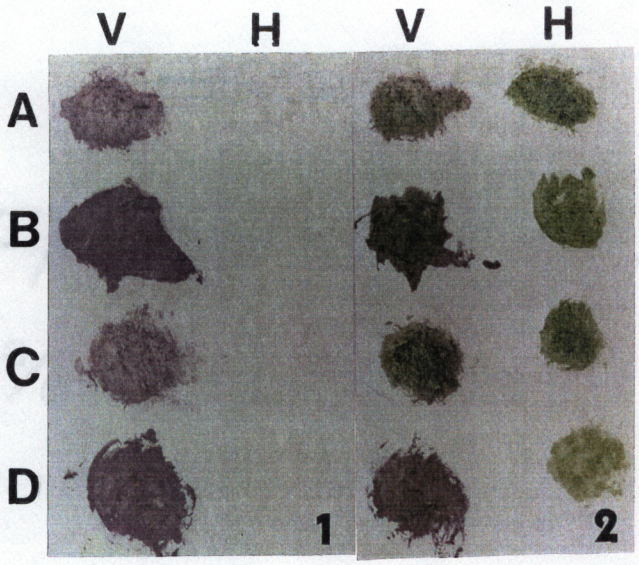
DETECTION OF BYMV, CYVV AND PSV USING TIBA

Sample preparation and application. A number of methods were experimented with sample preparation to find out which is the easiest and at the same time gives the best results. In the beginning leaf samples were rolled tightly while wearing gloves, and sections were made using a new razor blade, thereby following the Lin et al. (1990) method. The razor blade technique was tried for a couple of times, but the method was painful as the blade had to be rinsed in alcohol between samples or new blades had to be used for each and every sample. So I tried the squash technique,

where an applicator stick or a pointed toothpick was used to simply squash the tightly rolled sample on the membrane. Both applicator stick and toothpick were easy to use, but they created a small dent on the membrane while applying the samples. Therefore I stopped using the application aids and tried to blot the rolled samples directly with my gloved hands. As before leaves were rolled tightly while wearing gloves and the rolled leaf was torn in half and the torn leaf edge was pressed directly onto the membrane. Care was taken to avoid touching any part of the glove to either the membrane or the torn leaf edge. If the samples were torn off carefully, it was found that there was absolutely no need to change the gloves between samples. But if by any chance there was contamination on the glove, either by pressing or tearing off the samples, there were some false reactions (data not shown). Generally clover samples consisted of 9 leaves. All the results shown with tissue blots were prepared by tearing the sample.

Effect of Triton X-100 in removing residual green. Membranes were found to bind chlorophyll, which in excess, interfered with the interpretation of the results (Lin et al. 1990). I found that residual green color could be effectively removed by rinsing the membranes soon after blotting in 5% Triton X-100 (Fig. 4). Though Fig. 4 shows results obtained only with CYVV, similar results were obtained with other antisera. The time needed for rinsing varied with membrane type. Generally it was found that if the blots are pressed heavily on the membrane, it took a longer time to remove

Fig. 4. Effect of 5% Triton X-100 rinse on removal of residual green with clover yellow vein virus (CYVV). Membrane rinsed prior to the blocking step (Panel 1). Membrane not rinsed (Panel 2). Virus isolates cultured in the following hosts were blotted on both the membranes as follows. VA = CYVV-Pratt in subterranean clover, VB = CYVV-Pratt in Dwarf Gray Sugar pea, VC = CYVV-WC\ST2 in white clover, VD = CYVV-WC\ST2 in *Nicotiana benthamiana*. H column = corresponding healthy leaf.


V
H
V
H
A
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C
D
1
2

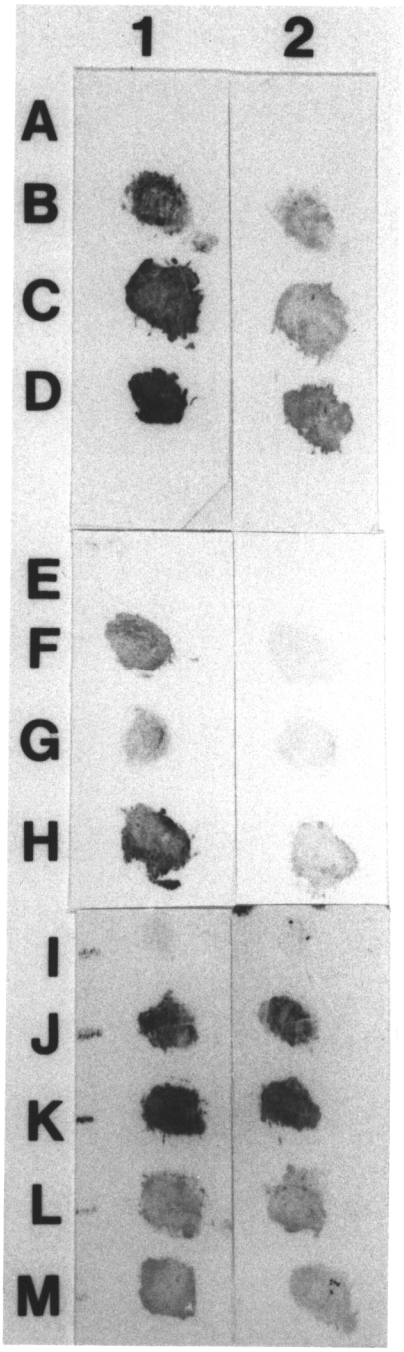
the green. On an average it took about 5-15 min to remove all the green residual color on a freshly blotted membrane. It took about 15 min or more to remove the green from a blotted membrane stored over a period of time.

Detection of BYMV, CYVV and PSV using antiserum at 10^{-4} and 10^{-5}. Tissue blots could detect the three viruses (Fig. 5) even at 10^{-5} antisera dilution. The intensity of the spot was darker at 10^{-4} dilution whereas at 10^{-5} they were lighter. Since the spots were darker at 10^{-4} dilution further tests were performed using only this dilution. Neither antiserum dilution gave any appreciable reaction with healthy leaves of several species.

Detection on different membranes. A number of different membranes were tested for their ability to detect the three viruses (Fig. 6). Generally speaking, all the membranes were capable of binding sufficient viral antigen for the detection of different viruses. However the nitrocellulose was preferable to the rest of the membranes because it gave no purple background color. The filter paper, ordinary notepad and Whatman filter paper also gave relatively low background color. Nytran (data not shown) always showed a low background color for up to a week or two, and then slowly turned deep purple with time. This may have been because the membrane was old, and beyond the recommended shelf life of the material.

Detection of CYVV and PSV in different parts of white clover plant. The parts tested were: old leaves (OL) and its corresponding petioles (OP), newly emerging leaves (NL) and its

Fig. 5. Comparison of sensitivity of tissue immunoblot assay (TIBA) for the detection of bean yellow mosaic (BYMV), clover yellow vein (CYVV) and peanut stunt (PSV) viruses using two concentrations of virus specific whole polyclonal antisera: 1:10,000 (Column 1) and 1: 100,000 (Column 2). Primary antibody was: A-D, BYMV; F-H, CYVV and I-M, PSV. Samples blotted were A = healthy subterranean clover; B = BYMV-RC\B in subterranean clover; C = BYMV-RC\B in Dwarf Gray Sugar pea; D = BYMV-204-1 in Dwarf Gray Sugar pea; E = healthy white clover; F = CYVV-WC\ST2 in white clover; G = CYVV-WC\ST2 in broad bean; H = CYVV-Pratt in *Nicotiana benthamiana*; I = healthy white clover; J = PSV-WC\ST4 in white clover; K = PSV-WC\ST4 in subterranean clover; L = PSV-WC\ST4 in cowpea; M = PSV in cowpea.


1
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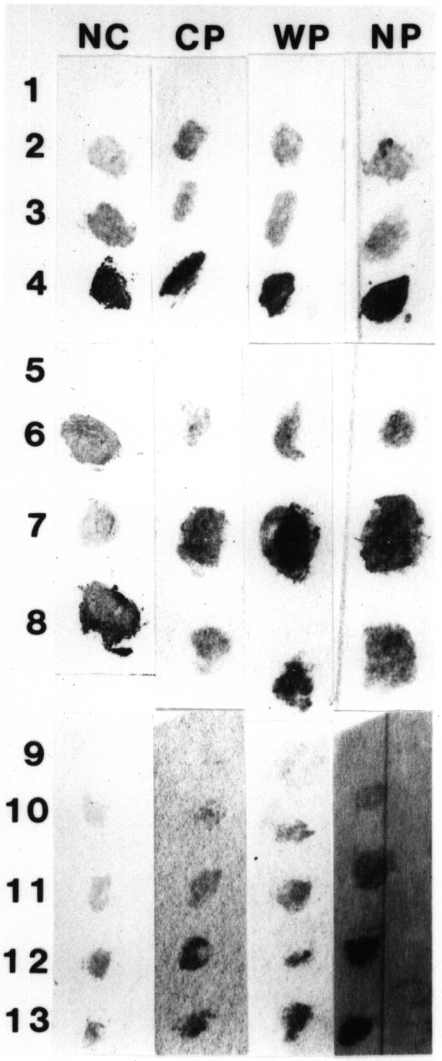
I
J
K
L
M

Fig. 6. Tissue immunoblot assay (TIBA) showing the detection of bean yellow mosaic (BYMV), clover yellow vein (CYVV) and peanut stunt (PSV) viruses using different membrane types. NC: Nitrocellulose, CP: Chromatography paper, WP: Whatman #1 filter paper, NP: Notepad paper (white). Primary antibody was: 1-4, BYMV; 5-8, CYVV; 9-13, PSV. Infected leaf samples all of the viruses were blotted on the membranes. 1 = healthy subterranean clover; 2 = BYMV-RC\W in subterranean clover; 3 = BYMV-RC\W in *Nicotiana benthamiana*; 4 = BYMV-204-1 in Dwarf Gray Sugar pea; 5 = healthy white clover; 6 = CYVV-WC\ST2 in white clover; 7 = CYVV-WC\ST2 in broad bean; 8 = CYVV-Pratt in *Nicotiana benthamiana*; 9 = healthy white clover; 10 = PSV in cowpea; 11 = PSV-WC\ST4 in cowpea; 12 = PSV-WC\ST4 in subterranean clover; 13 = PSV-WC\ST4 in white clover.


NC
CP
WP
NP
1
2
3
4
5
6
7
8
9
10
11
12
13

corresponding petioles (NP), stolons close to roots (ST-RT) and stolons close to growing tip (ST-GT). A diagram showing the parts assayed are shown in Fig. 7. CYVV was tested from WC\ST2 and PSV was tested from WC\ST4. Both were the original plant grown in the greenhouse for about 2 yrs. From WC\ST2, a total of 3 different leaves (each leaf consisted of 3 leaflets), their corresponding petioles and 3 stolons were collected from 3 sites on the pot (Site 1, 2 and 3). Site 4 consisted of different set of samples collected as a composite from all the three sites. A total of 3 different leaves, petioles and stolons were collected from each of the sites to represent site 4. Samples were collected in the similar manner for PSV-WC\ST4, but were only from 2 different sites (Site 1 and 2). Site 3 was a composite from both sites, as described for CYVV-WC\ST2, Site 4. By TIBA and i-ELISA it was found that the distribution of CYVV-WC\ST2 varied within the plant. All the 4 sites sampled from old leaves and petioles gave equal intensity in TIBA (Fig. 8, top panel; A and B) and moderately strong to strong reactions in i-ELISA (Table 4). The newly emerging leaves and petioles from all sites were negative in i-ELISA, but were very mildly positive by TIBA. Under the 10x power, a few distributed intensely blue spots could be seen (result not shown). Stolons from the growing tip and close to the roots were mildly positive in i-ELISA and TIBA in 2 samples and strongly positive in another. A composite sample from the 3 sites suprisingly, was only mildy positive by i-ELISA (Table 4, Site 4) but gave an intense spot in TIBA (Fig. 8, top panel; A, 4).

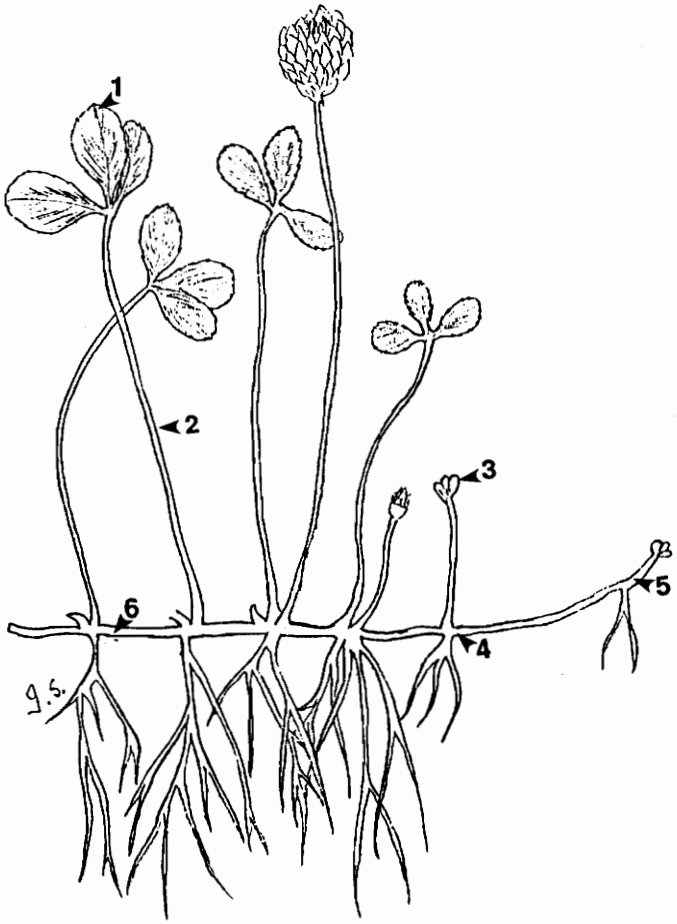
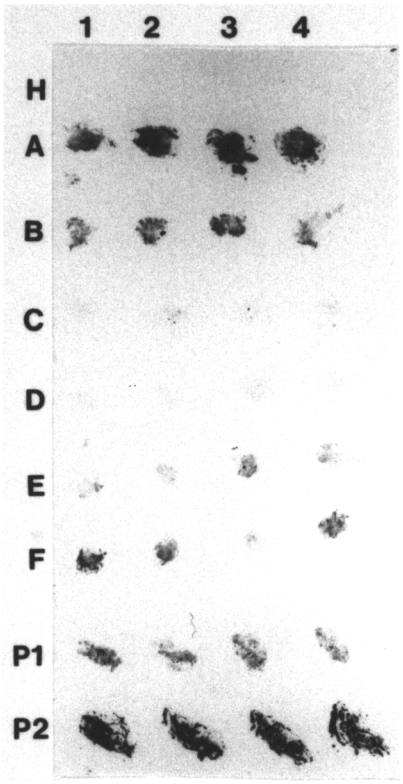


Fig. 7. Diagram showing the different parts of white clover. 1. Leaf, 2. Petiole, 3. New Leaf, 4. Stolon close the roots, 5. Stolon close to the growing tip, 6. Stolon

Fig. 8. Tissue immunoblot assay (TIBA) showing detection of clover yellow vein virus (CYVV) in different parts of white clover sample WC\ST2 (top panel) and peanut stunt virus (PSV) in different parts of white clover sample WC\ST4 (bottom panel). Sample WC\ST2 was detected with CYVV antiserum and WC\ST4 was detected with PSV antiserum. Samples were collected from different sites as described in methods. A = Old leaf; B = Old petiole corresponding to the leaf; C = New leaf; D = New petiole corresponding to the leaf; E = Stolon close to growing tip; F = Stolon close to roots; H = Healthy white clover; P1 = CYVV-Pratt in subterranean clover; P2 = CYVV-Pratt in *Nicotiana benthamiana*; P = PSV in cowpea.


1
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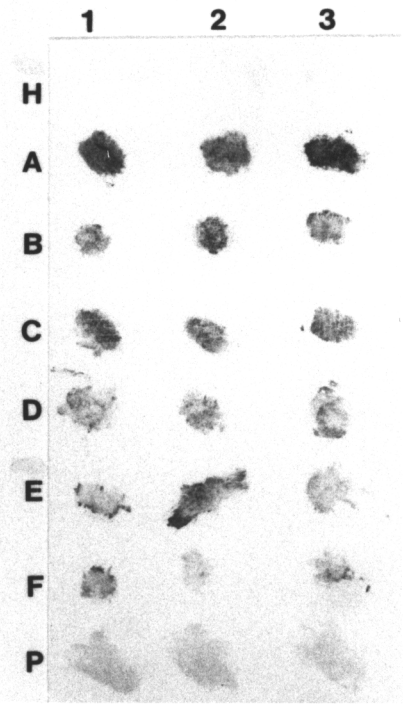
3
4
H
A
B
C
D
E
F
P1
P2


1
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A
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F
P

Table 4. Detection of clover yellow vein virus (CYVV) in different parts of WC\ST2 by indirect enzyme-linked immunosorbent assay

	Site[a]							
Sample[b]	1		2		3		4	
OL	0.659[c]	+++[d]	0.984	++++	0.723	+++	0.278	+
OP	0.608	+++	0.784	+++	0.590	+++	0.324	++
NL	0.165	-	0.163	-	0.068	-	0.035	-
NP	0.188	-	0.207	-	0.051	-	0.031	-
ST-GT	1.120	++++	0.561	++	0.471	++	0.108	-
ST-RT	1.189	++++	0.203	+/-	0.201	+/-	0.185	-
CYVV-Pratt	1.293	++++	1.284	++++	1.182	++++	0.988	+++

[a]Sites in a single pot of white clover from which samples were collected.
[b]Samples from different parts in the plant. OL = Old leaf, OP= Old petiole, petiole, NL = New leaf, NP = New petiole, ST-GT = Stolon close to growing tip, ST-RT = Stolon close to roots. CYVV-Pratt was cultured in *Nicotiana benthamiana*. incubation.
[c]Absorbance values read at 405nm after 60 min substrate incubation.
[d] Response given as + or -, based on + being at least the mean of the healthy reading plus 3 times standard deviation +/-weak, + mild, ++ moderate, +++ moderately strong, ++++ strong

PSV-WC\ST4 was found to be evenly distributed throughout the plant (Fig. 8, bottom panel). All the sites gave moderate to strong reactions in i-ELISA (Table 5). The intensity of the blue spots on the membrane corresponded reasonably well with i-ELISA results.

Red clover isolates were not tested as they were not in their natural hosts.

Detection in different hosts. Any type of host could be used for the detection of the viruses, provided it is infected with the virus (Eg. Fig. 6). Generally the intensity of the blot corresponded with the i-ELISA values (Eg. Fig. 8, top panel; A and Table 4). Blots made from WC\ST4 cultured in cowpea did not give an intense spot (Fig. 8, bottom panel; P). BYMV-RC\B cultured in sub clover (Fig. 5, B) and Dwarf Gray Sugar pea (Fig. 5, C) gave bright spots and were comparable to the intensity of the spot produced by BYMV-204-1 culture in Dwarf Gray Sugar pea (Fig. 5, D).

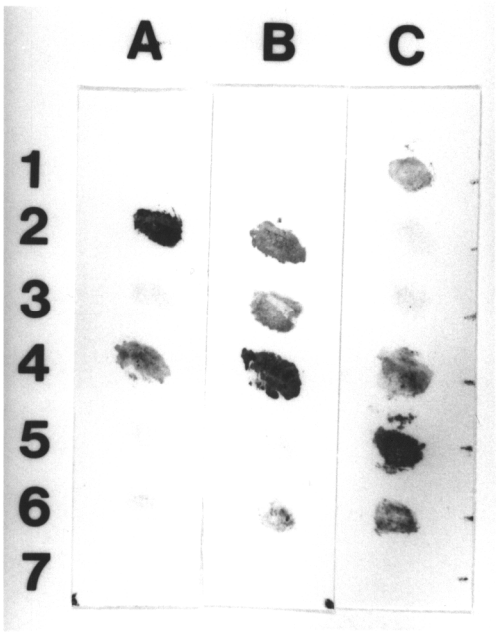
Detecting viruses in a mixed infection. The sample WC\ST5 was used to test for this as it was found to be naturally doubly infected with CYVV and PSV. Both the viruses were detected from the naturally infected white clovers (Fig. 9; B, 6 and C, 6), mechanically inoculated sub clover (Fig. 9; B, 5 and C, 5) and broad bean (Fig. 9; B, 4 and C, 4). This indicates that even in a mixed infection, if the correct antiserum is used against the virus/es present, the individual virus could be detected.

Table 5. Detection of peanut stunt virus (PSV) in different parts of WC\ST4 using indirect enzyme-linked immunosorbent assay

Sample[b]	Site[a] 1		2		3	
OL	0.539[c]	++[d]	0.585	++	0.553	++
OP	0.660	+++	0.580	++	0.790	+++
NL	0.883	+++	0.798	+++	0.764	+++
NP	0.685	+++	0.596	++	0.596	++
ST-GT	0.645	+++	1.092	++++	0.713	+++
ST-RT	0.490	++	0.633	+++	0.658	+++
PSV	0.759	+++				

[a] Sites in a single pot of white clover from which samples were collected.
[b] Samples from different parts in the plant. OL = Old leaf, OP = Old petiole, NL = New leaf, NP = New petiole, ST-GT = Stolon close to growing tip, ST-RT = Stolon close to roots. PSV was cultured in cowpea.
[c] Absorbance values read at 405nm after 60 min substrate incubation.
[d] Response given as + or -, based on + being at least the mean of the healthy reading plus 3 times standard deviation +/-weak, + mild, ++ moderate, +++ moderately strong, ++++ strong

Fig. 9. Tissue immunoblot assay (TIBA) showing the ability to distinguish between viruses using three different antisera and the ability to detect more than one virus in mixed cultures. Primary antibodies at 1:10,000 were: column A, Bean yellow mosaic virus; column B, Clover yellow vein virus; column C, Peanut stunt virus. Healthy and virus infected leaf samples were blotted on all three membranes. 1 = PSV in cowpea; 2 = BYMV-204-1 in Dwarf Gray Sugar pea; 3 = CYVV-Pratt in *Nicotiana benthamiana*; 4 = WC\ST5 in broad bean; 5 = WC\ST5 in subterranean clover; 6 = WC\ST2 in white clover; 7 = healthy white clover.


A
B
C
1
2
3
4
5
6
7

Specificity and cross-reactivity in detecting the three viruses by TIBA. In tissue blots by using PSV antiserum for virus detection, only PSV was detected, demonstrating that the antiserum was quite specific (Fig. 9; C, 1-7). But with BYMV and CYVV antisera, a one way cross-reaction was definitely seen. That is, CYVV antiserum reacted with both CYVV (Fig. 9; B, 2) and BYMV (Fig. 9; A, 2), and thus detects both viruses. Fortunately BYMV antiserum reacts weakly with CYVV (Fig. 9; A, 3), and thus distinguishes between the two viruses. Simultaneous use of BYMV and CYVV antiserum allows the identification of a sample as only BYMV and not CYVV.

COST AND FEASIBILITY ANALYSIS OF ELISA AND TIBA

Essential items of equipment are given in Table 6. The capital equipment cost involved to establish ELISA is more than for TIBA. For ELISA the total cost is approximately $16,500, whereas for TIBA the cost is only less than half that amount.

The expendable supplies include the items that are used once during the test and are then discarded. Under this section chemicals for buffers, substrates, solid phase (microtiter plates and membranes) and antibodies are included. In a 96-well microtiter plate, 44 duplicate samples can be applied considering the fact that proper controls are included. Hence 44 samples can be tested in a plate for a minimum of $1.26. Whereas on a 15 cm by 15 cm nitrocellulose up to 400 samples could be blotted. The calculated cost to test the equivalent of 44 ELISA samples is $0.80. The calculated cost of the substrate, is $0.34 for 20 mls

TABLE 6. Comparison of costs for establishing enzyme-linked immunosorbent assay (ELISA) and tissue immunoblot assay (TIBA) technique in a laboratory

	ELISA Amount in	TIBA dollars
1. Capital Cost		
a. Refrigerator		
0-14C range	2592	2592
-12to-37C range	3120	3120
b. Weighing Balance 0-240gm	1495	1495
c. pH meter Accumet 910 and electrode	788	788
d. Water bath	505	505
e. Computer and programs	2000	optional
f. ELISA reader	4150	—
g. Tissuemizer	1350	1350
h. Pipetman		
1-20ul	200	200
100-1000ul	200	200
i Glasswares, plastic bottles	700-900	700-900
j. Petri-dish	—	44/case of 500
2. Total Expendables		
a. Pipet tips	41.50/pk of 100 42.50/pk of 100	41.50/pk of 100 42.50/pk of 100
b. Gloves	40/case of 1000	40/case of 1000
c. Chemicals req	200	200
d. Microtiter plate	1.26 @126/100	—
e. Membranes		
NCM,0.45-um 15x15cm,5/pk	—	36.40
Nytran, 0.2um 20x20cm, 5/pk	—	57
f. Substrate pNP	17/1gm	—
NBT	—	57/1gm
BCIP	—	13/100mg
g. GAR F(ab'$_2$) Alk. phos.	77/0.5ml	77/0.5ml
h. Antibody from American Type Culture Collection	50-100	50-100

for one ELISA plate and $0.84 for TIBA (20 mls). Approximately 50 - 75 samples can be tested by TIBA using 20 mls of buffer solution of substrate. Antibodies are used at similar dilutions for both assays but with tissue blots the solution can be frozen and reused for up to 4 - 5 times. Intensity of the spots, however, decreases as the antiserum is reused. Reusing the antiserum with ELISA can also be done, but the process is laborious as the liquid has to be drawn out from each well. Similarly, the GAR F(ab'$_2$) is used at the same dilution, but with tissue blots the solution can be refrigerated and used up to 4 - 5 times. Intensity of the spot reduces but not sufficiently to interfere with the results.

In conclusion, the calculated cost of ELISA or TIBA on a per sample basis is the same. But the initial equipment costs involved with ELISA is much greater when compared to TIBA. Moreover ELISA is labor intensive, especially grinding and plating out of samples, and requires more than one day to complete. TIBA on the other hand is not labor intensive as there is no sample preparation and results can be obtained within 4 hrs.

CHAPTER 5

DISCUSSION

Bean yellow mosaic (BYMV), clover yellow vein (CYVV) and peanut stunt (PSV) viruses were successfully detected for the first time from naturally infected clovers in Virginia and from mechanically inoculated host species by tissue immunoblot assay. Sample application was simplified by simply tearing the tissue rather than using razor blades to make cuts as reported by Lin et al. (1990) and Hsu and Lawson, (1991). This is the first report in which residual green color caused by direct tissue blotting was removed using Triton X-100. Similar success has been reported by Powell (1987), with dot blots, not with tissue blots. Christie and Edwardson (1977) have used 5% Triton X-100 to remove plastids while preparing tissue for light microscopy staining.

A number of different membranes could be used for virus detection. Others have previously reported the use of nitrocellulose (Banttari and Goodwin, 1985; Lin et al., 1990; Hsu and Lawson, 1991) or filter paper (Haber and Knapen, 1989). However this is the first report where ordinary notepad paper has been used with TIBA to detect viruses. Nitrocellulose is recommended because of its superior lower background.

CYVV antiserum reacted with both CYVV and BYMV, hence could detect both the viruses but not distinguish between them. Fortunately, BYMV antiserum reacted strongly with BYMV and only weakly with CYVV, thus detected both the viruses and also

distinguished between them. I recommend using both of the antisera at the same time against the samples to be tested, and in this way an identification can be made. In practice, however, since CYVV exclusively infects white clover and BYMV red clover, the cross-reactivity will not diminish the utility of the method even if only one antiserum is used.

This is the first time indirect ELISA (i-ELISA) has been used to detect potyviruses by workers in the S228 project. The results corresponded with results obtained in tissue blot assays. Cross-reactivity of CYVV and BYMV antisera occurred as expected, but did not pose a major problem. From Tables 2 and 3, it is clear that at a 1:50 sap dilution, CYVV and BYMV cannot be distinguished, but at a higher sap dilution the viruses can be differentiated (Fig. 2 and 3). Hence with i-ELISA it was absolutely necessary to use sap dilutions to distinguish the viruses. Similar results have been reported with DAS-ELISA (McLaughlin et al., 1984).

Other workers have also found cross-reactions to occur between the two viruses, both using both polyclonal and monoclonal antibodies (Barnett et al., 1987; Bos et al., 1974; Reddick and Barnett, 1983). Monoclonal and polyclonal antibodies directed towards the virus-specific amino termini of coat proteins also do not show any clearcut distinction (Barnett et al., 1987). Work done with C-DNA probes can distinguish some strains, but generally the results correspond with those obtained using DAS-ELISA (Abu-Samah and Randles, 1983; Barnett et al., 1987). Results

obtained by C-DNA suggest that there is a group of related viruses within the potyvirus group, similar to what is seen using ELISA. A clear distinction can be made only using molecular techniques (Tracy et al., 1992). Such a study was not part of this research and can be investigated in the future.

The best time to assay for viruses, based on my study, would be spring through summer. The concentration of viruses, particularly in clovers in greenhouse, decreased during winter, indicating it was not a good time for testing. It is not clear as to why the concentration of the greenhouse clovers decreased during winter, but it may be related to slower growth of the plants, the effect of photoperiodism, or the fact that viruses have an up and down cycle in some hosts. More work needs to be done with the clovers either in the greenhouse, or more preferably in naturally growing conditions, to confirm this part of the study.

The best part of the plant to assay are the old leaves and corresponding petioles or stolons. Although PSV-WC\ST4 was evenly distributed in the white clover, I cannot affirm and say that such a trend will be seen with all white clover plants infected with PSV. Since the test was done only during the spring months, it is uncertain whether the concentration of the virus in all parts will be the same during other months.

Indirect ELISA was preferable to DAS-ELISA in this study because it enabled the detection of viruses with varied serotypes. This was extremely important since this is the first time anyone

has reported the characteristics of BYMV and CYVV isolated from clover in Virginia. Therefore i-ELISA was used as it enables the detection of a wider range of virus strains, and is advantageous to DAS-ELISA in that it does not require an additional antibody conjugate, which is laborious and time consuming to prepare and may alter antibody reactivity.

Therefore tissue blot is more advantageous than i-ELISA in that the blotted membranes can be prepared even in the greenhouse or in the field by persons with just a few instructions (Lin et al., 1990). Blots can be stored even up to 2-3 wks before they are processed (Lin et al., 1990). I was also able to store blotted membranes up to 3-4 wks before developing (data not shown). Mailing of sample or ELISA plates have been done in the Project (McLaughlin et al. 1984), but sample preparation for TIBA is definitely cheaper and easier than preparing samples for ELISA. The assay is easy to perform and can be completed in the laboratory within 4 hrs, and could be easily adapted to do it anywhere. ELISA takes a complete day or two to complete depending on the type of virus detection. Thus for routine diagnostic work in the laboratory, large-scale indexing programs or sampling procedures, TIBA is definitely a better technique to use than ELISA. The only disadvantage is that TIBA is a qualitative assay whereas ELISA is quantitative. But if required both of these techniques can be made vice-versa, that is ELISA can be qualitatively analyzed using the naked eye to judge the color intensity, and TIBA can be quantified using a densitometer.

In my study I have found that both the assays are useful in their own ways. On sampling the 17 clovers collected from different locations using TIBA (results from only selective samples are shown) and i-ELISA, I found that 5 white clovers were positive for CYVV and 4 were positive for PSV. White clovers were negative for BYMV as expected. Both of the red clovers were positive for BYMV. In general, the reactivity of the Virginia isolates of BYMV and CYVV was nearly identical with those of the known isolates.

It was very easy to detect PSV, especially based on symptoms produced on cowpea and single lesions on Tennessee Green Pod bean and *Chenopodium* (Tolin and Miller, 1975). The symptoms produced by the CYVV and BYMV isolates were confusing (Bos et al., 1977, 1974; Barnett et al., 1987). All the white clover samples that tested positive for CYVV showed similar symptoms on broad bean and were more like those described by (Bos et al., 1977) and a BYMV-like virus isolated in Virginia (Hunst and Tolin, 1982). But the symptoms on *Nicotiana benthamiana* varied to a certain degree from being mild to moderate in showing mosaic symptoms. Only CYVV-WC\GS4 showed moderate necrosis. On *C. amaranticolor*, CYVV-WC\ST2 showed complete necrosis which was more severe than WC\ST5 and differed from CYVV-Pratt (Barnett et al., 1987). But on *C. quinoa* the symptoms matched those described by Barnett et al. (1987). On Dwarf Gray Sugar pea, Alaska pea and Bush Blue Lake bean, CYVV-WC\ST2 and WC\ST5 showed similar symptoms. When they were tested against CYVV-Pratt antiserum, Bush Blue Lake was

negative (two times) whereas the two peas were positive. This indicates that WC\ST2 and WC\ST5 must be infected with another virus. Host range and serological tests showed that WC\ST5 was positive for PSV and CYVV. Hence it could be possible that PSV was isolated in Bush Blue Lake bean from WC\ST5. Similar reports of PSV infecting Bush Blue Lake bean have been made from Virginia (Tolin and Polston, 1978). Regarding WC\ST2 it can only be said that a virus, possibly PSV, infected the Bush Blue Lake bean. However WC\ST2 was negative to PSV by i-ELISA (Table 2 and 3) and TIBA (data not shown).

There were distinct variations among the two red clover samples that tested positive for BYMV-204-1. The Blacksburg BYMV-RC\B showed symptoms on Dwarf Gray Sugar pea that resembled the symptoms caused on the same host by the type isolate BYMV-204-1. Symptoms caused by BYMV-RC\B on *Chenopodium* species resembled BYMV-204-1, but on subterranean clover and broad bean they were slightly different. In subterranean clover there was not a ruffled leaf appearance and in broad bean the mosaic was not severe. Even by serology BYMV-RC\B was found to be closely related to the type isolate. BYMV-RC\W from Winchester, however, showed only mild symptoms on subterranean clover and symptoms on Dwarf Gray Sugar pea did not exactly correspond with those induced by BYMV-204-1.

Generally it was found that subterranean clover was easy to inoculate and infect with the 3 viruses, but white clover was not. Subterranean clover was a good host for BYMV and PSV, but the

Virginia isolates CYVV-WC\ST2 and CYVV-WC\GS4 killed subterranean clover easily. PSV-WC\ST4 caused moderate symptoms on subterranean clover but definitely did not kill it. Similarly WC\ST5, which was affected with CYVV and PSV, also moderately infected subterranean clover. Based on this study it can be hypothetically said that CYVV affects subterranean clover severely and PSV does not, but when CYVV and PSV are both present in the same host, PSV attenuates CYVV and as a result symptoms are mild.

In conclusion, the Virginia clover virus isolates have several things in common with previously reported isolates, but are distinct in other ways. However, more work needs to be done, especially with BYMV and CYVV isolates, so that a clean type culture can be obtained. Work such as single lesion isolation, purification, complete characterization and comparison to the type isolates by quantitative ELISA with equal concentrations of virus, can definitely help in giving the isolates a true characterization and strain designation.

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APPENDIX

TISSUE BLOT TECHNIQUE FOR DETECTION OF VIRUSES: Srinivasan & Tolin, 1992.

MATERIALS AND SOLUTIONS:

Solid phase: Nitrocellulose membrane, 0.45-um pore size.

Containers: Tops of micropipette tip boxes, 12 x 8 x 3 cm (requires 30-40 ml). Regular Petri dishes (9 cm, requires 15-20 ml) or Small Petri dishes (5 cm, requires 8-10 ml).

Storage of antiserum: 15 or 50 ml plastic centrifuge tubes with tops.

Miscellaneous: Forceps for moving membrane; Plastic gloves; pencil for marking; Scissors for cutting membrane; Template and strip holder (optional).

Potassium Buffered Saline (KPS): 5X [Store at room temperature]
0.10M K_2HPO_4 + 0.75M NaCl, pH 7.4
Per 500 ml, 8.71g K_2HPO_4, 21.92g NaCl. Adjust with 1 N HCl.

KPS + Tween: 1X [Store at 4 C]
0.02M K_2HPO_4 + 0.15M NaCl + 0.05% Tween-20, pH 7.4
Per 1000 ml: 200 ml 5X KPS + 0.5 ml Tween-20.

5% Triton X-100: 5 ml Triton X-100 dissolved in 100 ml distilled water. Use one time, then discard.

Blocking solution:
1% non-fat dry milk (Carnation) + 0.5% Bovine Serum Albumin (BSA) in 1X KPS
Per 500 ml, use 100 ml 5X KPS + 5 g milk + 2.5 g BSA.
Freeze in aliquots of 40 ml each.
OR: Make 20 ml: 4 ml 5X KPS + 0.2 g milk + 0.1 g BSA.
Use one time, then discard.

Primary antibody:
Whole rabbit antiserum, used at an appropriate dilution according to titer. Dilute in 1X KPS, *without Tween*. Eg. soybean mosaic virus used at 1:10,000. Specificity may increase if cross-absorbed with healthy plant tissue. Make volume according to the container used. Can be reused up to 5 times if kept at -20 C in-between.

Secondary antibody:
Goat anti-rabbit, alkaline phosphatase-labeled (Sigma), if rabbit polyclonal is used as primary antibody. Dilute in 1X KPS, *without Tween*, usually to 1:2000. [NOTE: manufacturer recommends 1:1000].

This can also be reused up to 5 times, if kept at 4 C. DO NOT FREEZE.
Substrate buffer: 0.1M Tris, 0.1M NaCl, 0.005 M $MgCl_2$, pH 9.5
Per 200 ml: 20 ml of 1M Tris + 1.17 g NaCl + 0.5 ml of 2M $MgCl_2$.
OR: Make 40 ml: 4 ml of 1M Tris + 0.234 g NaCl + 0.1 ml of 2M $MgCl_2$.

Substrate - NBT: dissolve 14 mg in 300 ul methanol for 40 ml of substrate buffer. Keep it aside covered and away from direct sunlight.

Chromophore - BCIP: dissolve 7 mg in 50 ul DMSO for 40 ml of substrate buffer. Keep it aside covered and away from sunlight.
To 40 ml of substrate buffer, add NBT first, stir, then add BCIP. The NBT/BCIP must be made freshly and then used one time and discarded.

Stop buffer: 0.01M tris-HCl + 0.001M EDTA, pH 7.5
Per 50 ml: 0.5 ml 1M tris + 0.2 ml of 0.25M Na_2EDTA solution + 45 ml water. Adjust pH with 1 N HCl. Put in graduated cylinder to bring to 50 ml volume.
Note: Can be made fresh each time or made and stored in larger volume at 4 C.

PROTOCOL

The entire is procedure done on a lab bench at room temperature. It is important to wear gloves while handling dry nitrocellulose membranes. Cut membranes to desired size with clean scissors. Use forceps and hold membranes by the edge while transferring them between containers.
Set up a series of 10 containers, selected to match size of membranes used. Label them as follows:

A: Triton X-100
B: Rinse buffer
C. Blocking solution
D: Primary antibody
E: Rinse buffer
F: Secondary antibody
G: Rinse buffer
H: Substrate solution
I: Distilled water
J. Stop solution

1. Blotting. Blot cut or torn tissue onto fresh, untreated nitrocellulose membrane. Hold onto membrane only 1-2 sec. Plastic template may be used to assure proper spacing and uniform blot sizes. Blotted membrane can be processed within a few min, or held up to 4 weeks if sealed in a plastic bag.

2. Decolorizing step - A. Immerse membrane in 5% Triton X 100 in container A and soak, with gentle agitation, for 5-15 min until the green color disappears.

3. Rinse - B. Transfer membrane to container B. Agitate gently for 3 min.

4. Blocking step - C. Transfer membrane to container C and immerse in blocking solution for 30 min, with occasional gentle agitation.

5. Primary antibody step - D. Pick membrane up and allow to drain, then transfer it to container D with primary antibody. Leave for 1 hr, with gentle agitation. When finished, pour residual primary antibody back into centrifuge tube, and refreeze. Mark on tube that it has been used.

6. Rinse - E. Put membrane into container E with rinse buffer. Agitate gently for 10 min. Pour rinsing buffer out of the container and add new rinsing buffer to the same container. Repeat rinse 2 times for 5 min each.

7. Secondary antibody - F. Transfer membrane to container F. Add secondary antibody and leave for 1 hr, with gentle agitation. When finished, pour secondary antibody back into the centrifuge tube, and store at 4 C. Mark on tube that it has been used.

8. Rinse - G. Put membrane into container G with rinse buffer. Agitate gently for 10 min. Pour rinsing buffer out of the container and add new rinsing buffer to the same container.

9. Substrate - H. Put 40 ml of substrate buffer into container H. Add NBT, stir. Add BCIP, stir. Then add membrane. Leave until blue color develops to desired intensity -- no more than 15 min.

10. Water rinse - I. Dip membrane briefly, 3-5 sec., into distilled water in container I. Additional rinses will nearly stop the reaction.

11. Stop bath - J. To stop reaction completely, transfer membrane to container J with 0.01M tris-HCl, 0.001M EDTA, pH 7.5, for 10 min.

12. Dry. Remove membrane and place on a Kimwipe to remove residual liquid. Store in the dark. Blue color fades and background darkens in light.

DATA RECORDING SHEET

TISSUE BLOTS: DATE:_____

Purpose of Test: Blot Code:_____

Membrane Type: _____ Conditions:_____

Primary Antibody:

Identity _____ Dilution _____ Times Used_____

Secondary Antibody:

Identity _____ Dilution _____ Times Used_____

Substrate : _____

Column A		Column B		Column C	
Sample	+\-	Sample	+\-	Sample	+\-
1					
2					
3					
4					
5					
6					
7					

COMMENTS:

VITAE

Indira Srinivasan was born in Bombay, India on 6th March 1963. She graduated from the University of Madras in 1984 with a Bachelor of Science Degree in Botany. During the year 1986 she did her Post Graduate Diploma in Medical Laboratory Technology, in the same University, and graduated in 1987. She married Dr. Ramji Srinivasan and came to Blacksburg, Virginia in 1989, and pursued a Master of Science degree at Virginia Polytechnic Institute and State University under the guidance of Dr. Sue Tolin the same year. On August 2nd. 1990, she was blessed with the birth of their son Nishant.