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