GENETIC STUDIES ON RESISTANCE TO ALFALFA MOSAIC VIRUS (AMV) AND TOLERANCE TO WHITE CLOVER MOSAIC VIRUS (WCMV) IN RED CLOVER (*Trifolium pratense* L.)

by

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M. Sc. Virology/Agronomy
Plant Science

ABSTRACT

Progenies of red clover were screened for resistance (R) to alfalfa mosaic virus (AMV) and for tolerance (T) to white clover mosaic virus (WCMV) through mechanical inoculations with the appropriate virus, and selection based on visual symptoms and ELISA (enzyme-linked immunosorbent assay). The use of intact leaf disks, instead of leaf extracts, as test samples for ELISA resulted in reliable detection. Average rates of tolerance to WCMV in progenies increased in two successive generations from 24.5% (T X T) to 34.2% (T/T X T/T) and remained almost constant, (8.4 and 9.7%) in T X S and T/S X T/S respectively. Percent tolerance in progenies increased linearly as the number of "doses" of tolerance in parents increased. Tolerance to WCMV behaved as if it were controlled by multiple genes showing additive effects. Seed transmission of WCMV was not detected. One generation of screening for resistance to AMV also suggested multigenic inheritance. Percent resistance in progenies reached 47.8% in one cross (R/R X R/R), was near 30% in R/R X R/S crosses and R/S X R/S, and close to 16% in R/S X S and R/R X S crosses. Percent resistance in progenies increased as the number of "doses" of resistance in the parents increased, however, the relationship was quadratic.

ETUDES GENETIQUES SUR LA RESISTANCE AU VIRUS DE LA MOSAIQUE DE LA LUZERNE (AMV) ET LA TOLERANCE AU VIRUS DE LA MOSAIQUE DU TREFLE BLANC (WCMV) CHEZ LE TREFLE ROUGE (Trifolium pratense L.)

M. Sc. Virologie/Agronomie
Phytologie

RESUME

Des descendances de trèfle rouge ont été criblées pour la résistance (R) au virus de la mosaique de la luzerne (AMV) et pour la tolérance (T) au virus de la mosaïque du trèfle blanc (WCMV) par inoculation mécanique avec le virus approprié, et par sélection basée sur les symptômes et le test ELISA (enzyme-linked immunosorbent assay). L'utilisation de rondelles de feuille au lieu d'extraits de feuille pour le test ELISA a donné des résultats fiables. Les taux de tolérance moyens ont progressé chez les descendants de deux générations successives, de 24.5% (T X T) à 34.2% (T/T X T/T) et sont demeurés à peu près constants (8.4 et 9.7%) pour T X S et T/S X T/S respectivement. Les taux de tolérance dans les progénitures ont augmenté de façon linéaire avec le nombre de "doses" de tolérance des parents. La tolérance au WCMV s'est comportée comme si elle était déterminée par plusieurs gènes additifs. La transmission du WCMV par la graine n'a pas été décelée. Une génération criblée pour la résistance au AMV a aussi suggéré une nature multigenique. Les taux de tolérance ont atteint 47.8% dans un croisement (R/R X R/R), autour de 30% pour les croisements R/R X R/S et R/S X R/S, et près de 16% pour les croisements R/S X S et R/R X S. Les taux de résistance dans les progénitures ont augmenté avec le nombre de "doses" de résistance des parents, toutefois, la relation était quadratique.

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ABBREVIATIONS OF VIRUS NAMES

AMV	Alfalfa mosaic virus
BCMV	Bean common mosaic virus
BYMV	Bean yellow mosaic virus
CYVV	Clover yellow vein virus
CYMV	Clover yellow mosaic virus
PCMV	Pea common mosaic virus
PSV	Pea streak virus
RCVMV	Red clover vein mosaic virus
SBMV	Southern bean mosaic virus
WCMV	White clover mosaic virus

1. INTRODUCTION

Red clover (Trifolium pratense L.) is the most widely grown of all true clovers and one of the most important forage legumes in the world (Barnett and Diachun, 1985; Taylor, 1985). Originating in South-Eastern Europe and Asia Minor, it became the first forage legume cultivated in Europe, and was introduced to North America by European settlers (Fergus and Hollowell, 1960). Grown alone or mixed with grasses, red clover is adapted to a wide range of soil types, pH levels, and environmental conditions. Other important characteristics include its relative ease of establishment (seedling vigor), and low seed cost compared to some other forage legumes. These features have made it widely used for hay, silage, pasture and soil improvement in most of the temperate regions of the world (Barnett and Diachun, 1985).

Red clover is best adapted where summer temperatures are in the range of 21 to 24 °C (Kendall, 1958) and adequate moisture is available during the growing season. In North America, it is grown in Eastern humid regions from Quebec and Ontario to Tennessee and North Carolina, and produced as a winter annual south of this range. It is also grown in the Pacific Northwest, principally under irrigation, for seed production (Taylor, 1985). Red clover is also produced in Northern Europe, Asia, New Zealand and Australia. Approximately 20 million ha of red clover are grown in the world, including 7 million in North America. Annual hay yield

in the US is around 4.0 tonnes/ha (Smith et al., 1985). In Quebec, more than 44% of the total amount of legume seeds sold in 1981 were red clover (Statistique Canada, 1981).

Although physiologically a perennial, red clover is generally productive for only 2 to 3 seasons (Smith et al., 1985; Zeider et al., 1971). This relative nonpersistence is attributed to a combination of factors including: improper management, environmental conditions, insect pests and diseases (Leath et al., 1971). Various root and foliar fungal pathogens, and viruses are known to reduce the longevity of red clover stands (Smith et al., 1985). Viruses have negative effects on several yield components of forage legumes (Barnett and Diachun, 1986). Two of the most common viruses infecting red clover are white clover mosaic (WCMV) and alfalfa mosaic (AMV) (Clark and Barclay, 1972; Hampton, 1967). Development of resistant varieties remains the most appropriate approach for the control of virus diseases.

The main objective of this research was to study the inheritance of tolerance to WCMV and resistance to AMV in red clover. To achieve this, the following sub-objectives had to be fulfilled:

i. Assessment of the rate of seed transmission of white clover mosaic virus (WCMV) from both tolerant and susceptible red clover plants, systemically infected, and grown under greenhouse conditions.

ii. Development of mass inoculation techniques for AMV and WCMV.

iii. Adaptation of reliable and rapid serological testing methods for screening large progeny populations for susceptibility to AMV and WCMV.

2. LITERATURE REVIEW

2.1 EFFECTS OF VIRUS DISEASES ON FORAGE LEGUMES

Perennial forage legumes can be infected by a wide variety of plant viruses, which cause diseases ranging from insidious infections to stunting or necrosis of entire plants. Overall crop losses due to viruses can be attributed to a combination of distinct effects (Barnett and Diachun, 1985). Forage yields are affected by a reduction of foliar growth (Gibson et al., 1981; Scott, 1982a); by a lower winter hardiness, persistence and vigor (Alconero et al., 1986; Pratt, 1967); by an increased susceptibility to pathogens of the root and crown rot complex, especially species of Fusarium (Denis and Elliot, 1967); and by a lower production root nodules which are also less efficient (Gibson et al., 1981; Guy et al., 1980; Khadhair et al., 1984).

Old forage legume stands often have a higher proportion of virus-infected plants than young stands (Ostazeski et al., 1970). Infected plants may be symptomless and provide a reservoir from which other plants can be infected. Leath and Barnett (1981) reported that virus disease incidence in red clover was increased rapidly during the first and second years, before stabilizing when 30 to 54 % of the plants were infected.

Early work by Diachun and Henson (1956) showed that

after stress of high temperature or cool weather, bean yellow mosaic virus (BYMV) could reduce stands of red clover clones by as much as 100 percent. Goth and Wilcoxson (1962) reported that seed formation of BYMV-infected red clover plants was about 10% of that of healthy plants. Smith and Maxwell (1971) reported that infection by the same virus in a controlled environment resulted in reductions in chlorophyll content, shorter shoots, higher nitrogen concentration, higher moisture content in the stems, higher leaf-to-stem ratio, and lower dry matter yield per plant.

The effects of white clover mosaic virus (WCMV) on the yield of red clover alone or in mixture with ryegrass were evaluated in field plots. Clover yields were affected more in grass-clover mixtures (up to 70%) than in pure stands. Yield reductions were more important with higher infection levels. With the addition of nitrogen fertilizer, the grass component compensated for lower clover yields due to infection (Scott, 1982a).

Khadhair et al. (1984) reported the effects of WCMV infection on various processes involved in nitrogen fixation in red clover. Nitrogenase activity, leghemoglobin content, and Rhizobium populations were reduced while nitrate reductase and acid phosphatase activities were increased.

2.2 SEED TRANSMISSION OF VIRUSES IN RED CLOVER

Since studies on tolerance to viruses involve crosses

between symptomless, systemically infected parents, and the classification of their progenies based on their reaction to mechanical inoculations, there is a risk of mis-classifying genotypes, which may be infected due to seed transmission. Therefore, a brief review of seed transmission of viruses in red clover is pertinent.

Some of the earliest reports of seed transmission of viruses concern red clover. Since Dickson and McRostie (1922) observed mosaic symptoms on young progenies of virus infected red clover plants, seed transmission of several viruses in red clover has been investigated (Hampton, 1963, 1967; Stuteville and Hanson, 1964a; Varma and Gibbs, 1966; Hampton and Hanson, 1968). Hampton (1963) assayed 66 seedlings from red clover seeds produced in a commercial seed field containing severely virus-infected plants. By host reaction, serology and physical properties, he determined that 19 of the seedlings (28.8%) contained virus: Four (6%) had WCMV, five (7.6%) had CYMV, and 10 (15%) had a non identified virus resembling CYMV. Hampton and Hanson (1968) assayed 1,800 seedlings from six different seed lots and found infection in 1 to 28 % of the seedlings. Causal agents were not characterized. However, Varma and Gibbs (1966) detected no virus infection in seedlings grown from 89 commercial seed crops from six different red clover varieties. Stuteville and Hanson (1964a) grew 8,300 seedlings from 111 seed-source plants infected with one or more of five viruses (BYMV, RCVMV, PSV, PCMV and AMV), and found no evidence of transmission. The authors suggested the effect of environment

during the time seeds are produced on virus occurence, concentration and survival, as possible causes for their negative results. Viruses may also differ in their ability to survive in clover seeds.

Some viruses capable of producing striking symptoms may produce only latent infections when seed borne. Lister and Murant (1967) showed that strawberry seedlings infected by seed transmission (with raspberry ringspot and tomato blackring viruses) showed no symptoms after 2 years, while mother plants showed normal symptoms. Hampton and Hanson (1968) found some red clover plants, infected from seeds, that remained symptomless for 200 days. These studies indicate the importance of further tests, in addition to visual observations, in seed transmission studies.

2.3 VIRUSES INFECTING RED CLOVER

The most prevalent viruses infecting red clover in Eastern Canada and the US are bean yellow mosaic (BYMV), alfalfa mosaic (AMV), red clover vein mosaic (RCVMV), clover yellow vein mosaic (CYVM), white clover mosaic (WCMV), pea streak virus (PSV), and clover yellow mosaic (CYMV) (Gates and Bronskill, 1974; Hanson and Hagedorn, 1961; Khadhair, 1983; Leath and Barnett, 1981; Mueller, 1965; Pratt, 1961, 1968; Stuteville and Hanson, 1965). Table 2.1 lists viruses reported to infect red clover in various parts of the world. Many of the viruses listed have been reported in North America and Europe (e.g. CVMV, PSV, CYVM), or have a worldwide

Table 2.1 Viruses which infect red clover naturally with their geographical distribution and main symptoms (adapted from Barnett and Diachun, 1986).

Virus group & Virus	Geographical Distribution	Symptoms
Viruses with rod-shaped particles		
Carlavirus Group		
Red clover vein mosaic v.	Europe, Canada, USA	mosaic, streaks stunt
Pea streak virus	" " "	none or mosaic
Potexvirus Group		
Clover yellow mosaic v.	Canada, USA	mosaic, streaks
White clover mosaic v.	Canada, USA, Europe New Zealand, Australia	mosaic or none
Potyvirus Group		
Bean yellow mosaic v.	worldwide	mosaic
Clover yellow vein v.	Canada, Britain, USA	mosaic or none
Tobravirus Group		
Pea early browning v.	Europe	mottle, stripe
Viruses with spherical particles		
Comovirus Group		
Red clover mottle v.	Europe	mottle
Cucumovirus Group		
Subterranean clover red leaf virus	Australia, N.Zealand	red leaf margins or none
Nepovirus Group		
Arabis mosaic virus	Europe	faint mottle or none

Table 2.1 (continued)

Virus group and virus	Geographical Distribution	Symptoms
Tobacco ringspot v.	USA	mottle or none
Peanut stunt v.	USA, Europe, Japan	mosaic, stunt
Dianthovirus Group		
Clover primary leaf necrosis v.	Canada	mottle
Red clover necrotic mosaic v.	Europe, Australia	veinal necrosis stunting
Ilarvirus Group		
Tobacco streak v.	USA	mottle
Luteovirus Group		
Bean leafroll v.	Europe, USA	vein yellowing
Soybean dwarf v.	Japan	none
Pea enation mosaic virus Group		
Pea enation mosaic v.	worldwide	mosaic, enation
Ungrouped		
Clover mild mosaic v.	Sweden	mosaic
Viruses with bacilliform particles		
Alfalfa mosaic virus Group		
Alfalfa mosaic virus	worldwide	mosaic, necrosis chlorosis

distribution (e.g. WCMV, AMV and BYMV).

The relative occurence of viruses appears to shift over the years. Pratt (1968) reported that PSV was the most common virus in red clover in Eastern Canada. Gates and Bronskill (1974) ranked BYMV first in Essex County, Ontario, and Khadhair (1983) found that WCMV was the most prevalent in the same crop growing in the Ottawa area in recent years.

2.3.1 Alfalfa mosaic virus

Alfalfa mosaic virus (AMV) is one of the most studied plant viruses. Its pathological, epidemiological and biochemical-biophysical properties have been much studied. An extensive review of AMV properties was published by Hull (1969).

2.3.1.1 Biological and Physicochemical properties

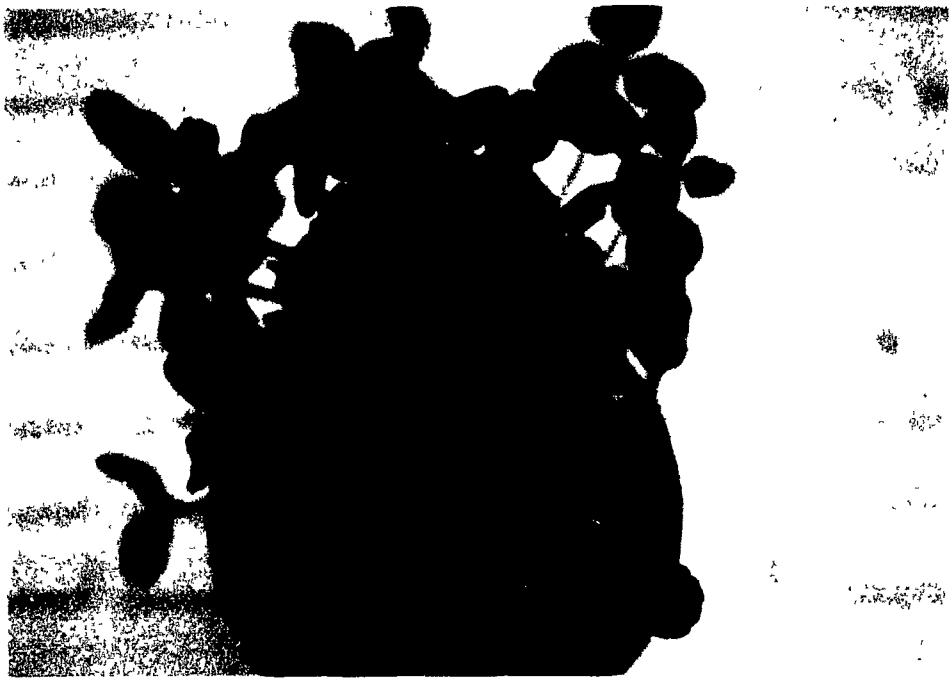
The virus was first described in 1931 (Weimer, 1931) and was first isolated from red clover in 1935 (Pierce, 1935). AMV is classified in a monotypic group without an approved group name. It has a worldwide distribution and is considered as a serious disease of white clover and a potentially serious disease of red clover (Hagedorn and Hanson, 1963; Kreitlow and Price, 1949; Malak, 1974). AMV was the most common virus infecting alfalfa during the 1979-1983 period in Alberta (Hiruki, 1987). In Eastern Canada, a high occurrence of infection was detected in alfalfa (Gates and Bronskill, 1974)

and white clover (Pratt, 1968). It was the most frequently isolated virus in red clover in Washington (Hampton, 1967). Besides North America, the occurrence of AMV in red clover was reported in Czechoslovakia (Musil and Matisova, 1967), Hungary (Beczner, 1978), and Japan (Akita, 1981; Inouye, 1969).

Alfalfa mosaic virus is one of the causes of "mosaic" in red and white clover (Jaspars and Bos, 1980). In red clover it also can cause mottling with important leaf distortion. However, symptoms vary with the strain of virus and the growing conditions (Barnett and Diachun, 1986). Figure 2.1 shows a red clover plant infected with AMV. Having a very wide host range, AMV occurs naturally in 150 species from 22 families, and is transmissible to 599 species from 68 families. In the Leguminosae, it infects 156 species in 32 genera (Edwardson and Christie, 1986a).

AMV is readily sap transmitted and can be transmitted in a nonpersistent manner by at least 14 aphid species (Jaspars and Bos, 1980). Transmission by various dodder species (Cuscuta spp) has also been reported (Schmelzer, 1956). Seed transmission was detected at rates as high as 48% from individual infected alfalfa plants (Hiruki, 1987) and as much as 10% in commercial alfalfa seeds (Jaspars and Bos, 1980), but it was not detected in red clover seeds (Stuteville and Hanson, 1964a). It is considered as the main source of spread of AMV in alfalfa (Hiruki, 1987). More seed transmission occurs through pollen than through ovules (Hemmati and McLean, 1977). Occurrence of seed transmission reduces when seeds are stored

Figure 2.1 A red clover plant infected with AMV showing severe leaf distortion.



in a freezer for a period of 6 weeks compared to seeds stored at 4 °C (Frosheiser, 1964). The main diagnostic species for AMV are listed in Table 2.2 with their respective symptoms. Tobacco cultivars which are hypersensitive to tobacco mosaic virus (e.g. Samsun NN and White Burley) are good sources of virus for purification (Hull, 1969; Jaspars and Bos, 1980). In these hosts, virus concentration reaches high peaks in 3 to 15 days after inoculation before declining to very low levels. Best virus yields are generally obtained when leaves are harvested 7 to 15 days after inoculation.

AMV is composed of three to four bacilliform particles of different length (56, 43, 35 and 30 nm long X 16 nm diam.), each containing a different species of ssRNA of messenger polarity. Numerous strains of AMV have been distinguished (Jaspars and Bos, 1980). Infections by a mixture of functional components by two strains, can yield pseudo-recombinant strains with properties of both parents (Van Vloten-Doting et al. 1970). The repeated passage of an isolate of AMV from potato through tobacco resulted in a change in symptom type induced by that isolate (Hull, 1969).

AMV is moderately immunogenic and no serological relationship has been found with other viruses. The dilution end point, which is the dilution limits between which infectivity is lost, is between 10^{-3} and 10^{-4}, but can be higher (Jaspars and Bos, 1980). Infectivity in sap is best retained when phosphate buffer (0.01 to 0.1 M) of pH 7.0 to 7.5 is used for leaf extraction.

Table 2.2 Main diagnostic species for AMV, with their most common syptoms (adapted from Jaspars and Bos,1980)

Species	Symptoms
Chenopodium amaranticolor and C. quinoa	Chlorotic or necrotic local lesions; systemic chlorotic and necrotic flecking.
Nicotiana tabacum	Necrotic or chlorotic local lesion; Systemic mild mottle, bright chlorotic vein banding; ringspots.
Ocimum basilicum	Systemic yellow mosaic.
Phaseolus vulgaris	Many strains give necrotic local lesions others, chlorotic local lesions, or none; systemic mild mottle; vein necrosis and leaf distortion.
Pisum sativum	In most cv. local lesions/wilting of inoculated leaves with stem necrosis and plant death.
Vicia faba	Most strains give black necrotic local lesions; mild mottle; stem necrosis and plant death.
Vigna unguiculata	Necrotic local lesions and no systemic symptoms for most strains; no local lesion and various systemic symptoms for others.

2.3.1.2 Purification

AMV was the first aphid-transmitted virus to be purified (Ross, 1941). A modified Steere's butanol-chloroform method is generally used to purify AMV (Hull et al., 1969; Van-Vloten Doting et al., 1970). Infected leaves can be frozen more than a year before purification, without effect on yield or component composition (Smith, 1972). An emulsion is formed when two volumes of 1:1 chloroform-butanol are added to one volume of homogenized plant material. The emulsion is broken by centrifugation, and the aqueous phase is ultracentrifuged for a relatively long period to sediment all components. Up to 1.5 g of virus per Kg of tissue can be obtained. Further purification and separation of the various components is done by centrifugation in sucrose density gradients (Van Vloten-Doting et al., 1968). The infectivity of purified virus, which tends to decrease rapidly, can be maintained by the addition of 0.001 M EDTA (ethylenediamine tetra-acetate) (Bol and Kruseman, 1968).

2.3.2 White clover mosaic virus

White clover mosaic virus (WCMV) is a member of the potexvirus group. It was first described by Pierce (1935), and its properties were reviewed by Bercks (1971). Khadhair (1983) reviewed the derivation of its name. Johnson (1942) suggested that white clover mosaic was caused by a complex of two viruses, pea mottle and pea wilt viruses. The presence of two

viruses was confirmed later, and Pratt (1961) suggested the names of WCMV for pea wilt and CYMV for pea mottle virus.

2.3.2.1 Biological and physico-chemical properties

WCMV commonly occurs in North America, Europe, New Zealand and Japan (Bercks, 1971; Inouye, 1969). It is recognized as the most prevalent contact-transmitted virus in red and white clover (Clark and Barcl.y, 1972). Reduction of dry matter yield of up to 50% and of seed yield of up to 80% was associated with WCMV-infections in the same species (Barnett and Gibson, 1977; Fry, 1959; Tapio, 1970 as cited by Scott, 1982a). WCMV usually causes mosaic and mottle of varying severity in clovers (Bercks, 1971). A light green interveinal stripe or fleck generally appears on new leaves 1 to 3 weeks after inoculation (Smith, 1972). These symptoms may become indistinct several weeks later. Some isolates are latent in white and alsike clover, but cause a mild chlorotic mottle or occasional necrotic flecks in red clover (Bercks, 1971; Smith, 1972). Figure 2.2 shows a red clover plant infected with WCMV. Infections of white clover by WCMV resulted in reductions of the number of seeds per head and per floret, lower seed weight, lower number of heads per plant (Barnett and Gibson, 1975), lower leaf yield and plant height (Fry, 1959), and lower number of root nodules per plant (Guy et al., 1980).

Figure 2.2 Mosaic symptoms on a red clover plant infected with WCMV.

WCMV has been reported to infect around 100 species in 37 genera of nine families, including 76 species in 20 genera of the Leguminosae (Edwardson and Christie, 1986b).

Seed transmission from infected red clover was reported at the rate of 6% (Hampton, 1963), and thus is considered as the main source of primary infection (Barnett and Diachun, 1985). WCMV is one of the very few legume viruses for which no vector has ever been established. Some reports of transmission by aphids and dodder have been made, but later studies have failed to confirm these results (Barnett and Diachun, 1985; Bercks, 1971). The virus spreads from plant to plant by contact between healthy and infected plants. Man and animals contribute to this process during cultivation and harvest (Scott, 1982a).

The most common diagnostic species for WCMV are listed in Table 2.3. Varieties of Phaseolus vulgaris L., and Pisum sativum L. can be used as propagation hosts for virus purification.

WCMV is a flexuous rod-shaped particle of 480 X 13 nm containing single stranded RNA. Several strains with minor differences are distinguished on the basis of symptom differences on various hosts. The virus is moderately to strongly immunogenic. Serological differences between strains are minor if any. The virus is distantly related to other members of the potexvirus group. Dilution end-point usually reaches 10^{-5} to 10^{-6} (Bercks, 1971).

Table 2.3 Main diagnostic species for WCMV, with their most common symptoms (adapted from Bercks, 1971).

Species	Symptoms
Trifolium spp.	Weak and diffuse, sometimes irregular mosaic; Occasionally latent, sometimes giving necrotic flecks.
Phaseolus vulgaris	Chlorotic spots on inoculated leaves, often forming necrotic patches on leaf veins. Chlorosis on veins of systemically infected leaves.
Vicia faba	Ring-like or necrotic local lesions. Systemic mosaic, sometimes with necrosis.
Vigna unguiculata	Small necrotic lesions or chlorotic spots on inoculated primary leaves. Systemic mosaic, sometimes with vein-banding.
Pisum sativum	Wilting of inoculated leaves. Systemically infected leaves with vein-clearing or diffuse mottling. If wilt progresses upward, the plant dies.
Cucumis sativus	Inoculated cotyledons develop yellow-green spots or white local lesions. Diffuse yellow spots on systemically infected leaves.

2.3.2.2 Purification

Two classical methods for purification of WCMV are often used. The first one involves homogenization of infected bean leaves in a buffer containing 0.2% ascorbic acid and 0.2% sodium sulphite, followed by an ether extraction. After addition of carbon tetrachloride to the aqueous phase, and two cycles of high and low speed centrifugation, pellets are suspended in 0.01 M phosphate buffer pH 7.0 (Wetter, 1960). The other method consists of homogenization of pea leaves in 0.1 M phosphate buffer followed by three cycles of high and low speed centrifugation (Fry et al., 1960). Khadhair and Sinha (1982) purified WCMV with a procedure that was first used for carlaviruses (Veerisetty and Brakke, 1978). It involves homogenization of tissues in phosphate-citrate buffer, clarification of the extract with CaH_2PO_4, precipitation of the virus with polyethylene glycol (PEG), and differential centrifugation steps. Purification is completed by a sucrose density gradient centrifugation.

2.4 CONTROL OF VIRUS DISEASES

Virus diseases are traditionally controlled by manipulation of cultural practices to prevent infection (Barnett and Diachun, 1986). More recently, oil sprays and reflective mulch to control vectors have been used for the control of some insect transmitted viruses. None of these measures is practical for most perennial forage production systems. Seed-borne viruses, easily spread mechanically or by

vectors, are the most difficult to control, especially in perennial crops submitted to repeated cultural operations or grazing. It is recognized that the use of resistant varieties is the most practical and economical measure for virus disease control when resistance is available and can be utilized in a breeding program (Barnett and Diachun, 1986).

2.5 RESISTANCE TO VIRUSES IN PLANTS

2.5.1 General considerations

The terms "tolerance" and "resistance" used in this thesis follow the definitions given by Fraser (1985a, 1986). Tolerance refers to a resistance to symptom formation, or the ability to support virus multiplication without showing visible or severe symptoms. Since infections without visual symptoms can result in yield reductions as important as in susceptible plants (Kooistra, 1968), the term tolerance should ideally designate the ability to withstand infection without significant effects on yield components. Resistance refers to the ability to inhibit the virus reproductive cycle or the development of pathogenic effects in the host. Resistance can also be used as a more general term, including resistance to vectors, to seed transmission, to virus establishment, and tolerance.

Breeding plants for resistance can be done by empirical methods without detailed knowledge of the genetic basis of the resistance (Fraser, 1986). However, a knowledge

of the genetic mechanisms enables more appropriate decisions about the breeding strategy, and can provide useful information in understanding the mechanisms of resistance at the biological and physiological levels.

Classical genetic analysis of resistance to viruses involves crossing of resistant and susceptible parents, and determining the reaction of the F1, F2, and backcross generations to inoculation with the virus. In the great majority of studies on plant resistance to viruses, reviewed by Fraser (1987), and summarized in Table 2.4, segregation ratios for different host reactions have given evidence for simple Mendelian control. Out of 63 virus-host combinations considered, 29 are controlled by a single dominant gene; 10 are controlled at a single locus which is gene-dosage dependent; 11 by apparently recessive genes. In five cases, control is possibly oligogenic, and eight examples show possible effects of modifier genes.

Polygenic resistance and modifier effects can occur in two different forms. In one form, resistance can depend on cooperative or cumulative action of many genes, all involved in the mechanism. In the other form, modifiers, which are not directly involved with resistance can affect the antiviral activity of one or more major resistance genes (Fraser, 1985b). Evidence for polygenic resistance and modifiers came from two types of experiments: Classical genetic analysis of genetic ratios, and cytogenetic analysis of lines with modified chromosome complements. Contradictory results concerning

Table 2.4 Genetics of resistance to viruses in crop species. (adapted from Fraser, 1987).

Genetic basis	Number of host-virus combinations
Single dominant gene	29
Incompletely dominant (gene-dosage dependent)	10
Apparently recessive	11
Sub-total: monogenic	50
Possibly oligogenic	5
Monogenic with possible modifier genes or effects of host genetic background	8
Sub-total: oligogenic	13
Total number of host-virus combinations in sample	63

polygenic resistance have been obtained in some cases. Early studies on resistance to cucumber mosaic virus (CMV) in cucumber (Shifris et al., 1942) and to bean yellow mosaic virus (BYMV) in Phaseolus vulgaris (Baggett and Frazier, 1957) suggested that resistance was caused by two or three complementary genes and modifiers. Later studies clearly demonstrated that a single gene was in fact responsible for resistance in both cases (Wasuwat and Walker, 1961; Provvidenti and Schroeder, 1973). The latter results were obtained when environmental conditions were more rigourously controlled. The complex mechanisms proposed earlier were probably attempts to explain genotype-environment interactions in purely genetic terms (Fraser, 1985b; 1986).

2.5.2 Breeding red clover for resistance to viruses

Among forage legumes, species that have received more attention by breeders tend to exhibit more tolerance to viruses than others (Barnett and Diachun, 1986). Alfalfa, and white and red clover have been intensively selected by breeders. Although many viruses can infect alfalfa, only a few can cause damage, and it is difficult to infect alfalfa and white clover by mechanical inoculation with some of their common viruses (Barnett and Gibson, 1977).

Red clover has a diploid chromosome number of 14 (n=7). It is a cross pollinated species with a strong gametophytic self-incompatibility. The self-incompatibility is a one locus S-allele system which prevents selfing by reducing

the growth rate of pollen tubes on plants which have the same S allele (Taylor and Smith, 1979). Tetraploid forms (2n=48) have been developed in Europe, but are not used in North America, probably due to lower seed yields and little overall yield advantage over diploids (Smith et al., 1985).

The first step in developing resistant varieties involves the identification and selection of individual resistant plants (Taylor and Ghabrial, 1986). Populations of red clover consist of disparate genotypes, heterozygous for many traits, with striking plant-to-plant differences in morphology, and in response to virus infections (Barnett and Gibson, 1975; Diachun and Henson, 1956; Scott, 1982b; Stuteville and Hanson, 1964b). Diachun and Henson (1956) showed that differences in virus symptom expression, within red clover field populations, were due to genetic differences between plants.

Individual red clover plants resistant to several viruses have been observed (Barnett and Gibson, 1975; Diachun and Henson, 1956, 1960; Hanson and Hagedorn, 1961; Khan et al., 1978; Stuteville and Hanson, 1964b), but immunity to other common viruses has not been detected (Alconero, 1983; Barnett and Gibson, 1975; Scott, 1982b). In most cases, resistance is controlled by one to a few genes (Diachun and Henson, 1974; Khan et al., 1978). Khan and coworkers (1978) reported that resistance to single strains of red clover vein mosaic virus (RCVMV) was under the control of a single dominant gene.

Diachun and Henson (1974) showed that there were three types of resistance to clover yellow mosaic virus (CYMV), each controlled by a different dominant gene. The red clover cultivars Arlington and Kenstar have been released as being resistant to bean yellow mosaic virus (BYMV) (Smith et al., 1973; Taylor and Anderson, 1973). Other cultivars, such as Lakeland and Penscott, possess some levels of tolerance or resistance to other viruses (Barnett and Diachun, 1986). A program to introduce resistance to a virulent isolate of BYMV is in progress (Taylor et al., 1986). All ten clones, constituents of the cultivar Kenstar, were crossed with a hypersensitive plant resistant to virus isolate 204-1. After five backcross generations and selection, resistant plants were inter-crossed and progenies were field tested. The new population, although resistant to 204-1, was highly susceptible to a new soybean strain of BYMV. Since Diachun and Henson (1960) reported different reactions of red clover clones to different BYMV strains, these results are not surprising. Breeding for tolerance can be more appropriate than for resistance or hypersensitivity, for viruses occuring as several strains. It is also appropriate to determine whether tolerant plants might be tolerant to more than one virus strain, and if plants infected with one isolate are cross-protected against other strains. However, using tolerant breeding material can be hazardous since it can result in a high occurrence of symptomless carriers from which the virus can spread to non-tolerant plants or varieties.

Recent successful interspecific hybridizations between Trifolium pratense and T. sarosiense Hazsl., and between T. repens and T. ambiguum Bieb., using embryo rescue techniques, may result in new possibilities for red clover breeders. The resulting hybrid genotypes often carry resistance genes to several common clover viruses, provided by T. sarosiense and T. ambiguum genomes (Barnett and Diachun, 1985). Complete resistance to WCMV was only detected in T. ambiguum in a study on resistance in different clover species (Barnett and Gibson, 1975).

2.5.3 Screening for resistance

The first and perhaps the most important step in a breeding program involves screening of large plant populations to identify sources of resistance. Screening methods often involves testing of populations under greenhouse conditions followed by field studies. Greenhouse studies give indications on the overall resistance reaction. Comparisons between field resistance and greenhouse resistance should ideally be done to correlate plant responses under such distinct environments (McLaughlin and Scott, 1986).

2.5.3.1 Virus detection and identification

Due to the sub-cellular nature and the small size of viruses, specialized procedures are needed for their detection and identification. Diagnostic indicator hosts are used, but symptoms are not always reliable. Different strains of a virus

may cause different reactions on the same host or even have a different host range (Walkey, 1985), therefore, the inoculation of a wide range of hosts is recommended. Other characteristics such as serology, particle size and morphology, DNA/RNA genome, coat protein molecular weight and amino acid composition are used to identify viruses. Serology is probably the most widely used method. For definitive identification of any virus, several characteristics must be studied, while for routine detection, one characteristic may be sufficient (McLaughlin and Scott, 1986).

2.5.3.2 Mechanical inoculations

Plants to be inoculated should be large enough to be handled, and as young as possible for maximum susceptibility (McLaughlin and Scott, 1986). Growing conditions should be optimal for a rapid growth. Fertilization must be provided if plants are kept for long periods. Ideally, plants to be inoculated should be grown in a closely controlled environment with temperatures between 18 and 25 °C, high humidity and moderate light intensity. These conditions usually optimize susceptibility to infection and symptom expression (Walkey, 1985). Plants grown in normal greenhouse conditions tend to give different responses to infection as seasonal conditions change. To minimize these fluctuations, shading and cooling during the summer, and supplementary lighting during the winter, are essential.

Mechanical inoculation consists of the introduction of

infective virus or viral RNA into sub-lethal wounds made through the leaf surface. When a virus establishes itself in a living cell, infection occurs. Inoculum is generally in the form of sap obtained by grinding infected plant tissues in a suitable buffer, usually at the proportion of 1 g tissue per 5 to 10 ml of buffer. Phosphate buffers (0.02 to 0.1 M), pH 7 to 8, have been used routinely by many workers (McLaughlin and Scott, 1986). Optimal conditions vary for each virus-host combination, but generally low pH inactivates infectivity of most viruses (Gibbs and Harisson, 1976). Other chemicals [e.g. sodium diethyldithiocarbamate (Na-DIECA), 2-mercaptoethanol] may be added to counteract deleterious effects of crude sap extracts and enhance infectivity. A light abrasive such as celite (diatomaceous hearth) may be added to the inoculum, or the leaves of the plants to be inoculated can be dusted with carborundum (silicon carbide) or carundum (aluminium oxide) (McLaughlin and Scott, 1986).

Inoculations are done by gently rubbing the leaf surface with the finger or an object wetted with the inoculum. The pressure necessary to wound the cells without killing them, varies with the plant species, the age and condition of leaf, and the additive(s) present in the inoculum.

Genetic studies on resistance may involve inoculation of large populations with the same inoculum. Air brushes of the type used by artists can be used but the pressure and distance to the leaf from the nozzle require careful standardization

(Mackenzie et al., 1966). Several types of pressure inoculating devices have been successfully used for many types of viruses. They mainly consist of air compressors coupled to liquid guns (Mackenzie et al., 1966; Scott, 1982a). Scott (1982a) reported 100% infection of WCMV in red clover using a pressure of 414 KPa (60 psi). Pressures of 400 to 500 KPa are recommended to obtain uniform infection rates (Mackenzie et al., 1966).

Several pre- and post-inoculation treatments can affect the success of mechanical inoculations. Placing the plants in darkness or in a shaded environment for 24 hours before inoculation generally increases susceptibility. Washing inoculated leaves with water immediately after inoculation also enhances infection rates by most viruses (Walkey, 1985).

2.5.3.3 Symptoms

The first step to detect infection is the observation of symptoms. Changes in the infected plants may be conspicuous, such as alterations in leaf coloration and shape, or plant size, or they may be subtle and occur gradually, (McLaughlin and Scott, 1986). Symptoms on various hosts for the main legume viruses of the northern temperate zone were reported by Hampton et al. (1977). Barnett and Diachun (1985) reviewed the symptoms induced by virus infections in Trifolium species.

2.5.3.4 Serology

Many serological techniques have been developed for the detection of plant viruses (Van Regenmortel, 1982). Virus antiserum is normally prepared from the blood of animals immunized with purified virus preparations. Rabbit is the most widely used animal, but other rodents, goat, horse and chicken may also be used. The purity of the virus injected is critical, since it will determine the specificity of the antiserum. Plant components should be totally absent to avoid production of non-viral antibodies. Any serological test must include control samples of healthy tissues along with tested plants to detect non-specific reactions. Among available serological tests, the microprecipitin, latex agglutination, immunodiffusion, ELISA (enzyme linked immunosorbent assay), and immunosorbent electron microscopy (ISEM) are used with forage legume viruses (McLaughlin and Scott, 1986). The immunodiffusion and the ELISA were used in this study.

2.5.3.4.1 Immunodiffusion tests

All these tests involve the diffusion of either or both antigen and antibodies through a semi-solid medium (gel), to form a visible precipitin line where they meet. The simplicity of the protocols make these tests suitable for assaying many samples easily with little preparation. For elongated virus particles, difficulties of diffusing through the gel can be overcome by decreasing the gel concentration, or by physical or chemical degradation of the virus particles

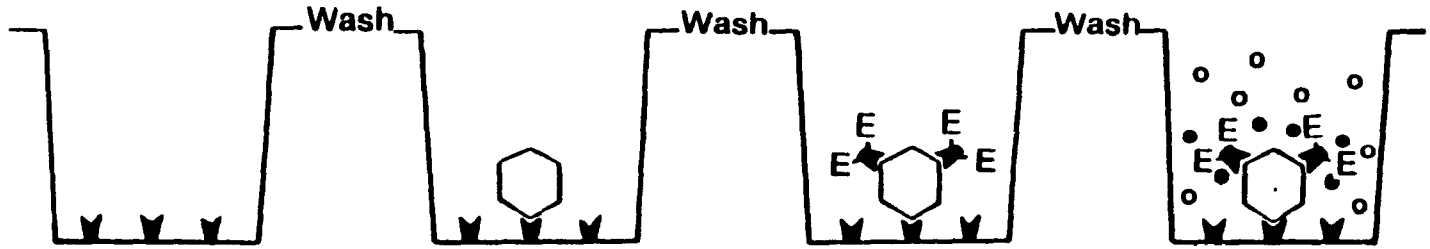
(Purcifull and Batchelor, 1977; Shepard, 1972).

Gels are made of 0.5 to 1.5% agar, buffered with phosphate or sodium chloride solutions containing sodium azide, an anti-microbial agent. The inclusion of sodium dodecyl sulfate (SDS), an anionic detergent, is often appropriate to degrade elongated viruses (Purcifull and Batchelor, 1977; Uyemoto et al., 1972)

For the Ouchterlony double diffusion test, wells of 5 to 6 mm diameter and 4 to 8 mm apart are cut into a gel layer in a Petri dish. Antigen is placed in one well, antiserum in the other, and diffusion is allowed over a certain period of time. A positive reaction produces a precipitin line between the two wells where the relative concentration of the reactants is optimal. The test allows simultaneous testing of various antigens with a given antiserum, but it is not highly sensitive, and requires the use of relatively large amounts of reactants.

2.5.3.4.2 The enzyme-linked immunosorbent assay (ELISA)

Clark and Adams (1977) described the adaptation of the double antibody sandwich form of the ELISA test (DAS-ELISA) (Voller et al., 1976) for the detection of plant viruses. This method, which is shown in Figure 2.3, became rapidly the most widely used serological test for plant virus detection (Clark et al., 1986; Hill, 1984).



1. Specific antibody adsorbed to plate
2. Add test sample containing virus
3. Add enzyme-labelled specific antibody
4. Add enzyme substrate

Fig. 2.3 Diagrammatic representation of the enzyme immuno-assay. (After Clark & Adams, 1977.)

In this multi-step method, specific antibodies are adsorbed onto a solid surface in wells of polystyrene microtiter plates. Unbound antibodies are rinsed away. Test samples suspected to contain the corresponding antigen are incubated in the sensitized wells, and antigen recognized by the adsorbed antibodies are bound. Unbound or non-specific antigens are rinsed away. A second antibody preparation (the same antibody used for coating but conjugated with an enzyme), the conjugate, is added to the bound antigen, forming the double antibody sandwich. Unbound conjugates are rinsed away. In the wells lacking antigen, no conjugate is retained. The double antibody sandwich is detected by the addition of a suitable chromogenic substrate solution, which forms a colored product in the presence of the enzyme. Visual observation reveals the presence of antigen in the test sample while spectrophotometric measurements can be related quantitatively to the relative virus concentration in the test sample (Van Regenmortel, 1982). In negative tests (without the specific antigen), the antibody sandwich does not form. Therefore, the enzyme is not present to induce color change in the substrate solution.

ELISA is generally as sensitive or more so (1 to 10 ng/ml) than most other methods for virus detection in plants (Clark et al., 1986). The test is also relatively rapid, specific and reliable. However, preparation of the test samples, which normally involves the homogenization of plant tissues, traditionally with mortars and pestles, represents a major limiting factor in terms of time and labour (Hill, 1984),

and may limit the scope of a study. Alternative homogenizing and crushing devices such as roller presses (McLaughlin et al., 1984), ball homogenizers and drilling devices (Mathon et al., 1987) have been proposed. Marco and Cohen (1979), Romaine et al. (1981), and Menassa et al. (1986) proposed the substitution of disks of intact leaf tissues for homogenized tissue extracts. Longer incubation of test samples and shaking of the plates were proposed to increase the sensitivity of the assay. Other parameters can also be modified to allow the use of intact leaf disks (Romaine et al., 1981): using a large number of disks per well, extending the substrate reaction, and changing the concentration of the reagents.

Preparation of reagents and use of ELISA for the detection of forage legume viruses are described by McLaughlin and Barnett (1978, 1979), and McLaughlin et al. (1981, 1984).

3. MATERIALS AND METHODS

3.1 VIRUS MANIPULATION

3.1.1 Virus maintenance and host assays

A culture of white clover mosaic virus (WCMV), originally obtained in a survey of the Ottawa area during the summer of 1979 (Khadhair and Sinha, 1982) was maintained in red clover plants in a greenhouse, after multiplication from a single lesion on Gomphrena globosa L.. The alfalfa mosaic virus (AMV) culture was originally collected in the Ste-Anne-de-Bellevue area during the summer of 1982, and also maintained in red clover plants. Host ranges were studied for both isolates to assess purity. Hosts from Leguminoseae, Amaranthaceae, Cucurbitaceae, Chenopodiaceae, Labiaceae, and Solanaceae (Table 3.1) were grown from seeds and inoculated at an early stage with infected red clover leaves ground in mortar and pestle, in five times their weight of 0.1 M potassium phosphate buffer, pH 7.0 containing 0.01% 2-mercaptoethanol and diatomaceous earth (Celite, Johns Manville). Both viruses were assayed on 10 different species. Multiplication hosts used as sources for purification were Pisum sativum cv Little Marvel, in the case of WCMV, and Nicotiana tabacum cv White Burley or Samsun NN for AMV.

Table 3.1 Differential hosts inoculated with AMV and WCMV.

Plant Family	Species
Chenopodiaceae	Chenopodium amaranticolor C. quinoa
Cucurbitaceae	Cucumis sativus
Labiaceae	Ocimum basilicum
Leguminoseae	Pisum sativum (cv Lincoln and Little Marvel) Phaseolus vulgaris (cv Bountiful, Pinto US No 1, and Tendergreen) Vicia faba Vigna unguiculata
Solanaceae	Nicotiana tabacum (cv Sansum NN, Havana 38, and white Burley) N. glutinosa

3.1.2 Virus purification

3.1.2.1 Alfalfa mosaic virus

AMV was purified as described by Hull et al. (1969), followed by a sucrose density gradient centrifugation (Van Vloten-Doting et al., 1968). Each 100 g of systemically infected tobacco leaves harvested 12 days after inoculation, was homogenized at 4 °C in 100 ml of 0.01 potassium phosphate buffer pH 7.1 with 1.0 g of ascorbic acid. The pH was readjusted to 7.1 by adding a few ml of 50% K_2HPO_4. This suspension was re-homogenized with 100 ml of 1:1 chloroform-butanol for 1 minute. The emulsion was broken by centrifugation at 5,000 rpm (6,050 g) for 5 minutes in a Sorvall rotor (Type SS-34). The aqueous phase was withdrawn and

centrifuged at 36,000 rpm (approximately 120,000 g) for 2.5 hours in an angle 65 rotor in a Spinco model L ultracentrifuge. The pellet was resuspended in 0.01 M phosphate buffer pH 7.1 and centrifuged again at low speed (10,000 rpm for 10 min.) and high speed (36,000 rpm for 2.5 hours). The pellet was resuspended in 10 ml of 0.01 M potassium phosphate buffer pH 7.1 containing 0.001 M EDTA (Bol and Kruseman, 1968). Virus concentration was determined with a Unicam SP 800A ultraviolet Spectrophotometer, using the following formula:

$$\text{Concentration (mg/ml)} = \frac{\text{(Abs. at 260 nm) (dilution)}}{(5.1 + 4.8 + 4.8 + 4.7)/4},$$

which takes into account the absorbance at 260 nm of all four RNA species (Jaspars and Bos, 1980).

Decolorized 60% sucrose (600 g/l) stock solution was used to prepare 10 to 40% sucrose density gradients in 30 ml polyallomer tubes. Successive volumes of 7, 7, 7, and 5 ml of 40, 30, 20, and 10% sucrose (density at 4 °C of 1.156, 1.117, 1.079, and 1.039 respectively) in 0.01 M NaH_2PO_4 (pH 7.0) were carefully layered in the tubes and allowed to diffuse overnight to form a smooth gradient. Two ml of approximately 1.0 mg/ml virus suspension were loaded onto each column and centrifuged in a SW 25.1 rotor at 22,000 rpm (52,600 g) for 2.5 hours. After centrifugation, the sucrose columns were fractionated with an Isco model D density gradient fractionator connected to an Isco model UA 5 absorbance monitor, by pumping a 50% sucrose solution through a hypodermic syringe inserted in the bottom of the tube (Fig. 3.1) (Brakke, 1963). This forced the gradient

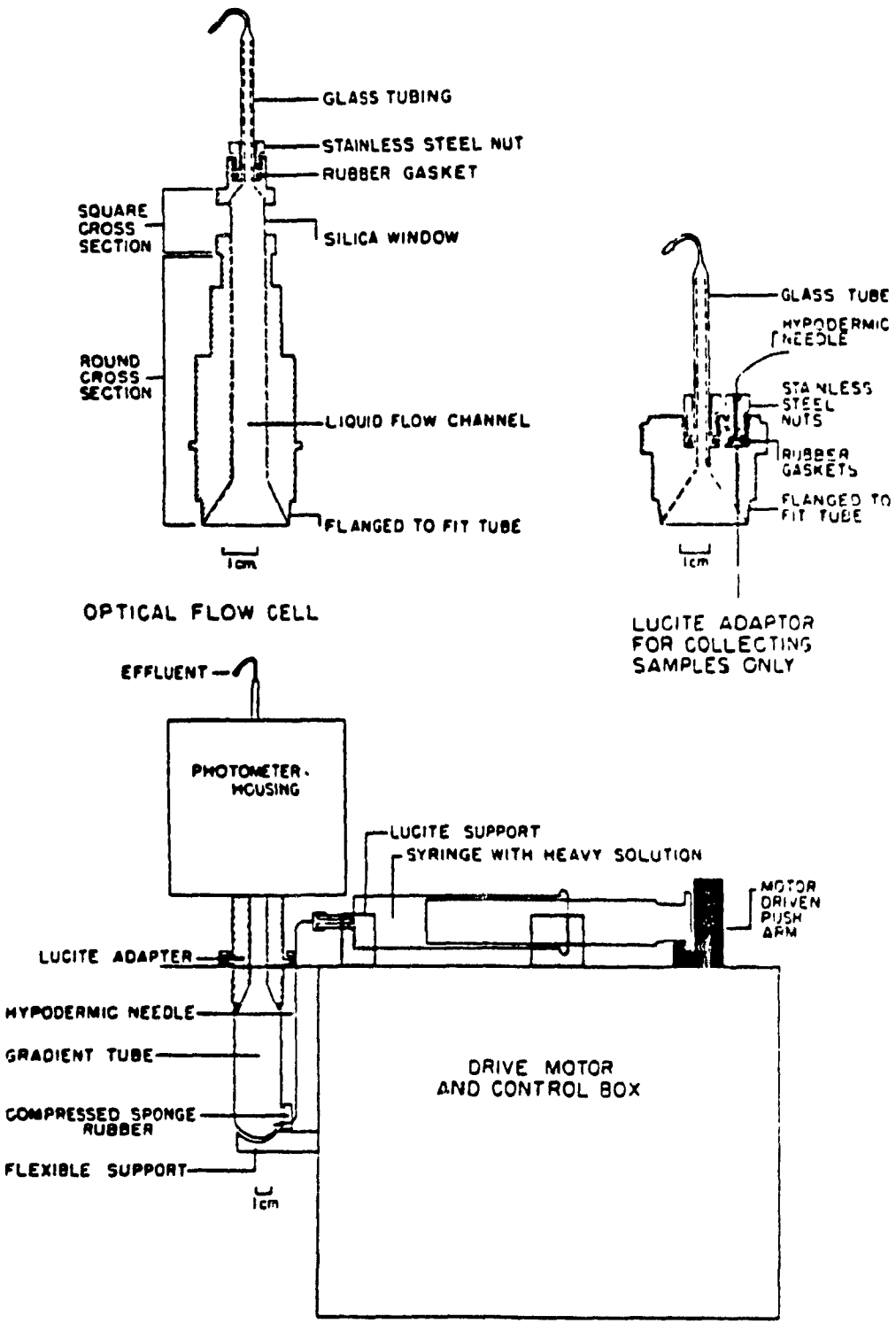


Fig 3.1 Schematic representation of ISCO gradient column fractionator and flow densitometer (BRAKKE, 1963).

content to pass through the flow cell of an ultraviolet optical unit coupled to the absorbance monitor, which localized and recorded positions of the virus zones. The collected fractions were pooled and diluted 4 times in 0.01 M phosphate buffer pH 7.1. The suspension was centrifuged for 3 hours at 36,000 rpm in the angle 65 rotor. Resulting pellets were resuspended in a small volume of PO_4 buffer. The ultraviolet absorbance was read to determine the final concentration of the purified virus before storage at -20 °C.

3.1.2.2 White clover mosaic virus

WCMV was purified from frozen infected pea leaves and stems harvested 7 days after inoculation as described by Khadhair and Sinha (1982). Plant material was homogenized in two parts (w/v) of 0.165 M phosphate and 0.018 M trisodium citrate (pH 9.0) and one part of chloroform. The homogenate was strained through two layers of cheesecloth and centrifuged in a Sorval SS 34 rotor at 10,000 rpm (12,100 g) for 10 minutes. The supernatant was clarified with calcium phosphate formed *in situ* by slow and simultaneous addition of one-twentieth volume 0.2 M Na_2HPO_4 and one-hundredth volume of 1.0 M $CaCl_2$ with constant stirring for 15 to 20 minutes. The resulting suspension was centrifuged for 10 minutes at 10,000 rpm and the virus was precipitated from the supernatant by dissolving 6% (w/v) solid polyethylene glycol 6,000 (PEG). The precipitated virus was then pelleted by centrifugation at 10,000 rpm for 15 minutes, and resuspended in extraction buffer

(one tenth of the original volume of sap) containing 1.0% Triton X-100 (alkylaryl polyether alcohol). The suspension was centrifuged at 10,000 rpm for 10 minutes, layered on a pad of 10 ml of 25% sucrose and centrifuged in an angle 65 rotor at 36,000 rpm for 1.5 hour. Pellets were resuspended in diluted extraction buffer (one tenth of the original molarity) and centrifuged for 10 minutes at 8,000 rpm. The supernatant was then submitted to a sucrose density gradient (10-40% sucrose) centrifugation and fractionated as described for AMV except that sucrose solutions were prepared in 0.0165 M Na-phosphate and 0.0018 M Na-citrate buffer pH 8.9, and centrifugation at 22,000 rpm was done for a shorter period (105 minutes). Final virus concentration was determined with the formula (Bercks, 1971):

$$\text{Concentration (mg/ml)} = \frac{(\text{Abs at } 260 \text{ nm})\ (\text{dilution})}{3.6}$$

The virus was stored at -20 °C.

3.1.3 Serology

Rabbit antiserum to both viruses was obtained from laboratory stocks. Crude normal serum and antisera were used in double diffusion tests. Samples of antisera to WCMV, AMV, and clover yellow mosaic virus (CYMV) were obtained from O.W. Barnett (Barnett and Gibson, 1975) for comparison purposes with our isolates.

3.1.3.1 The Ouchterlony agar double diffusion test

Immunodiffusion agar plates were prepared by pouring 10 ml of melted agar solution into plastic Petri dishes. The following three media were prepared (Uyemoto et al., 1972; Van Regenmortel, 1982):

SALINE: 0.8% Noble agar (Difco)
0.85% NaCl
0.1% NaN_3

PHOSPHATE: 0.8% Noble agar
0.01 M Phosphate buffer pH 6.5
0.1% NaN_3

SDS: 0.85% Noble agar
0.5% SDS (sodium docedyl sulfate)
0.02% NaN_3.

After solidification at room temperature, the plates were marked into four quadrants. Groups of wells were cut into each quadrant in a hexagonal arrangement and sucked out with the upper end of broken pasteur pipets (Canlab #5202) having an external diameter of 6.0 mm, and connected to a vacuum water line. Central wells were filled with antiserum or normal serum and peripheral wells with diluted purified virus suspension or comminuted plant tissues in the appropriate buffer (saline, phosphate or SDS).

3.1.3.2 The ELISA test

The "double antibody sandwich" method of the enzyme-linked immunosorbent assay (DAS-ELISA) was done as described by Clark and Adams (1977) with several modifications (McLaughlin and Barnett, 1978; Mclaughlin et al., 1981). The reagents for ELISA are described in Appendix I.

3.1.3.2.1 Extraction of gamma-globulins from crude antiserum

Chromatography of aliquots of whole antiserum through a 1 X 20 cm column of DEAE [(diethyl aminoethyl) Affi-gel blue (Biorad)] was used for extraction of gamma-globulins. The serum was dialysed against three changes of 0.02 M Tris-HCl and 0.028 M NaCl pH 8.0 at 4 °C during a total of 24 hours. Fifteen ml of DEAE gel (ratio gel-serum 7.5:1) was successively pre-washed, in a Buchner funnel, with 5 volumes of 0.1 M acetic acid (pH 3.0) containing 1.4 M NaCl and 40% (v/v) isopropanol, and with 10 bed volumes of starting buffer (0.02 M Tris-HCl and 0.028 M NaCl, pH 8.0). The gel was then poured in the column and eluted with five more volumes of starting buffer. The column of gel was allowed to settle for several hours and the hydrostatic pressure was adjusted to obtain a flow rate of 1.0 ml per minute. Glycerol was added to the antiserum to a final concentration of approximately 10% (v/v). An aliquot of 2.0 ml was layered onto the column which was eluted with three bed volumes of starting buffer. The effluent, spectrophotometrically analysed (OD_{280}) with an Isco model UA2 analyser connected to an Isco model 610 "Lab graph" recorder, was

collected in 2.0 ml fractions in an Isco model 270 fraction collecter. Fractions containing gamma-globulins were pooled and further purified by saturated ammonium sulfate precipitation (Clark and Adams, 1977). An equal volume of saturated $(NH_4)_2\ SO_4$ was slowly added to the suspension at room temperature with gentle stirring. The pH was adjusted to 7.8 with 1.0 N NaOH. The mixture was held for 60 minutes at room temperature and centrifuged for 10 min. at 10,000 rpm. The resulting pellet was resuspended in 2.0 ml of half strength phosphate buffered saline (PBS, see Appendix I). The concentration of gamma-globulins was estimated by measuring the absorbance at 280 nm, and adjusted to 1.0 mg/ml (OD_{280} = 1.4). Purified gamma-globulin was stored in 1.5 ml micro test tubes at -20 °C.

3.1.3.2.2 Conjugation of alkaline phosphatase with gamma-globulin

Five parts of alkaline phosphatase type VII-T (Sigma # CP-6774) were added to two parts (w/w) of purified gamma-globulin (1.0 mg/ml) and dialysed in 500 ml of PBS buffer containing 0.2% glutaraldehyde (4.0 ml of 25% in 496 ml buffer), during 4 hours at room temperature (McLaughlin et al., 1981). Further dialysis against three changes of PBS buffer (500 ml) was done at 4 °C during 24 hours to remove the excess glutaraldehyde. Prior to storage at 4 °C, 5.0 mg of bovine serum albumin (Sigma # A2153) was dissolved per ml of conjugate suspension.

3.1.3.2.3 Detection of infection

To determine the optimum reagent dilutions for the test, a "checkerboard" similar to the one proposed by McLaughlin and Barnett (1978) was designed (Fig. 3.2).

For routine detection, Falcon microtest assay plates (# 3912) were sensitized with 1.5 ug/ml of anti-WCMV or 5.0 ug/ml anti-AMV purified gamma-globulin in carbonate coating buffer for 2 hours at 37 °C. After washing with PBS-TWEEN buffer, the plates were stored at -20 °C in plastic bags with a wet paper towel for later use. Test samples consisted of either 10% (w/v) ground red clover leaf tissues in PBS-TPO buffer or three to nine (diameter = 3 or 5 mm) intact leaf disks per well (fresh weight 1.0 and 2.3 mg respectively) immersed in 200 ul of the same buffer. Wells of the peripheral rows and columns were generally not used to minimize the "plate effect" described by Clark and Adams (1977). Leaf disks were cut with paper punches (McGill Co. Morengo ILL.) from stacked leaflets and were handled with dissecting needles. For sample incubation, plates were covered and placed on a reciprocating shaker (100 cycles per minute) at 4 °C for a period of 16 to 18 hours. Enzyme-conjugated gamma-globulins were diluted in PBS-TPO buffer 1000-fold for WCMV and 250-fold for AMV, and incubated at 37 °C for 2 to 3 hours. Substrate solutions (p-nitrophenyl phosphate, Sigma # 104-105) were prepared at 1.0 mg/ml in substrate buffer. The enzyme substrate reaction was allowed to proceed at room temperature for 30 min. (WCMV) or 1 hour (AMV) and was stopped by adding 50 ul of 3.0 N NaOH to the

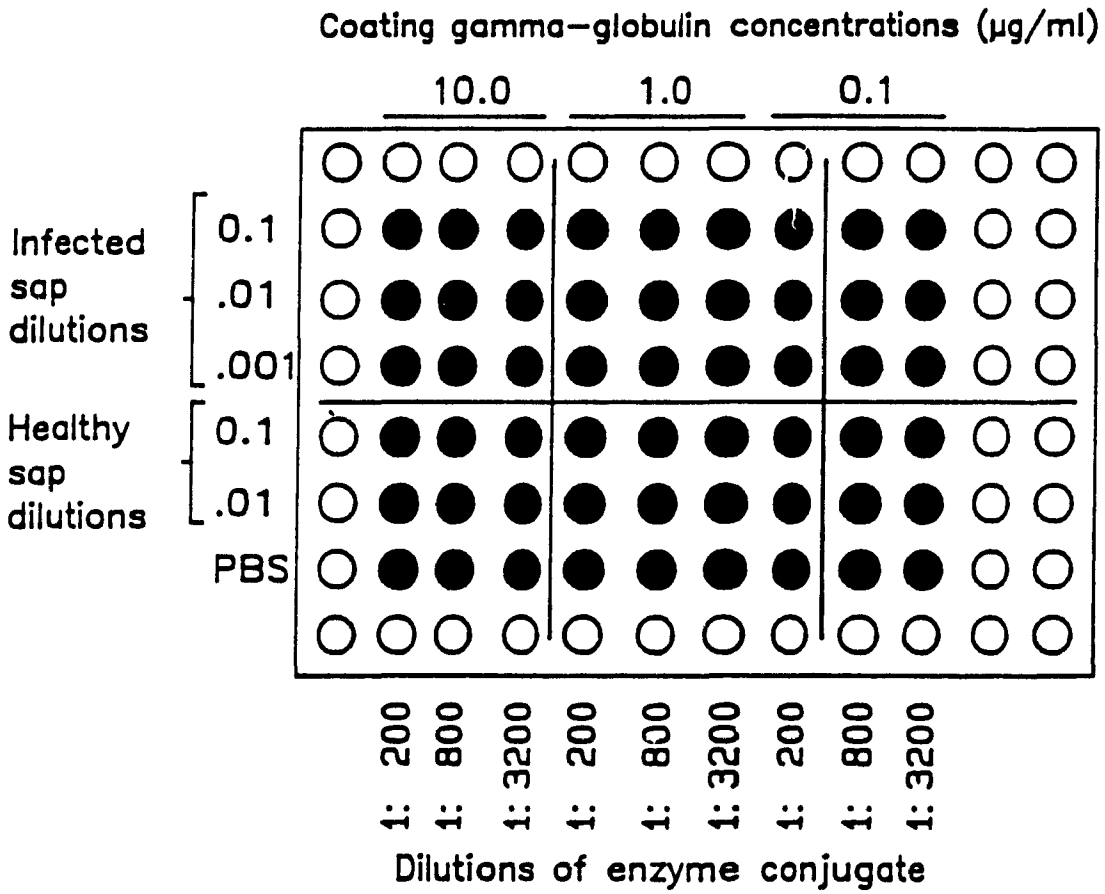


Figure 3.2 The ELISA "checkerboard". Serial dilutions of reagents and test samples in respective rows and columns to determine optimum concentrations for high sensitivity and low non-specific reactions.

wells. Absorbance was read at 405 nm with a Titertek (Multiscan MC) microplate reader. The test was considered positive when the absorbance value was superior to the mean absorbance value of negative controls plus three times their standard deviation. Samples resulting in dubious absorbance values were re-tested until conclusive results were obtained. The sensitivity of the ELISA system was determined by assaying serial dilutions of the purified viruses from 0.1 to 10^{-6} mg/ml in sample buffer for AMV and 0.1 to 10^{-7} mg/ml in both buffer and healthy red clover sap for WCMV.

To evaluate the leaf disk sampling method for the detection of WCMV, one, three, and six disks were immersed in the same volume of PBS-TPO buffer used for grinding the respective number and size of disks. The effect of extending the enzyme-conjugate incubation time was also evaluated by comparing the absorbance values resulting from leaf disks and comminuted samples with 0.5, 1.0, 2.0, and 3.0 hours of conjugate incubation. Some experiments were done with infected greenhouse plants showing light mosaic symptoms during mid-summer (average temperature = 22-35 °C), while others involved material growing in cooler temperature (T = 15-24 °C) and showing more severe mosaic. For these experiments, test samples were completely randomized with at least eight replicates per plate. Analysis of variance (SAS-ANOVA) was carried out, and since all F tests were significant ($p < 0.01$), least significant differences and standard deviations were computed for each sample type.

3.2 GENETIC STUDIES ON RESISTANCE

3.2.1 Parental material

All plant material was maintained and manipulated in the greenhouse, where average winter temperatures were around 18 to 24 °C and summer temperatures between 22 and 34 °C. Red clover plants previously selected by a former student for resistance to AMV and tolerance to WCMV (parents and CO F_1) were used as parental material (Appendix II). Ten plants from Montcalm and Florex cultivars selected as tolerant to WCMV, and 16 vigorous plants selected from a pool of CO F_1 individuals resistant to AMV (cultivars Arlington, Montcalm, Florex, and Pacific) were used for the hybridization. Twelve plants, susceptible to the appropriate virus, were selected from nursery material (cvs Florex, Tristan, Macdonald College strains 117 and 123) on the basis of relative vigor and leaf pubescence. Parental plants involved in crosses with their respective numbers of progenies are listed in the "Results and Discussion", section 4.

Stem cuttings from all parents were rooted in indole butyric acid in a perlite-vermiculite medium (1:1) in an intermittent misting frame for a period of 3 weeks. Several weeks after transfer to soil, one plant of each genotype was mechanically inoculated, as described previously, with the appropriate virus to confirm its resistance/ susceptibility character.

3.2.2 Hybridization procedure

Parental material was transplanted in a soil medium of loam, peat moss, vermiculite and sand (4:1:1:1) and fertilized with a mixture of 10-52-10 (5 g/l) and 0-15-30 (2 g/l). All stems were cut back to about 3.0 cm above the soil surface and artificial light (sodium lamps) was provided from 18:00 to 22:00 h to extend the photoperiod to approximately 16 hours a day. Hybridizations of the first cycle were carried out from the end of August to the beginning of October 1987. A second cycle of crosses with WCMV-tolerant material was done in August and September 1988. Abundant and frequent watering was necessary to prevent wilting in warmer periods. Insects, mites, and fungal pathogens were routinely controlled.

3.2.2.1 Pollinations

Flower heads with about half of the florets open were preferentially chosen for pollination. Reciprocal crosses were done between 10:00 h and 15:00 h during sunny days with methods similar to those described by Taylor (1980). The narrow end of a toothpick was further thinned and covered with small amounts of black fibres obtained by scraping a piece of felt. This end was inserted between the standard and the keel petals and a downward pressure was applied to force out the staminal column causing the pollen to adhere to the pollinating instrument (Figure 3.3). Groups of 3 to 6 florets were visited before transferring the pollen to, and harvesting new pollen from, the other parent of the reciprocal. When moving from one parent to

Figure 3.3 Harvesting pollen from red clover florets for hybridization with a toothpick covered with felt fibers.

Figure 3.4 Hybridized red clover plants on a greenhouse bench under supplemental lighting.

the other, the string bearing the identification tag was inserted between pollinated and unpollinated florets. The pollinating instrument was soaked in alcohol for about 10 min. and dried for 30 min., and hands were washed with soap and warm water between each cross, to avoid pollen mixtures. Figure 3.4 shows hybridized red clover plants in the greenhouse.

3.2.2.2 Harvest and storage

Six to eight weeks after pollination, entire heads were harvested and stored at room temperature for complete drying for at least 2 weeks. Seeds were hand threshed, counted and stored at -20 °C for varying periods.

3.2.3 Culture and mass inoculation of progenies

The day before sowing, seeds were scarified for a few seconds between two sheets of 120 grit sandpaper. They were placed on a wet filter paper in a Petri dish, and allowed to swell overnight. At sowing, unswollen seeds were re-scarified if less than 100 swollen seeds were available. Progenies were grown in plastic flats containing 72 individual cells (Kord products, Bramalea, Ont.) in the soil medium previously described (Figure 3.5). Red clover seedlings were grown for a period of 8 to 10 weeks before a first inoculation, with frequent waterings to avoid dehydration due to the small volume of soil medium. Fertilization with 20-20-20 (5.0 g/l) was initiated fortnightly from the second month. Inoculum of WCMV was prepared by grinding systemically infected pea leaves (cv

Figure 3.5 Plastic flats (72 cells) used for culture of progenies.

Little Marvel), either fresh or frozen, in five volumes of 0.01 M potassium phosphate buffer pH 7.0 containing 0.01% 2-mercaptoethanol, and passing the homogenate through a double layer of cheesecloth. Celite (Johns Manville) was added at 0.5 g per 100 ml of extract. The use of an ice container to maintain infectivity, and frequent stirring of the suspension to avoid sedimentation of the abrasive, were necessary during inoculation. Inoculum of AMV was prepared much the same way except that only fresh infected tobacco (cv White Burley or Samsun NN) ground in three volumes of buffer gave satisfactory results.

Inoculations were done by spraying the inoculum over the seedlings with an artist's air brush (Badger- USA model 350) coupled to an air compressor (Gelman, model 13152). Since this did not yield pressures in the order of 60 to 80 psi (412-550 KPa) as recommended by Mackenzie et al. (1966), virus penetration was favored by roughly smearing the surface of sprayed flats with hands (Figures 3.6 and 3.7).

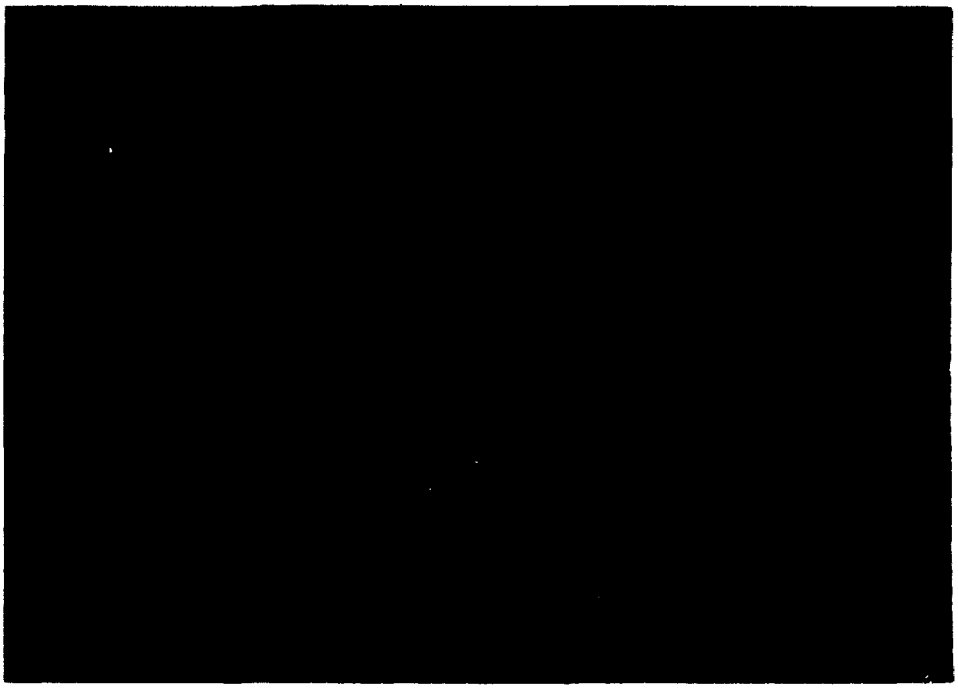
In addition to progeny populations, three flats (216 seeds) of each of the cultivars Florex, Montcalm, and Tristan were sown and screened for WCMV-tolerance.

3.2.4 Screening red clover populations

Virus-caused symptoms were visible from 2 to 3 weeks after WCMV inoculations and 4 to 6 weeks after AMV inoculations. Removal of infected plants was done 1 or 2 weeks

Figure 3.6 Spraying inoculum on young red clover seedlings with an artist air brush coupled to an air compressor.

Figure 3.7 Smearing seedlings after spraying to favor virus penetration in tissues.



after the first appearance of symptoms. Symptomless seedlings were rearranged in the flats to conserve space, and re-inoculated. Three cycles of inoculation and selection were carried out for each population. Plants remaining symptomless were submitted to an ELISA test, using the leaf disk sampling method (replicated samples), to detect if any tolerance to AMV and immunity to WCMV occurred. Seedlings were trimmed back to minimize evapo-transpiration, especially during warm summer periods. Since populations screened for AMV-resistance were grown for longer periods, 3 to 4 cm of soil medium was added after 2 to 3 months underneath the cells in the flats, to maintain vigor and prevent dehydration of the seedlings.

3.2.5 Statistical analysis

The uniformity between reciprocals of each cross was determined with the homogeneity of binomial proportion test described by Ostle and Mensing (1975). The chi-square test of independence (Daniel, 1978) was performed to determine the homogeneity within each category of crosses. Analysis of variance (General Linear Procedure - SAS - GLM) was carried out with transformed arcsin $\sqrt{\%}$ data, for different genotypes categories. Significant differences between categories were located with the Duncan's New Multiple - Range test. Linear regression analysis of transformed (Arcsin) % resistant / tolerant progeny on the number of "doses" of resistance / tolerance in parent genotypes was performed and plotted. Doses refer to the number of times resistant/tolerant plants were involved in crosses to give those progenies. Although

each cross is unique and there are no replicates within cross categories, the analysis was performed to provide some indications on the significance of the grouping in those categories.

3.3 SEED TRANSMISSION OF WCMV

3.3.1 Screening progenies from tolerant parents

Seedlings from the C1 F_1 and C2 F_1 generations from WCMV-tolerant parents (which were systemically infected) were visually inspected for mosaic symptoms prior to inoculation. The ELISA leaf disk test was also done on 1,140 seedlings from 24 different C1 F_1 crosses with duplicated samples in adjacent wells of the microplates.

3.3.2 Screening progenies from susceptible parents

During the spring of 1988, 30 WCMV-infected plants exhibiting severe mosaic symptoms were repotted, trimmed, and fertilized weekly with a low nitrogen soluble fertilizer mixture (10-52-10 at 5 g/l, and 0-15-30 at 2 g/l). They were maintained under an extended photoperiod, as described before, to stimulate flowering. At blooming, they were divided into two groups and placed in the screened cages (1.0 X 1.0 X 1.0 m) shown in Figure 3.8 Three bumble bees (Bombus spp.) were captured and placed in each cage, with water and a sugar solution. The bees were replaced with new ones when mortality

Figure 3.8 Screened cages used for random pollination of severely WCMV-infected red clover plants with bumble bees.

occurred to obtain uniform pollination, during a period of 4 weeks. Maximum day temperatures reached around 34 °C at several occasions in the greenhouse during this period. Fertilized heads were harvested as described before, and pooled for individual mother plant. Progenies were grown in 96-celled flats, and labelled with maternal origin. These resulted in approximately 90 seedlings per mother plant which were grown and observed for visual symptoms during a period of 8 weeks. ELISA tests (leaf disks) were done on 720 seedlings, which were progenies of 10 randomly selected mother parents.

4. RESULTS AND DISCUSSION

4.1 PURIFIED VIRUS ULTRAVIOLET ABSORBANCE SPECTRA

After purification with sucrose density gradient centrifugation, fractions containing the virus were localized through their ultraviolet absorbance at 254 nm, and collected. Figures 4.1 and 4.2 reproduce the spectra for AMV and WCMV respectively. Note the three major absorbance peaks for AMV in Fig. 4.1 corresponding to different particle sizes of the virus.

4.2 ASSESSMENT OF THE PURITY OF VIRUS CULTURES

Host reactions, electron microscopy and serology confirmed the identity and purity of the virus isolates despite their maintenance in living plants for more than three years.

4.2.1 Differential host reactions

Differential hosts inoculated with both AMV and WCMV showed symptoms corresponding to published descriptions (Bercks, 1971; Hampton et al., 1978; Jaspars and Bos, 1980). Figures 4.3, 4.4, 4.5 and 4.6 show some of the typical symptoms observed on infected hosts and Table 4.1 lists the principal symptoms detected.

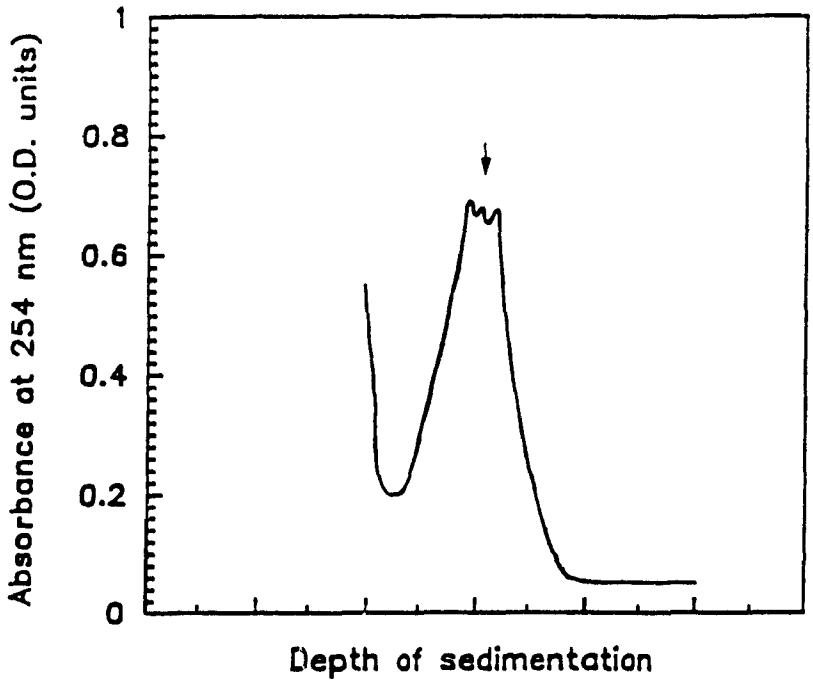


Figure 4.1 UV absorbance pattern (254 nm) of AMV during fractionation after density gradient centrifugation Fractionation from SW 25.1 rotor tubes at 5.0 ml/min Chart speed of 60 cm/h, and 1.0 OD range.

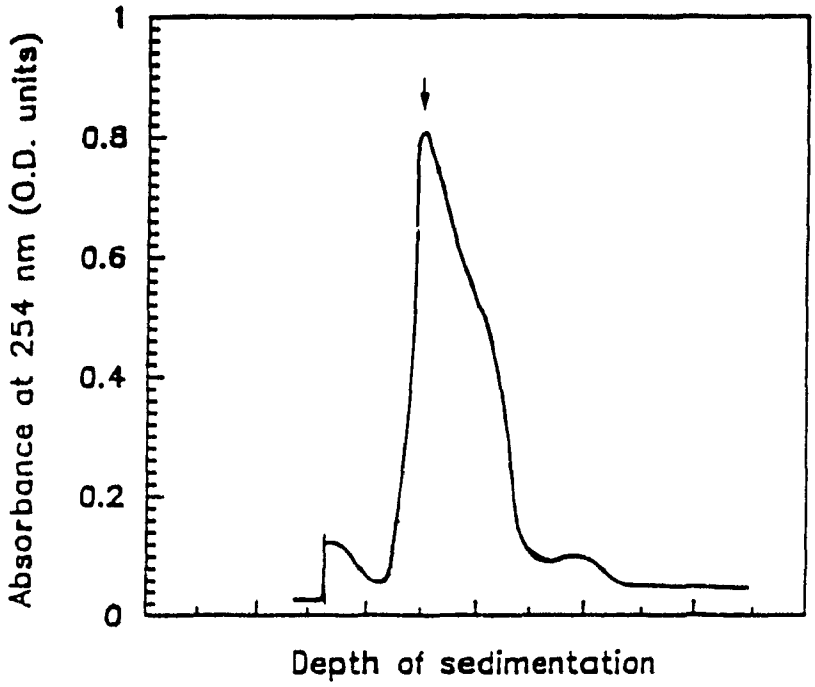


Figure 4.2 UV absorbance pattern (254 nm) of WCMV during fractionation after density gradient centrifugation Fractionation from SW 25.1 rotor tubes at 5.0 ml/min Chart speed of 60 cm/h , and 2.0 OD range.

Figure 4.3 Systematically infected Chenopodium amaranticolor with AMV showing flecking and leaf distortion (right).

Figure 4.4 Systemic necrosis of Vicia faba infected with AMV.

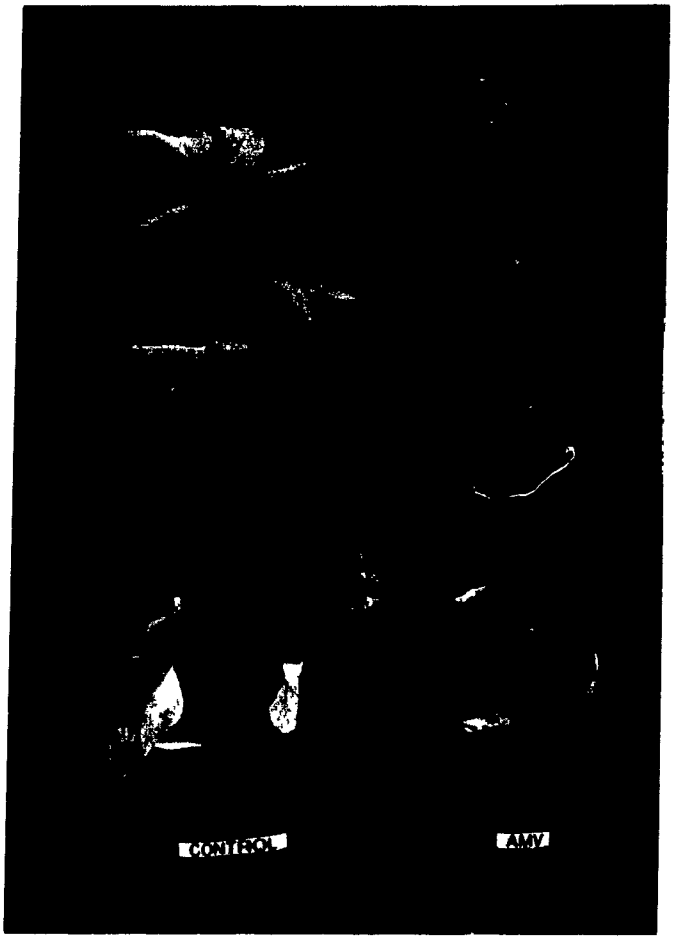

CONTROL
AMY

Figure 4.5 Systemic chlorosis of Nicotiana tabacum (cv White Burley) infected with AMV.

Figure 4.6 Systemic syptoms on Phaseolus vulgaris infected with WCMV showing typical star-shaped chlorotic lesions.

Table 4.1 Differential host reactions to mechanical inoculations with AMV and WCMV.

Diagnostic species	Inoculum	Host reactions
Chenopodium amaranticolor and C. quinoa	AMV :	-Necrotic local lesions -Systemic flecking -Leaf distortion
	WCMV :	---- (1)
Cucumis sativus	AMV :	-Chlorotic local lesions -mosaic
	WCMV :	-Diffuse light green local lesions
Nicotiana tabacum (cv Sansum NN, Havana 38, and White Burley)	AMV :	-Local chlorosis or necrosis of varying severity -Systemic mottle, vein banding and chlorotic ringspots.
	WCMV :	----
N. glutinosa	AMV :	-Local necrosis, mosaic
	WCMV :	----
Ocimum basilicum	AMV :	-Pronounced systemic yellow mosaic
	WCMV :	----
Phaseolus vulgaris (cv Bountiful, Pinto #1, and Tendergreen)	AMV :	-Chlorotic local lesions
	WCMV :	-Mosaic -Star-shaped chlorotic lesions
Pisum sativum (cv Lincoln)	AMV :	-Chlorotic local lesions
	WCMV :	-Chlorotic local lesions, mosaic

Table 4.1 (Continued)

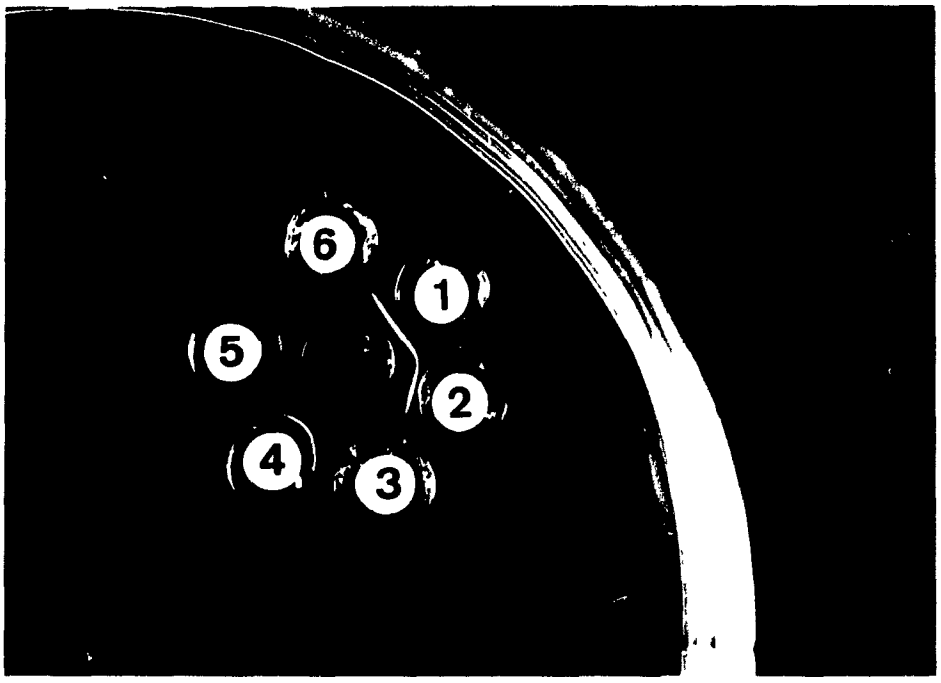
Diagnostic Species	Inoculum	Host Reactions
Pisum sativum (cv Little Marvel)	AMV :	-Chlorotic local lesions
	WCMV :	-Local lesions, wilting -Plant death
Vicia faba	AMV :	-Necrotic local lesions -Stem necrosis -Wilting of young leaves
	WCMV :	-Necrotic local lesions -Necrotic ringspots
Vigna unguiculata	AMV :	-Small necrotic local lesions
	WCMV :	-Chlorotic local lesions -Systemic interveinal chlorosis

(1) ---- represents the absence of symptoms

4.2.2 Ouchterlony double diffusion tests

The double diffusion tests permitted the confirmation of the identity of our virus isolates and the absence of cross contamination between AMV and WCMV. The specificity of our antisera was also confirmed since no precipitate was detected with crude sap extracts of healthy plants from different species. Infected plant extracts reacted positively with antisera from our laboratory stocks, as well as with the antisera to the same viruses used by Barnett and Gibson (1978) and kindly provided by O. W. Barnett, which confirmed the serological relationships with our isolates. Figure 4.7 illustrates typical precipitates obtained from the reaction between homologous antigen and antiserum.

Figure 4.7 Positive reaction in an Ouchterlony double diffusion plate between homologous antigen and antiserum in saline agar. Central well contains AMV crude antiserum; peripheral wells contain test samples: (1) and (2) purified AMV at different concentrations; (3) sap of red clover infected with unidentified virus; (4) tobacco sap infected with AMV; (5) healthy red clover sap; (6) healthy tobacco sap.


6
1
5
2
4
3

4.2.3 Electron microscopy

Observation of plant sap with TEM related the presence of flexuous rods with WCMV infections. Photographs of negatively stained purified preparations of both AMV and WCMV, after several months of storage at 20 °C are presented in Figures 4.8 and 4.9.

4.3 VIRUS DETECTION WITH THE ENZYME-LINKED IMMUNOSORBENT ASSAY

4.3.1 Sensitivity of the ELISA

Serial dilutions of AMV and WCMV indicated that concentrations of 10 ng/ml could be detected with the ELISA. Figure 4.10 gives a comparison between dilutions of WCMV in PBS-TPO buffer and in crude healthy red clover sap. Figure 4.11 compares serial dilutions of AMV in PBS-TPO buffer, using different concentrations of coating gamma-globulin. The ELISA for detection of WCMV gave consistently higher absorbance values than the AMV assay in the same conditions. However, the latter was at least as sensitive as the former since the computed upper limit for negative tests (mean absorbance value of negative controls plus three times their standard deviation) was also lower for AMV than for WCMV due to lower background reactions. This threshold absorbance value was 0.045 for AMV and 0.227 for WCMV. The lowest concentrations detected were between 1 and 10 ng/ml for AMV diluted in PBS-TPO buffer (with coating antibody (Ab) concentrations of at least 1 ug/ml. WCMV

Figure 4.8 Electron micrograph of purified AMV preparation after storage for 16 months at 20 °C. The preparation was fixed with formaldehyde and stained with 2% uranyl acetate.
Bar represents 200 µm.

Figure 4.9 Electron micrograph of purified WCMV after storage at for 14 months at -20°C. The preparation was stained with 2% potassium phosphotungstate (PTA; pH 7).
Bar represents 500 µm.

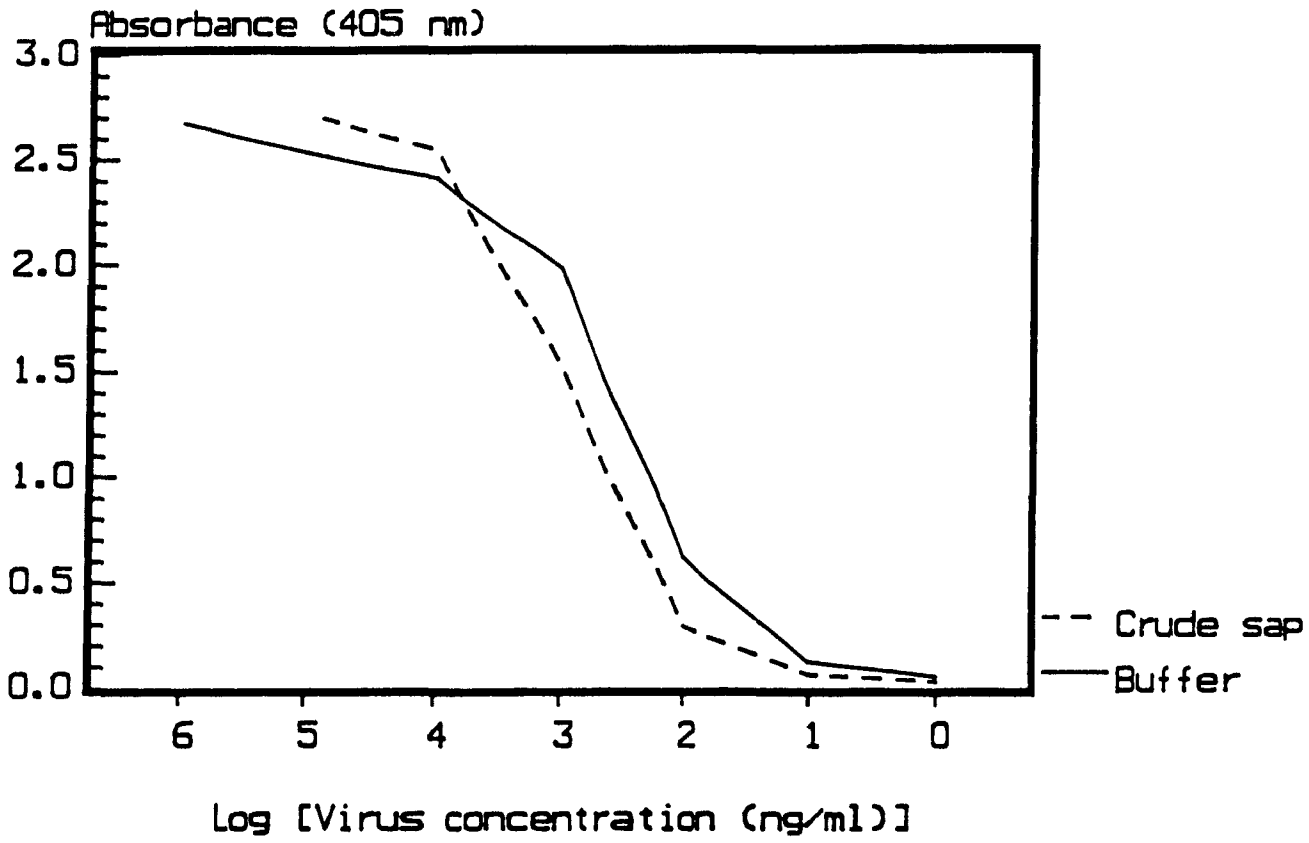


Figure 4.10 ELISA absorbance values for serial dilutions of a purified preparation of WCMV in PBS buffer and healthy red clover sap. Coating Ab (1.5 mg/ml) incubated 2 hours at 30°C. Sample incubated 18 hours at 4°C while shaken at 100 RPM. Enzyme conjugate diluted 1:1000 and incubated 3 hours at 37°C. Substrate reaction of 30 min. at 1.0 mg/ml.

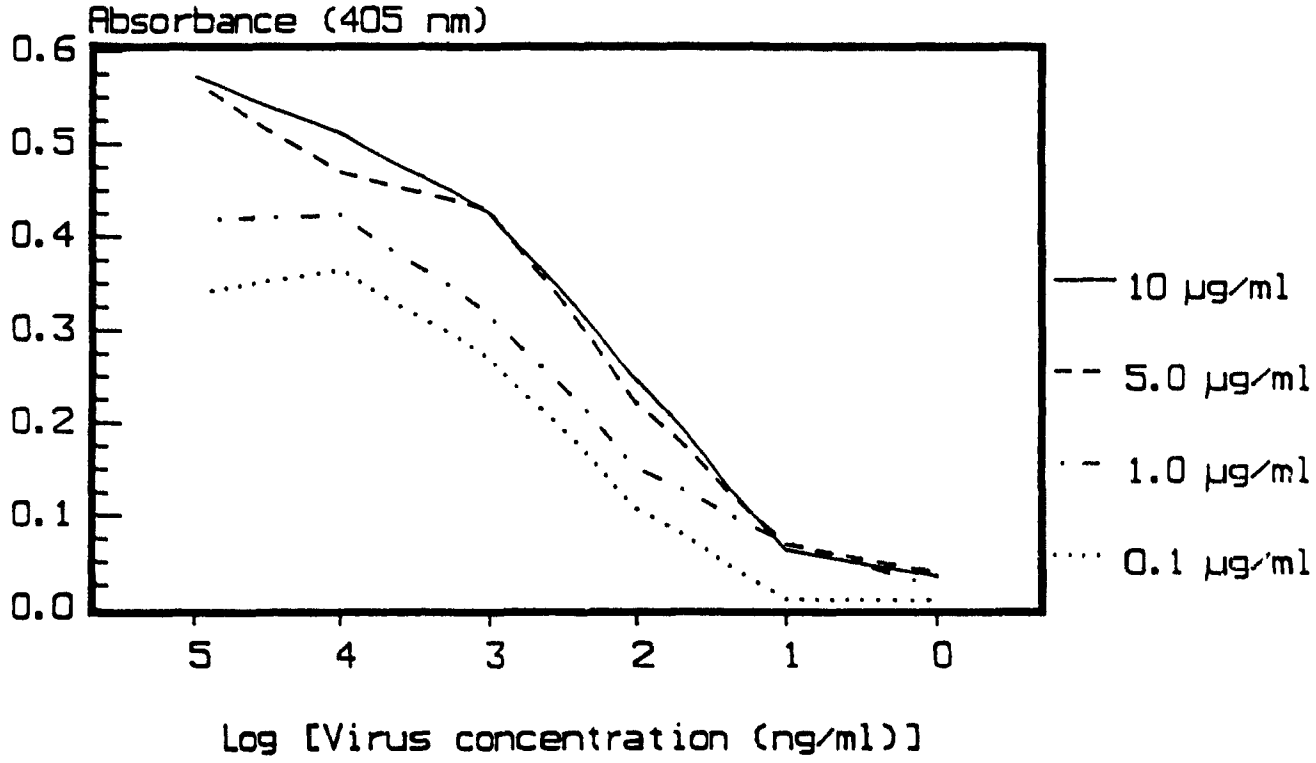


Figure 4.11 ELISA absorbance values for serial dilutions of a purified preparation of AMV using different concentrations of coating gamma-globulins. Coating Ab incubated 2 hours at 30°C. Samples incubated 18 hours at 4°C while shaken at 100 RPM. Enzyme conjugate diluted 1:1000 and incubated 3 hours at 37°C. Substrate reaction of 45 min. at 1.0 mg/ml.

was detected at concentrations around 10 ng/ml when diluted in buffer, and between 10 and 100 ng/ml when diluted in crude sap.

Differences in sensitivity for ELISA might be due to differences in immunogenicity between the 2 viruses, to the relative proportion of immunoglobulins (IgG, IgM, IgA) in each antiserum, or in any other small difference in the experimental procedure (Van Regenmortel, 1982). Detection of concentrations in the order of 1 to 10 ng/ml with ELISA is generally considered as a lower limit for most viruses (Van Regenmortel, 1982; Clark et al., 1986).

In these experiments where the sensitivity of the ELISA was assessed, intact leaf disks used as test samples for WCMV detection gave the same absorbance values as around 1,000 ng/ml of virus in crude sap for three disks (A_{405} = 1.58), and between 100 and 1,000 ng/ml for two and one disk (A_{405} = 1.12 and 0.67 respectively). At high concentrations of virus (over 10^4 ng/ml), the sensitivity curves tended to level off in both systems. This might be due to a saturation of binding sites on adsorbed antibodies.

4.3.2 Routine detection of infection with the ELISA using intact leaf disks

The detection of WCMV was highly reliable with this sampling method, using a small number of disks per well (three to six). For the detection of AMV, increasing enzyme-conjugate

concentrations, increasing the number of disks (nine to twelve) and extending the substrate reaction time to 60 or 90 minutes gave good results. Re-assaying samples that gave dubious absorbance values further enhanced the reliability of both systems.

4.3.2.1 Effect of the number of disks per well for WCMV detection

By comparing different numbers of leaf disks with the equivalent amount of ground material, it was found that increasing the number of disks led to a linear increase of absorbance values. Absorbance values from intact disks ranged from 11.9% (1 disk) to 57.0% (6 disks) of the values obtained with corresponding amounts of ground leaf material (Figure 4.12). In some experiments, the mean absorbance value from a single infected disk was not significantly different from healthy control means (L.S.D. $P = 0.05$), but was always superior to the computed upper limit for negative tests.

The usual ELISA procedures involve dilutions of test samples in the range of 2 to 10 % (wt./vol.) (Clark et al., 1986). All samples used in our leaf disk experiments were within this range except the single disk, which corresponded to a 1.57 % dilution. This low concentration was still sufficient to obtain maximum absorbance from comminuted samples (Fig. 4.12), which suggests the presence of a relatively high concentration of WCMV particles in infected leaves. The correlation detected between number of disks and absorbance

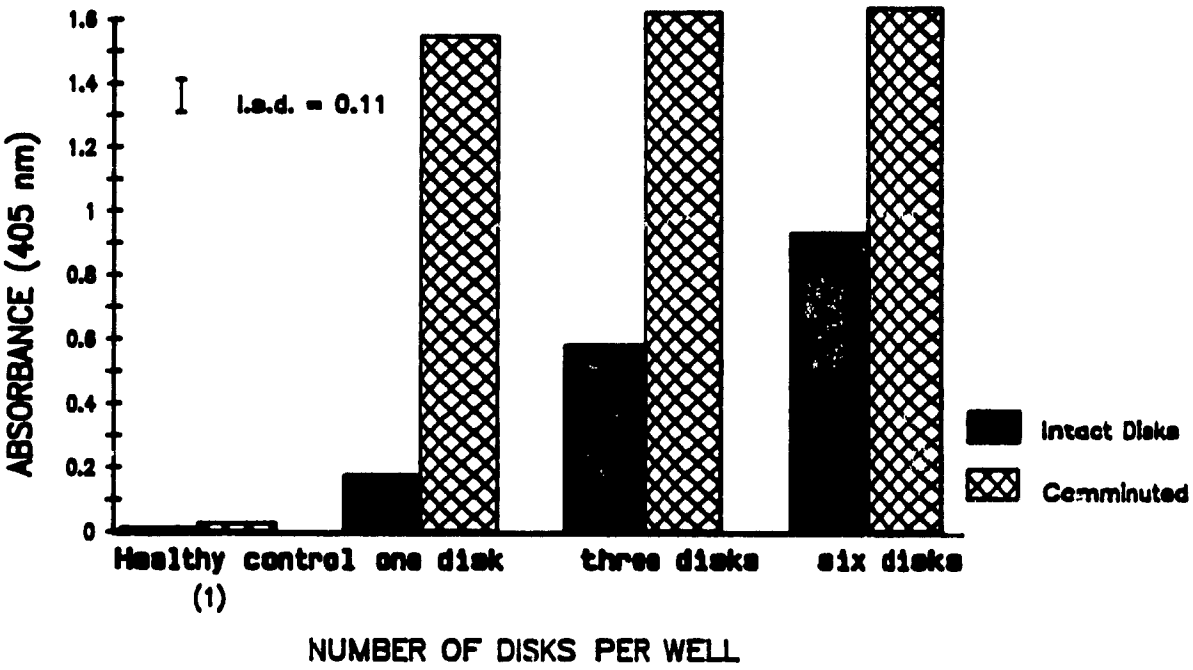


Figure 4.12 Effect on absorbance values for ELISA of the number of intact 5 mm red clover disks per well in comparison with the equivalent amount of comminuted leaf tissue.
L.s.d.= Least significant difference (p = 0.05).
(1) Healthy control samples consist of three disks either intact or comminuted.

values confirms previous reports (Menassa et al., 1986; Romaine et al., 1981).

4.3.2.2 Effect of enzyme-conjugate incubation time and symptom severity

In experiments with material from plants with light mosaic symptoms, three 6 mm. disks were compared to the equivalent amount of comminuted tissue with conjugate incubation times of 0.5, 1.0, 2.0, and 3.0 hours (Figure 4.13). There was a strong correlation between mean absorbance values from infected disks and incubation time (r= 0.96 to 0.99). At all incubation times, mean absorbance values from infected disks were significantly different from healthy controls (P = 0.05). Incubation of more than 1 hour resulted in absorbance values superior to 2.0, the upper limit of the plate reader, for all homogenized tissue samples. Two and 3 hours incubation of enzyme conjugate with infected disks resulted in mean absorbances of 50.2 % and 62.7 % of the highest quantified (A_{405} = 2.0) mean value detected for comminuted samples (mean absorbance = 1.69 with 1.0 hour incubation).

When leaf material showing more severe mosaic symptoms was used, absorbance values from intact disks reached 59.5% and 60.8% of those from comminuted tissues at 0.5 and 1.0 hour incubation respectively (Figure 4.14). A 1 hour incubation with comminuted tissues and a 2 or 3 hours incubation with intact disks resulted in several absorbance values superior to 2.0. A 2 hour incubation with disk samples gave approximately

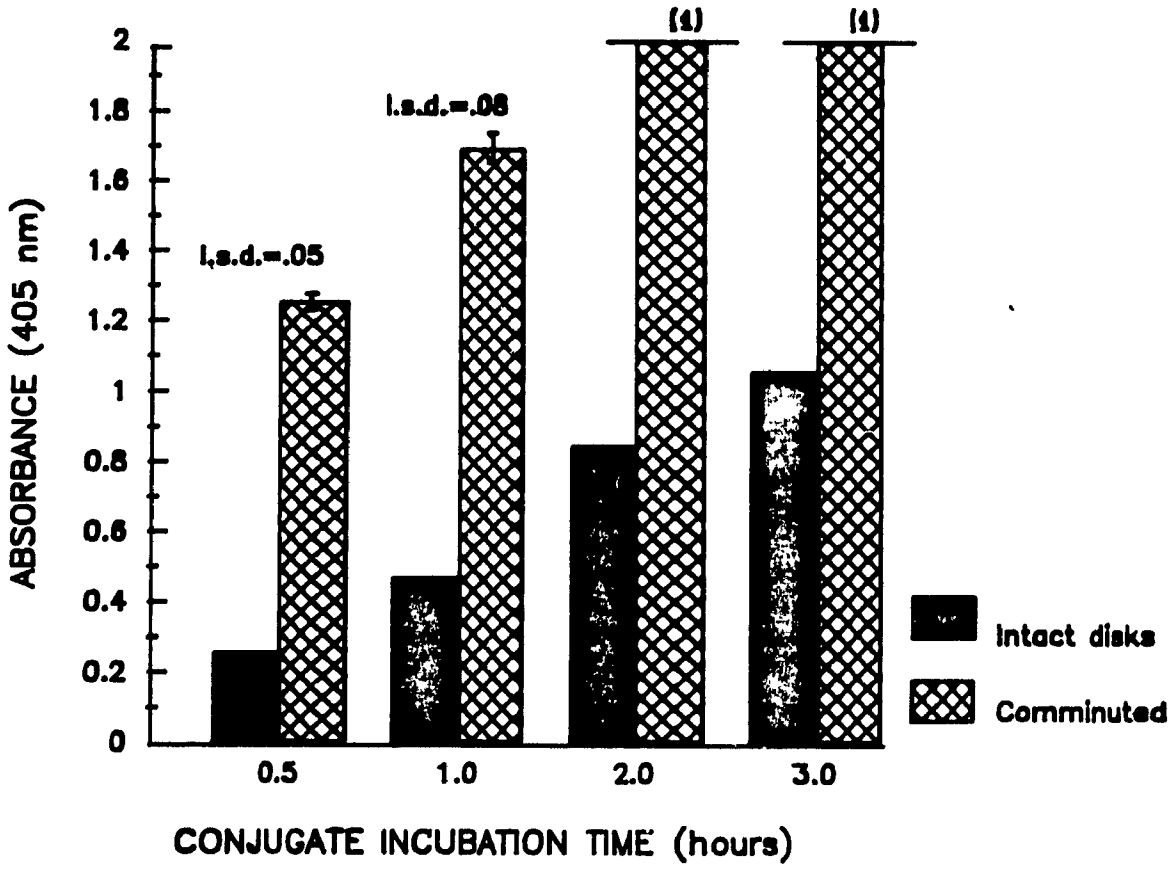


Figure 4.13 Effect on absorbance values for ELISA of enzyme conjugate incubation time at 37°C using intact or comminuted leaf disks from WCMV—infected red clover plants showing moderate symptoms. Each pair of bars represents one ELISA plate. Least significant differences for individual pair of bars. Samples of three 6 mm disks.
(1) All 15 absorbance values superior to 2.0, the upper limit for the microplate reader.

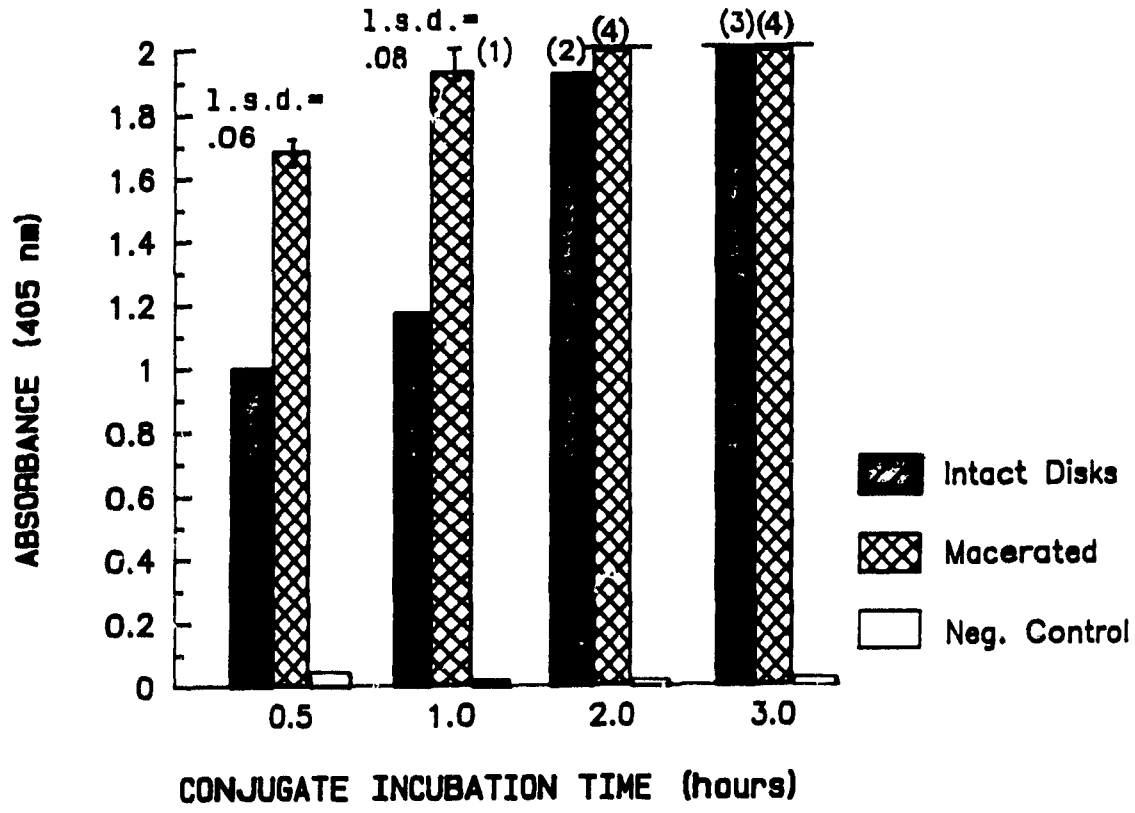


Figure 4.14 Effect on absorbance values for ELISA of enzyme conjugate incubation time at 37°C using intact or comminuted leaf disks from infected red clover plants showing severe mosaic symptoms.
Samples of three 6 mm disks.
(1) Six absorbance values out of 15 superior to 2.0.
(2) Nine absorbance values superior to 2.0.
(3) Thirteen absorbance values superior to 2.0.
(4) All 15 absorbance values superior to 2.0.

the same results as a 1 hour incubation with comminuted tissues.

McLaughlin et al. (1981) showed that different parameters of the ELISA procedure can be modified to change the sensitivity of the test. When substrate reaction time or substrate concentration was augmented, noticeable increases in background reaction were observed. Clark and Adams (1977) and McLaughlin et al. (1981) reported that absorbance values increased with enzyme-conjugate incubation time in an almost linear pattern for the first 2 or 3 hours. Our experiments gave similar results, and the increase in absorbance was not accompanied by increases in non-specific reaction (Figs. 4.13 and 4.14).

Although the experiments with different conjugate incubation times cannot be compared statistically, a pronounced increase in absorbance values for all disk samples was detected when material grown in cooler temperatures and showing more severe mosaic symptoms was used (Fig. 4.14). This may be the consequence of lower virus concentrations reflected by milder symptoms when plants are grown in higher temperatures (Walkey, 1985; Ford, 1973).

Standard deviations of absorbance values were consistently higher for infected disk samples than for infected homogenates. This may reflect an uneven distribution of virus particles within sampled tissues. It can be concluded that the leaf disk method is unreliable for quantitative studies even if

it can provide some indication of relative virus concentrations in particular situations (Marco and Cohen, 1979). The method can probably be used in qualitative studies for a wide variety of plant virus-host combinations with simple modifications of the ELISA procedure. The number of disks per well and the enzyme-conjugate concentration and incubation time appear to be simple and efficient parameters to manipulate in order to increase the sensitivity of an ELISA system using disk samples.

Since the punches were not washed between individual samples, some carry-over was detected when a healthy plant was sampled immediately after an infected one. Reports on simplified ELISA sample preparation involving presses or homogenizers suggest washing the instruments between each sample (McLaughlin et al., 1984; Mathon et al., 1987). Because only a small portion of a punch contacts injured or broken cells, the washing and drying operations can advantageously be omitted at the cost of repeating the assay of plants from which dubious absorbance values were obtained. Since a single person can prepare between 200 and 400 samples in one afternoon, compared to around 50 when using mortars and pestles, the leaf disk sampling method greatly improved the screening capacity needed to realize this research.

4.4. SEED TRANSMISSION OF WCMV

All progenies listed in Tables 4.8 to 4.13 were visually inspected for virus symptoms before inoculation. No infection attributed to seed transmission was detected out of more than 4,400 seedlings observed and 1,140 plants serologically tested (ELISA). Among progenies from susceptible infected parents inter-crossed with bumble bees, 1,440 plants observed from 18 different crosses and 720 ELISA tests did not show any presence of WCMV. These results suggest that seed transmission of WCMV in red clover is not likely to occur under conditions similar as those experimented in our study.

Since negative results are inconclusive, the relatively high rate of transmission generally recognized for WCMV (6 %) in red clover cannot be questioned. However, Hampton (1963), who reported this rate, based his conclusions on a sample of only 66 seedlings grown from field produced seeds on plants affected by mixed infections. A possible explanation of our results may be related to the occasional high temperatures reached in the greenhouse during pollinations and seed set. In addition to environmental factors, it is possible that mixed infections could have encouraged seed transmission in Hampton's study.

4.5 RESISTANCE TO AMV

The terms used in this study are adapted from the terminology generally used to designate cycles of recurrent selection (Fehr, 1987). Cycle O (CO) refers to the initial parents (CO P) and their progenies (CO F_1) (Sections 4.5.1 and 4.6.1 and Appendix II). Cycle 1 progenies (C1 F_1) refers to the offspring of crosses between two selected (tolerant/resistant) CO F_1 plants (R X R or T X T) or between one CO F_1 plant (T or R) and another susceptible (S) plant (R X S or T X S). C2 F_1 refers to the progeny of selected C1 F_1 plants, and so on.

4.5.1 Screening of commercial cultivars and C0 F_1 progenies

Since the work discussed in this section was done before the outset of this research, under conditions which were not recorded, the results are presented in Appendix II, Tables 1 to 4. This work is discussed here as it provided the base populations for the present study.

Populations of 60 plants of each of 4 diploid (Florex, Montcalm, Arlington, and Pacific) and 1 tetraploid (Sally) cultivars were screened for resistance to AMV. One resistant plant was detected in each of three cultivars (Arlington, Montcalm and Pacific) and two were observed in Sally. These results reflect a common occurrence of this character in a wide variety of red clover genotypes but at a relatively low frequency, which confirms a previous study by Hanson and

Hagedorn (1961).

Individual crosses between resistant plants (CO P) yielded progenies with significantly higher frequencies of resistance (19.8 % total for diploids) than crosses between resistant and susceptible (10.6 %) (chi-square at the 0.05 level), while crosses between susceptible plants yielded no resistant progenies. The chi-square test of independence revealed homogeneity within the resistant X resistant category and heterogeneity within the resistant X susceptible category (Tables 3 and 4, App. II). Despite some differences between reciprocals, they were heterogeneous (0.05 level) in only one C0 F_1 cross between a resistant Arlington and a susceptible Florex plant (A1/F2). Since there was no consistent trend, no inference could be made on possible cytoplasmic effects.

4.5.2 Classification of C1 F_1 progenies and independence of progeny tests

Several resistant CO F_1 plants were crossed with other resistant CO F_1 to give the R X R C1 F_1 progenies (Table 4.2) while others were crossed with susceptible plants from the cultivars Tristan (genotypes 01, 02, 03), MCC-123 (genotype 04) and Florex (genotypes 05 to 08) to give R X S C1 F_1 progenies (Table 4.3) A knowledge of the origin of the parents (Table 2b, Appendix II) allowed the distinction of categories of crosses.

Chi-square values were computed with frequencies of resistance in progenies to evaluate the homogeneity of

reciprocal crosses. These values appear in the last column of Tables 4.2 and 4.3. The chi-square test of independence was performed to evaluate the homogeneity of resistance frequencies within each category. The chi-square test of homogeneity was also used with total values of each category to locate significant differences between categories. The results of these tests are shown in Tables 4.4 and 4.5.

Table 4.2 Frequencies of resistance to AMV in C1 F_1 (R X R) progenies from crosses between two resistant CO F_1 plants (R X R) and Chi-square values for homogeneity of reciprocals.

Cross #	Category	Crossed G-type***	Total #	Res. #	Res. %	Chi-sq.** Recipr.
1	R/R // R/R	A1/P // P/A1 *	44	21	**47.7**	0.0
		P/A1 // A1/P	46	22	**47.8**	
Total	**(R/R // R/R)**		**90**	**43**	**47.8**	
2	R/R // R/S	A1/P // F3/A1	67	17	**25.4**	3.6
		F3/A1 // A1/P	67	26	**38.8**	
3	" "	A1/M // F6/M	78	30	**38.5**	2.8
		F6/M // A1/M	91	24	**26.4**	
4	" "	A1/P // M/F6	30	9	**23.1**	0.03
		M/F6 // A1/P	46	13	**21.3**	
Total	**(R/R // R/S)**		**379**	**119**	**31.4**	
5	R/S // R/S	F2/A1 // A1/F3	36	9	**25.0**	2.3
		A1/F3 // F2/A1	49	20	**40.8**	
6	" "	F2/A1 // F3/A1	61	23	**37.7**	5.5 (het)**
		F3/A1 // F2/A1	68	13	**19.1**	
7	" "	F6/M // M/F2	58	10	**17.2**	5.4 (het)**
		M/F2 // F6/M	86	30	**34.9**	
Total	**(R/S // R/S)**		**358**	**105**	**29.3**	

* Crosses are illustrated after Purdy *et al.* (1968). A single slash (/) represents a primary cross and a double slash (//) symbolizes a secondary cross.

** Chi-square values superior to 3.8 indicate heterogeneity of reciprocals at the 5% level of significance.

*** G-type refers to genotype

Table 4.3 Frequencies of resistance to AMV in C1 F_1 progenies from crosses betweeen one resistant (R) CO F_1 and one susceptible (S) plants (R X S), and chi-square values for homogeneity of reciprocals.

Cross #	Category	Crossed G-type	Total #	Res. #	Res. %	Chi-squ.* Recipr.
1	R/R // S	A1/M // O2	27	5	**18.5**	1.4
		O2 // A1/M	81	8	**9.9**	
2	" "	A1/P // O3	89	22	**24.7**	3.8
		O3 // A1/P	78	10	**12.8**	
3	" "	A1/P // O7	92	12	**13.0**	0.7
		O7 // A1/P	74	13	17.6	
Total	**(R/R // S)**		**441**	**70**	**15.9**	
4	R/S // S	A1/F3 // O2	75	13	**17.3**	0.12
		O2 // A1/F3	98	19	**19.4**	
5	" "	A1/F3 // O5	34	9	**26.5**	
		O5 // A1/F3	0			
6	" "	F3/A1 // O2	47	8	**17.0**	0.17
		O2 // F3/A1	77	11	**14.3**	
7	" "	F3/A1 // O4	59	7	**11.9**	3.2
		O4 // F3/A1	71	9	**12.7**	
8	" "	F2/M // O1	74	9	**12.2**	0.001
		O1 // F2/M	42	5	**11.9**	
9	" "	F6/M // O2	66	9	**13.6**	2.4
		O2 // F6/M	48	12	**25.0**	
Total	**(R/S // S)**		**691**	**111**	**16.1**	

* Chi-square values superior to 3.8 indicate heterogeneity of reciprocals at the 5% level of significance.

Table 4.4 Chi-square tests of independence in categories of C1 F_1 progenies (from tables 4.2 and 4.3) to evaluate the homogeneity within these categories.

Category	Resistant %	d.f.*	Computed Chi-square	Probability
R/R // R/R	47.8	1	0.0	> 0.995
R/R // R/S	31.4	5	5.9	0.25 - 0.50
R/S // R/S	29.3	5	14.3 ** (het)	0.01 - 0.025
R/R // S	15.8	5	8.6	0.10 - 0.25
R/S // S	16.1	10	9.7	0.25 - 0.50

* d.f. = degree of freedom = number of crosses -1.

** category heterogeneous at the 5% level of significance.

Table 4.5 Chi-square test of homogeneity between categories of C1 F_1 progenies to evaluate the difference between the totals of each category.

(R/R // R/R) and (R/R // R/S)	Chi-square	= 8.63 * (het)
(R/R // R/S) and (R/S // R/S)	" "	= 0.37
(R/S // R/S) and (R/S // S)	" "	= 25.38 * (het)
(R/R // S) and (R/S // S)	" "	= 0.11
(R/R // S) and (S/S)	" "	= 160.8 * (het)

* Pairs of categories with a chi-square value superior to 3.8 are heterogeneous at the 5% level of significance.

Table 4.6 Mean percent resistance among C1 F_1 progenies of the various categories of crosses.

N	Mean*	Category of cross
2	47.8a	R/R // R/R
6	31.4b	R/R // R/S
6	29.3b	R/S // R/S
11	16.1c	R/S // S
6	15.9c	R/R // S
4	0.00d	S/S

* Means with the same letter are not significantly different (P = 0.05)

The analysis of variance (AOV) table and the regression analysis appear in Appendix III. The only heterogeneous category in this C1 F_1 generation (Table 4.4) includes the crosses involving resistant plants from resistant X susceptible crosses (R/S // R/S). This is consistent with the results obtained with CO F_1 progenies where the R X S category, from which the parents for R/S // R/S crosses were selected, was the only heterogeneous category (Table 3, Appendix II). Reciprocals tend to be more heterogeneous in the same R/S // R/S category. Crosses which resulted in great reciprocal differences do not suggest a consistent trend.

Significant differences between categories determined

by the Duncan's test (Table 4.6) are consistent with the results of the chi-square tests using totals of categories (Table 4.5). The categories R/R // R/S and R/S // R/S in resistant X resistant crosses (Table 4.2) and R/R // S and R/S // S in resistant X susceptible crosses (Table 4.3) were not significantly different at the 0.05 level.

The regression analysis of the R X R progenies between the number of "doses" of resistance [i.e. the number of times a resistant plant was involved in crosses in previous generations (e.g. R/R // R/R = 4; R/S // R/S = 2)] and the transformed (Arcsin) percentage of resistance in progenies revealed a significant quadratic effect. This suggests that the level of resistance in progeny populations tends to level off after a certain accumulation of favorable genes (Fig 4.15). However, the small size of the R/R // R/R category (only 1 cross with reciprocal) limits the validity of this analysis. An equal number of observations in each category would be more meaningful. An analysis with only the averaged percent of categories entered as data, revealed a linear regression (R^2 = 0.96; P = 0.02; intercept not significantly different from 0). Several R/R // R/R crosses and a series of crosses between their resistant progenies would certainly yield meaningful information to determine the actual nature of the regression, and would indicate if more than 50% resistant plants in a progeny is attainable.

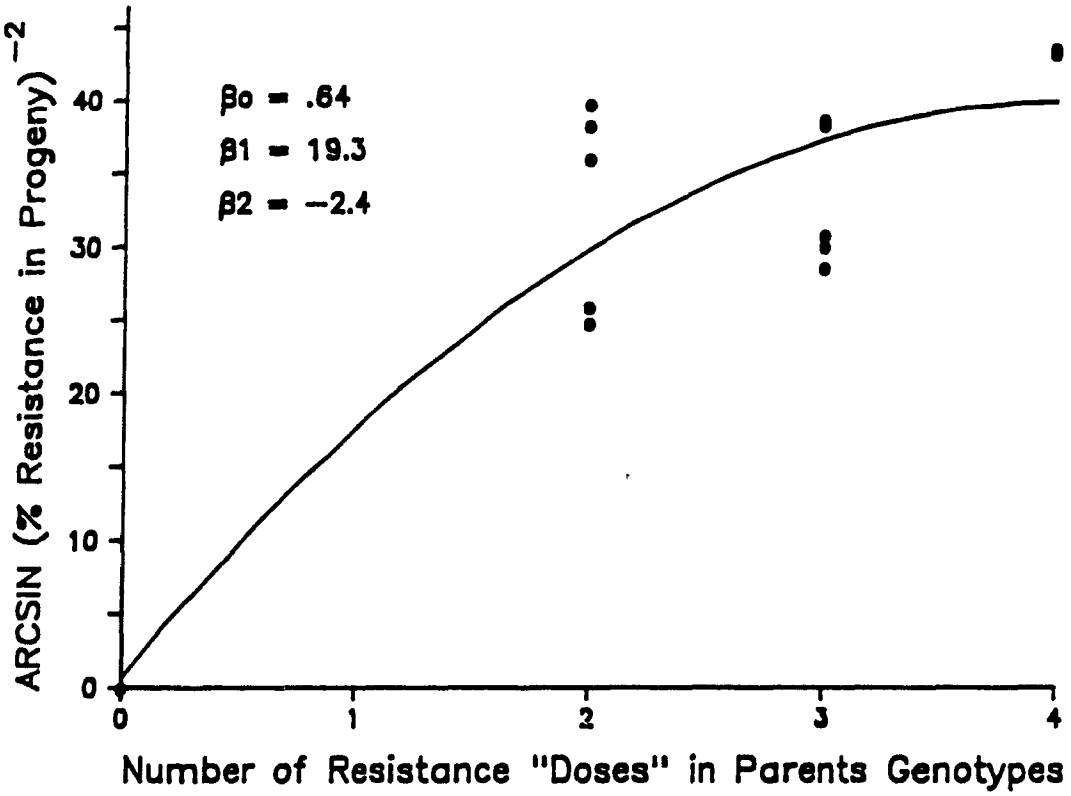


Figure 4.15 Relationship of number of resistant plants involved in previous generations (parents and C0 F1) to produce C1 F1 plants, to the percentage of plant resistance to AMV in this generation.

4.5.3 Theoretical considerations on resistance to AMV

The ratios of resistant : susceptible plants obtained in the CO F_1 and C1 F_1 progenies suggest that resistance to AMV in red clover is not simply inherited in terms of dominance and recessiveness. Results from crosses between resistant plants suggest that the character is either oligogenic and additive (quantitatively inherited), or is only expressed at a certain state of heterozygocity of several major loci. The level of heterozygosity of red clover cultivars complicates the genetic analysis for multigenic characters. In order to complete the analysis, information on the parents' genotypes and on reactions of 3 generations (CO F_1, C1 F_1 and C2 F_1) and some back-crosses generations would be needed. To this point, so many assumptions would have to be made to define a quantitative model that it would not be meaningful. However, the data suggest possible interpretations.

The heterozygous state of a few genes contributing to the expression of resistance can be retained as a possible interpretation. In the simplest model, if two main genes (say A and B) have to be heterozygous to confer resistance, resistant parents must be AaBb. A cross between 2 resistant plants would result in 4/16 resistant F1 plants (25%). The average resistance of F1 progenies from diploid plants (without considering data from the tetraploid cultivar Sally) was 19.8% which is reasonably close to 25%. However, this model would never allow more than 25% resistance in the offspring of any particular cross.

If any two out of three genes have to be heterozygous to confer resistance, frequencies of resistance in progenies would vary depending on the genotypes of the parents. In this case, the lowest frequency of resistance in progenies of a resistant X resistant cross would be 25% (e.g. AaBbCC X AaBbCC) and the highest frequency would be 75% (e.g. AaBbCC X AaBbcc), but in most cases , 50% resistance would be obtained. Crosses between plants heterozygous at all three loci (AaBbCc), between one plant heterozygous at three loci and one plant heterozygous at two loci (e.g. AaBbCc X AaBbCC) and between plants heterozygous at two different loci (e.g. AaBbCC X AaBBCc) would all result in 50% resistance in progeny.

Progenies of parents heterozygous at the same 2 loci would have 2 chances out of 9 to be 75% resistant, 6 chances to be 50% resistant and 1 chance to be 25% resistant, depending of the alleles present at the third locus.

With this model, progenies from a resistant X susceptible cross can be from 12.5% (e.g. AaBbCC X AaBBCC) to 75% resistant (e.g. AaBbcc X AaBBCC).

Such a model could help to interpret some of our data. With this type of inheritance, progenies from different R X S crosses would certainly have more chances of being heterogeneous, and 50 % resistance in progenies of R X R crosses would be common. However, the net progression obtained in the frequency of resistance from one generation to the other or within a generation with more "doses" of resistance

could not be explained. Thus, additive inheritance seems to better fit the data, but a few more crosses between all categories of resistant plants should be examined to draw any conclusion.

4.6 TOLERANCE TO WCMV

A major difficulty when working with tolerance to viruses is the subjectivity of classification. In the case of WCMV in red clover, the tolerance character refers to a lower level of symptom expression, which is mostly reflected by more vigorous plants and the absence of mosaic when compared with susceptible. However, tolerant plants are consistently less vigorous than healthy plants (slower growth and lower seed yield), and under particular environmental conditions may show slight mosaic symptoms or leaf deformation. Tolerance may also involve delays in symptom expression. Scott (1982b) reported important reductions of the proportion of symptomless WCMV-infected red clover plants between 1 and 2 months after mechanical inoculation of populations from 16 different cultivars.

4.6.1 Screening of commercial cultivars and CO F_1 progenies

For the reasons mentioned in the preceding section, the results from the initial screening and the selection for tolerance in the CO F_1 progeny, done before the present study, are listed in Appendix II and were not included in the analysis. All tolerant plants used as parents in this research

came from crosses between one tolerant plant (cv Montcalm) and one susceptible plant (cv Montcalm or Florex). The origin of the genotypes involved is defined in Table 4 of Appendix II.

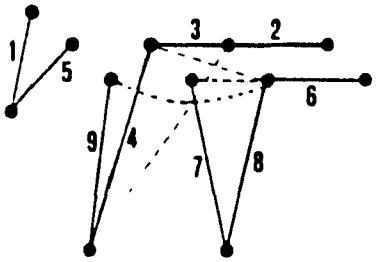
4.6.2 Classification of progenies (C1 F_1 and C2 F_1) and independence of progeny tests.

The results of screening C1 F_1 progenies from crosses between tolerant (T X T) with the chi-square values for homogeneity of reciprocals appear in Table 4.7. The results from crosses between tolerant C0 F_1 and susceptible plants (T X S) are given in Table 4.8. Susceptible plants were taken from Tristan (01), MCC-123 (02) and MCC-117 (03, 04). Diagrams at the right of Tables 4.7 and 4.8 illustrate crosses between tolerant genotypes for the next generation (C2 F_1) which are listed in Tables 4.9 and 4.10 respectively. Several intended crosses illustrated by discontinuous lines were not successful, probably due to the self-incompatibility resulting from the narrow genetic basis from which plants were obtained.

Tables 4.11 and 4.12 list the results obtained in screening backcross progenies. Results of screening sample populations from three commercial cultivars are given in Table 4.13. Table 4.14 gives the results of the chi-square tests of independence which measures the homogeneity of C1 F_1 and C2 F_1 categories.

Table 4.7 **Frequencies of tolerance to WCMV in C1 F_1 progenies from crosses between two tolerant C0 F_1 plants (T/T), and chi-square values for homogeneity of reciprocals.**

Cross #	Crossed G-type	Total #	Tol. #	Tol. %	Chi-squ. recipr.*	Next crosses Diagram **
1	A1/B1	41	12	**29.3**	0.14	
	B1/A1	43	11	**25.6**		
2	A2/B5	17	1		1.28	
	B5/A2	16	3			
3	B2/B3	14	3		0.90	
	B3/B2	22	8			
4	B4/C1	106	27	**25.5**	0.50	
	C1/B4	121	26	**21.5**		
5	B6/C1	92	32	**34.8**	0.88	
	C1/B6	64	27	**42.2**		
6	B1/D	22	5		2.69	
	D/B1	20	1			
7	B2/D	88	15	**17.0**	0.12	
	D/B2	54	8	**14.8**		
8	B5/D	38	10	**26.3**	1.09	
	D/B5	47	8	**17.0**		
Total	**(T/T)**	**805**	**197**	**24.5**		

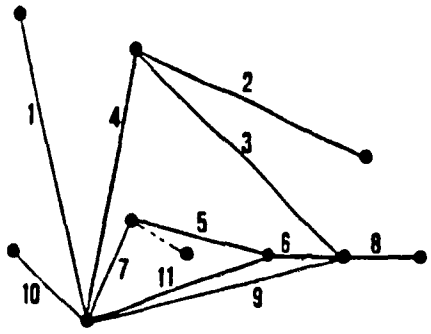


* Chi-square values inferior to 3.8 indicate homogeneity of reciprocals at the 5% level of significance.

** A black "●" represents a tolerant plant selected and used as a parent for the next generation of T X T crosses (C2 F_1). Numbered lines refer to "cross number" listed in Table 4.9. Discontinuous lines illustrate intended crosses between incompatible plants (sterile).

Table 4.8 **Frequencies of tolerance to WCMV in C1 F_1 progenies from crosses between a tolerant C0 F_1 plant and a susceptible plant (T X S), and chi-square values for homogeneity of reciprocals.**

Cross #	Crossed G-Type	Total #	Tol. #	Tol. %	Chi-squ. recipr.*	Next Crosses Diagram **
9	B4/01	84	12	**14.3**	1.32	
	01/B4	101	9	**8.9**		
10	B5/02	47	4	**8.5**	0.47	
	02/B5	55	7	**12.7**		
11	B6/03	111	7	**6.3**	0.10	
	03/B6	77	4	**5.2**		
12	C2/01	53	7	**13.2**	0.51	
	01/C2	46	4	**8.7**		
13	C1/01	106	15	**14.1**	3.41	
	01/C1	108	7	**6.5**		
14	C1/04	96	11	**11.5**	0.05	
	04/C1	105	11	**10.5**		
15	D/01	52	4	**7.7**	0.02	
	01/D	47	4	**8.5**		
Total	**T/S**	**1088**	**106**	**9.7**		



* Chi-square values inferior to 3.8 indicate homogeneity of reciprocals at the 5% level of significance.

** A black "●" represents a tolerant plant selected and used as a parent for the next generation of T X T crosses C2 F_1. Numbered lines refer to "cross #" listed in Table 4.10. Discontinuous line illustrate an intended cross between incompatible plants (sterile).

Table 4.9 Frequencies of tolerance to WCMV in C2 F_1 progenies from crosses between tolerant C1 F_1 plants produced from tolerant X tolerant CO F_1 plants (T/T // T/T), and chi-square values for homogeneity of reciprocals.

Cross #	Crossed G-type	Total #	Tol. #	Tol. %	Chi-square * Reciprocals
1	B3/B2 // B6/C1	20	7		
	Reciprocal	11	4		0.01
2	(B4/C1)a // (B4/C1)b	27	5	**18.5**	
	Rec.	24	8	**33.3**	1.47
3	(B4/C1)b // (B4/C1)c	65	32	**49.2**	
	Rec.	52	14	**26.9**	6.02* (het.)
4	(B4/C1)c // (B2/D)b	6	2		
	Rec.	19	9		0.36
5	(B4/C1)d // B6/C1	36	13	**36.1**	
	Rec.	24	8	**33.3**	0.05
6	(C1/B4)b // (C1/B4)c	19	6		
	Rec.	31	12	**38.7**	1.29
7	(C1/B4)a // (B2/D)a	105	35	**33.3**	
	Rec.	76	26	**34.2**	0.02
8	(C1/B4)b // (B2/D)a	9	5		
	Rec.	7	2		1.16
9	(C1/B4)d // (B2/D)b	10	0		
	Rec.	27	6	**22.2**	2.65
Total	**(T/T // T/T)**	**568**	**194**	**34.2**	

Incompatible Crosses:		Number of heads pollinated
10	(B4/C1)b // (B2/D)b	2
	Rec.	3
11	(C1/B4)b // (B4/C1)c	2
	Rec.	1
12	(C1/B4)b // (C1/B4)d	3
	Rec.	3
13	(C1/B4)a // (C1/B4)b	2
	Rec.	2

* Chi-square values superior to 3.8 indicate heterogeneity of reciprocals at the 5% level of significance.

Note: Crosses are illustrated after Purdy *et al.* (1968):
A single slash (/) represents a primary cross
and a double slash (//) symbolyzes a secondary cross.

Table 4.10 **Frequencies of tolerance to WCMV in C2 F_1 progenies from crosses between tolerant C1 F_1 plants produced from tolerant X susceptible CO F_1 plants (T/S // T/S), and chi-square values for homogeneity of reciprocals.**

Cross #	Crossed G-type	Total #	Tol. #	Tol. %	Chi-square * Reciprocals
1	01/D // B6/03	43	3	7.0	0.25
	Reciprocal	25	1	4.0	
2	03/B6 // C1/01	101	13	12.8	0.12
	Rec.	92	11	12.0	
3	03/B6 // (04/C1)a	56	6	10.7	0.002
	Rec.	48	5	10.4	
4	03/B6 // 01/D	50	7	14.0	0.49
	Rec.	43	4	9.3	
5	C1/01 // (04/C1)b	129	13	10.0	1.82
	Rec.	141	8	5.7	
6	(04/C1)a // (04/C1)b	47	3	6.4	0.77
	Rec.	24	3	12.5	
7	C1/04 // 01/D	110	6	5.4	0.47
	Rec.	89	7	7.9	
8	(04/C1)a // (04/C1)d	36	2	5.6	0.25
	Rec.	35	3	8.6	
9	(04/C1)a // 01/D	28	2	7.1	0.52
	Rec.	31	1	3.2	
10	(04/C1)c // 01/D	137	17	12.4	0.63
	Rec.	138	13	9.4	
11	(04/C1)b // 01/D	91	5	5.5	0.07
	Rec.	93	6	6.4	
Total	**(T/S // T/S)**	**1587**	**133**	**8.4**	

Incompatible Cross:		Number of heads pollinated:
12	G1/04 // (04/C1)e	2
	Rec.	2

* Chi-square values inferior to 3.8 indicate homogeneity of reciprocals at the 5% level of significance.

Table 4.11 Frequencies of tolerance in back-cross progenies involving a tolerant (T) C1 F_1 plant and one of its parent and chi-square values for homogeneity of reciprocals.

Category	Crossed G-types	Total #	Tol. #	Tol. %	Chi-square * recipr.
T/S // S	B6/03 // 03	69	1	1.5	0.01
	03 // B6/03	60	1	1.7	
T/S // T	B6/03 // B6	20	0		0.66
	B6 // B6/03	31	1	3.2	
T/S // S	01/D // 01	23	0		
	01 // 01/D	22	0		
S/T // S	04/C1 // 04	49	3	6.1	0.01
	04 // 04/C1	52	3	5.8	
S/T // S	04/C1 // 04	15	3		0.07
	04 // 04/C1	15	2		
T/T // T	B6/C1 // C1	10	3		0.30
	C1 // B6/C1	2	1		

Table 4.12 Frequencies of tolerance in back-cross progenies involving a susceptible (S) C1 F_1 plant and one of its parent and chi-square values for homogeneity of reciprocals.

Category	Crossed G-type	Total #	Tol. #	Tol. %	Chi-square * reciprocals
S/T // S	01/C1 // 01	18	1		0.63
	01 // 01/C1	11	0		
S/T // T	01/C1 // C1	119	7	5.9	2.22
	C1 // 01/C1	36	0		
T/T // T	C1/B6 // B6	57	9	15.8	0.26
	B6 // C1/B6	41	8	19.5	
T/T // T	C1/B6 // C1	67	13	19.4	0.32
	C1 // C1/B6	52	8	15.4	
T/T // T	B6/C1 // B6	17	2		1.73
	B6 // B6/C1	28	8		

* Chi-square values inferior to 3.8 indicate homogeneity of reciprocals at the 5% level of significance.

Table 4.13 Frequencies of tolerance in populations of commercial cultivars.

Cultivar	Total #	Tolerant #	Tolerant %
Florex	96	2	2.1
Montcalm	210	19	9.05
Tristan	211	5	2.4

Table 4.14 Chi-square test of independence in categories of C1 F_1 and C2 F_1 progenies (from Tables 4.7 to 4.10) to evaluate homogeneity within categories.

Category	Tolerant %	d.f.*	Computed chi-square	Probability
T / T	24.5	15	33.38**	< 0.005
T / S	9.7	13	11.14	0.50 - 0.75
T/T // T/T	34.2	17	21.51	0.10 - 0.25
T/S // T/S	8.4	21	17.57	0.50 - 0.75

* d.f.= degree of freedom = number of crosses - 1.

** Chi-square values superior to $X^2_{(.95,df)}$ are heterogeneous at the 5% level of significance.

Table 4.15 Mean percent tolerance among C1 F_1 and C2 F_1 progenies of the various categories of crosses.

N	Mean*	Category Of Cross
4	34.2a	T/T // TT
9	24.5b	T/T
14	9.7c	T/S
16	8.4c	T/S // T/S
3	5.0d	S

* Means with the same letter are not significantly different.

The AOV table and the regression analysis appear in Appendix III. For the analysis, the category of susceptible (S) represents the results obtained in screening populations of cultivars (Table 4.13). These were included in the analysis since they correspond to initial populations which were not previously selected for tolerance. Differences were significant between T/T // T/T (in Table 4.9) and T/T (in Table 4.7), but not between T/S (in Table 4.8) and T/S // T/S (in Table 4.10). Within a single generation (T/T and T/S or T/T // T/T and T/S // T/S), differences are consistently significant ($P = 0.05$).

One regression analysis was done per generation (C1 F_1 and C2 F_1). Results from Table 4.13 were included, as having 0 "dose" of tolerance, in both cases. The independent variable (X) was the number of tolerant (T) "doses" in the parents' genotypes, as defined for AMV and the dependent variable was the transformed (arcsin) percent tolerance in progenies. The regression coefficient (R^2) values were 0.73 and 0.68 for C1 F_1 and C2 F_1 respectively. Figures 4.16 and 4.17 illustrate the linear regression for each generation. The analyses appear in Appendix III.

All reciprocals, except one in the C2 F_1, were homogeneous, suggesting that there was no significant cytoplasmic effect on tolerance to WCMV. All categories were significantly different from each other except T/S and T/S // T/S which showed almost the same levels of tolerance. Chi-square tests and Duncan's tests showed the same results. The

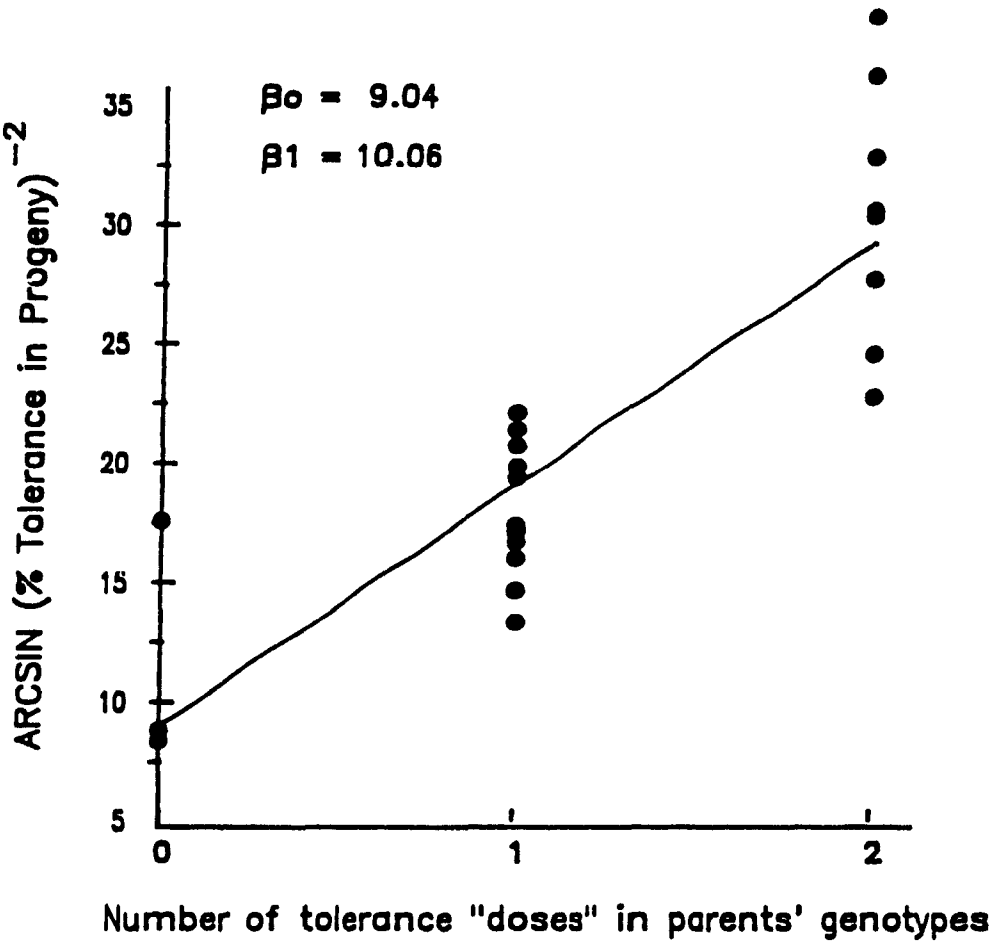


Figure 4.16 Relationship of number of tolerant plants involved in previous generations (parents and C0 F1) to produce C1 F1 plants, to the percentage of plant tolerance to WCMV in this generation.

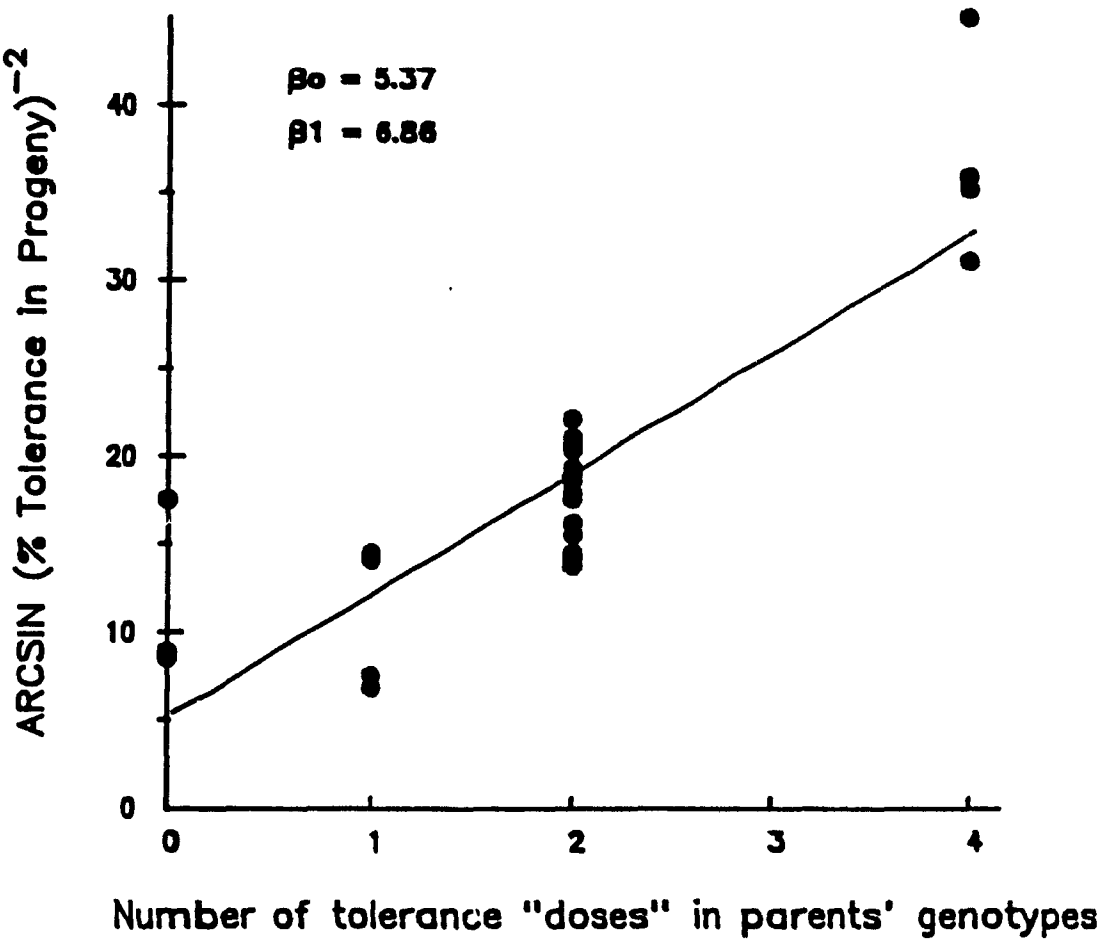


Figure 4.17 Relationship of number of tolerant plants involved in previous generations (parents, C0 F1 and C1 F1) to produce C2 F1 plants, to the percentage of plant tolerance to WCMV in this generation.

only heterogeneous category in the 2 generations was the T/T category of the C1 F_1 in which progenies had tolerance levels ranging from around 15 to 35%.

Regressions in each of the C1 F_1 and C2 F_1 generations are linear, suggesting a possible additive inheritance. In the C2 F_1 regression, the back-cross progenies carrying 1 "dose" of tolerance (T/S // S and S/T // S) were included.

Back-cross genotypes and the size of their progenies were limited due to the lack of vigor of most old infected material involved in the previous generation as parents. These plants although tolerant in several cases flowered very little, produced very little pollen and had a low seed set. Therefore, the results of Tables 4.11 and 4.12, although not very conclusive, provide some information. In Table 4.12, progenies from crosses between susceptible plants from a T X T cross and one of its parent (T/T // T) exhibited tolerance at the rate of 15 to 20% which is superior to the percent tolerance in progenies from tolerant parents in T/S (C1 F_1) or T/S // T/S (C2 F_1) categories, both under 10%.

It is interesting to note that crosses between tolerant plants of the C1 F_1 progeny from the T/S category (9.7% tolerance) did not yield significantly more tolerant progenies in the C2 F_1 generation (category T/S // T/S - 8.4%). This suggests that the gene dosage present in those (T/S) plants did not permit the improvement of the level of tolerance through selection. However, environmental factors might have

influenced symptom expression since selections in these 2 generations were done at one year interval.

4.6.3 Theoretical considerations on tolerance to WCMV

The ratios of tolerant : susceptible plants obtained may suggest a quantitative inheritance of tolerance to WCMV. The consistency of linear progression of tolerance levels with the increase of the number of tolerant genotypes in parental "pedigrees" (number or tolerant "doses") indicates an additive inheritance. However, it would be interesting to produce a further generation with some crosses between T/T // T/T plants. This would allow the determination of any significant quadratic effects, or limits on levels of tolerance in progenies. Assuming the linear regression model and using the regression parameters estimated from C2 F_1 data, we could extrapolate that more than 12 "doses" of tolerance would be necessary to reach a population totally tolerant to WCMV. The possibility of the additive model is enhanced by the behavior of progenies from tolerant X susceptible crosses (C1 F_1: T/S) and from tolerant progenies of theses crosses (C2 F_1: T/S // T/S). The initial proportion of tolerance genes must exceed a certain level for an effective selection. Multigenic inheritance of recessive or incompletely dominant genes often confer tolerance by reducing symptom severity (Fraser, 1985b). Tolerance to WCMV in red clover is likely to be of this nature.

One difficulty encountered in analysing the data was

that only 2 classes were defined (susceptible and tolerant) and the border line between them is often blurred. Furthermore, it would have been more appropriate to scale symptom severity (e.g. 0 to 5) or to classify progeny on the base of several morphological criteria since a wide range of visual symptoms (mosaic, stunting, leaf deformation, multiple budding from the crown area, etc..) were expressed. Quantifying tolerance through the evaluation of growth rate, or of virus concentration in tissues would have given more information. The ELISA test with traditional comminuted samples can be a useful technique to quantify virus levels in infected plants (Clark et al., 1986). In order to obtain consistent and reliable results, the sampling procedure must be carefully standardized. The period of time between inoculation and the test, the age of the leaf to sample, the weight of tissue sampled, and the growing condition of the tested plants would have to be rigidly uniform. Furthermore, this kind of investigation would require a tremendous amount of work and many fewer progenies and smaller populations could be handled.

There is a need for a growing environment which is much more uniform and constant than the conventional greenhouse for research on resistance/tolerance to viruses. Environmental effects on plant reactions to viruses is well documented, and poorly controlled environment has resulted in erroneous genetic interpretation in some cases (Shifris et al., 1942; Bagget and Fraser, 1957). The conditions in which progenies were grown for this research were difficult to standardize. Strong

gradients of light intensity and temperature were detected within sections of the greenhouse, and even within single growing benches. Minimal and maximal temperatures were recorded for a period of 45 days (February and March 1989) at both ends of a bench on which progenies were grown and screened for tolerance to WCMV. The average difference between minima and maxima during that period was nearly 14 °C on one thermometer, and maxima reached over 40 °C on a few occasions on both thermometers. Differences between the thermometers was important, reaching up to 12 °C in one occasion. Despite these factors, the results of this investigation are consistent and reflect an additive inheritance of tolerance to WCMV.

5. CONCLUSIONS

The ultimate objective of a study on resistance is to contribute to improve crop productivity. The understanding of the genetic mechanisms involved in plant's response tc infection is an important step forward. Once this is achieved, appropriate breeding strategies and selection methods can be defined more efficiently.

This study represents a significant contribution to the identification of the nature of tolerance to WCMV and to some extent, of resistance to AMV. In both cases, it has been established that selection is effective, and resistance levels are significantly raised when the appropriate genotypes are combined. Tolerance to WCMV behaves like an oligogenic character, and it should be relatively simple to introduce or concentrate the corresponding genes into breeding material through recurrent or mass selection. However, large populations must be used to avoid inbreeding. Agronomic studies should now be done to determine if there is a yield advantage in increasing tolerance to WCMV levels in commercial red clover cultivars. Resistance to AMV also seems to be a multigenic character, but further investigations are required to define its nature more accurately. More research is also needed to establish the correlation between field resistance and resistance in a controlled environment. We still must determine if resistance to a single strain of AMV or WCMV also confers resistance to other strains or affects other characters

such as resistance to other viruses or pathogens.

In addition to a better genetic knowledge, this study has fulfilled other important objectives. The ELISA leaf disk sampling method has been adapted with much success. Without the screening efficiency provided by this technique, the study would have been necessarily scaled down. Smaller amounts of data could have compromised the conclusions about the two rather complex characters studied.

Other contributions from this research concern the success of the mass inoculation method used, and the conclusions about seed transmission of WCMV in the greenhouse. Finally this research has permitted the identification of several resistant /tolerant red clover genotypes (further selected for vigor), which are likely to be attractive to red clover workers, as breeding material.

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APPENDIX I

Buffers for the enzyme-linked immunosorbent assay

Coating Buffer (pH 9.6)

1.59 g Na_2CO_3
2.93 g $NaHCO_3$
0.20 g NaN_3
make up to 1 litre with water

Phosphate Buffered Saline (PBS) Buffer (pH 7.4)

8.0 g NaCl
0.2 g KH_2PO_4
1.15 g Na_2HPO_4
0.2 g KCl
0.2 g NaN_3
make up to 1 litre with water

Washing Buffer

PBS + 0.5 ml polyoxyethylene sorbitan monolaurate (Tween-20) per litre PBS

Extracting and Diluting (PBS-TPO) Buffer (pH 7.4)

Washing Buffer (approx. 975 ml)
20.0 g polyvinylpyrrolidone (PVP 44,000)
2.0 g ovalbumin
make up to 1 litre with washing buffer

Substrate Buffer (pH 9.8)

800 ml water
97 ml diethanolamine
0.2 g NaN_3
adjust pH with 1.0 N HCl
make up to 1 litre with water

APPENDIX II

A. Resistance to AMV. Screening of commercial cultivars and CO F_1 progenies.

Table 1. Frequency of resistance to AMV in populations of commercial cultivars.

Cultivar	Total #	Resistant #	Symbols * Resistant	Susceptible
Florex (F)	60	0		F1 - F7
Arlington (A)	60	1	A1	A2 - A4
Montcalm (M)	60	1	M	
Pacific (P)	60	1	P	
Sally (S)	60	2	S1, S2	S3, S4
Total	**300**	**5**		

* **Symbols** refer to individual plants (resistant or susceptible) used as parents to produce CO F_1 progenies.

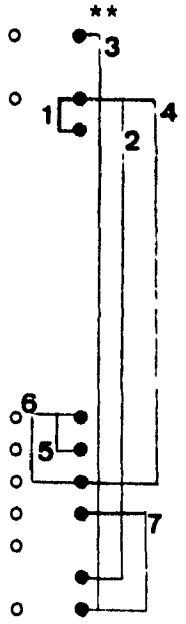
Table 2a. Frequencies of resistance in CO F_1 progenies (with pooled progenies of reciprocal crosses).

Category	Crossed G-type	Total #	Resistant #	Resistant %
R/R	A1/M*	73	16	**21.9**
"	S1/S2	79	10	**12.7**
Total R/R		**152**	**26**	**17.1**
R/S	S1/S3	60	11	**18.3**
Total R/S		**60**	**11**	**18.3**
S/S	F1/A2	59	0	**0**
S/S	A2/F2	87	0	**0**
"	S3/S4	99	0	**0**
Total S/S		**245**	**0**	**0**

* Crosses are illustrated after Purdy et al. (1968). A single slash (/) represents a primary cross, and a double slash (///) symbolizes a secondary cross.

Table 2b. Frequencies of resistance to AMV in CO F_1 progenies and chi-square values for testing homogeneity of reciprocals.

Category	Crossed G-type	Total #	Res. #	Res. %	Chi-square for reciprocals*
R/R	A1/M	47	7	14.9	0.66
	M/A1	42	9	21.4	
"	A1/P	52	12	23.1	0.77
	P/A1	44	7	15.9	
"	M/P	36	9	25.0	1.08
	P/M	39	6	15.4	
"	S1/S2	41	4	9.8	0.18
	S2/S1	48	6	12.5	
Total R/R		**349**	**60**	**17.2**	
R/S	A1/F2	45	0	0	8.60 *
	F2/A1	64	11	17.2	
"	A1/F3	35	8	22.9	2.10
	F3/A1	53	6	11.3	
"	M/F2	45	8	17.8	0.48
	F2/M	42	10	23.8	
"	M/F6	41	2	4.9	0.01
	F6/M	46	2	4.3	
"	P/A4	37	2	5.4	2.10
	A4/P	37	0	0	
"	P/F7	47	3	6.4	1.50
	F7/P	42	6	14.3	
"	S1/S3	40	8	20.0	1.40
	S3/S1	31	3	9.7	
"	S1/S4	13	1	7.7	0.03
	S4/S1	32	2	6.2	
"	S2/S3	32	2	6.2	0.33
	S3/S2	40	4	10.0	
"	S2/S4	36	1	2.8	0.13
	S4/S2	22	1	4.5	
Total R/S		**780**	**80**	**10.3**	
S/S	A2/F2	45	0	0	
	F2/A2	42	0	0	
"	S3/S4	49	0	0	
	S4/S3	50	0	0	
Total S/S		**186**	**0**	**0.0**	



* Chi-square values superior to $X^2_{(0.95, 1)} = 3.84$ indicate heterogeneous reciprocals at the 5% level of significance.

** A black "•" represents a resistant plant selected and used as a parent for the next generation of R X R crosses (C1 F_1); numbered lines refer to " Cross # " in Table 4.2. A white "o" represents a resistant plant which was crossed with a susceptible plant (C1 F_1: R X S) as shown in table 4.3.

Table 3. Chi-square test of independence in categories of CO F_1 progenies (from table 2a) to evaluate the homogeneity within these categories.

Category	d.f.*	Resistant %	Computed Chi-square	Chi-square (0.95) (d.f.)
R/R	7	17.2	**6.0**	**14.1**
R/S	19	10.3	**40.0**	**30.1** (het.)**

* d.f. = degree of freedom (number of crosses in category -1)

** (het.) = heterogeneous category at the 5% significance level.

Table 4. Chi-square test of homogeneity between categories of CO F_1 progenies to evaluate the difference between the total of each category.

R/R	vs	R/S	: Chi-square$_{(.95)}$	= **10.68** *
R/S	vs	S/S	: " "	= **36.80** *

* Chi-square values superior to 3.8 indicate heterogeneity of reciprocals at the 5% level of significance.

B. Tolerance to WCMV: Screening of commercial cultivars and CO F_1 progenies.

Table 5. Frequencies of tolerance in CO F_1 progenies from crosses involving a tolerant plant (M1) selected from a population of the cultivar Montcalm with two susceptible plants from Montcalm (M2) and Florex (F) populations.

Category	Crossed G-type	Total #	Tolerant #	Tolerant %	Symbols *
T/S	M1/M2	47	6	**12.8**	A1, A2
	M2/M1	43	9	**20.9**	B1, B2,..B6
"	M1/F	40	3	**7.5**	C1, C2
	F/M1	36	3	**8.3**	D
Total		**166**	**21**	**12.6**	

* **Symbols** refer to individual tolerant plants used as parents to produce C1 F_1 progenies (tables 4.7 and 4.8)

Chi-square tests for reciprocals:

M1/M2 and M2/M1 Chi-squ. = 0.90 *

M1/F and F/M1 " = 0.02 *

* Chi-square values inferior to 3.8 indicate homogeneity of reciprocals at the 5% level of significance.

Chi-square test of independence:

R/S (12.6 %) d.f. = 3. Chi-square = 4.23

The category is homogeneous at the 5% level of significance since Chi-square < $X^2_{(.95,\ 3)}$ (7.81).

APPENDIX III

Resistance to AMV in C1 F_1: Analysis of variance for R X R and R X S crosses.

General Linear Models Procedure

Dependent Variable: ARCSIN (% R)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	3904.344895	780.868979	42.49	**0.0001**
Error	25	459.396640	18.375866		
Corrected Total	30	4363.741535			

R-Square	C.V.	Root MSE	ARCS Mean
0.894724	17.40168	4.286708	24.6338710

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CATEGORY	5	3904.344895	780.868979	42.49	**0.0001**

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CATEGORY	5	3904.344895	780.868979	42.49	**0.0001**

Regression analysis of resistance to AMV for R X R crosses.

Model: MODEL1
Dependent Variable: ARCSIN (% R)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	3656.12172	1828.06086	51.162	**0.0001**
Error	13	464.50366	35.73105		
C Total	15	4120.62538			

Root MSE	5.97755	R-square	**0.8873**
Dep Mean	26.07375	Adj R-sq	**0.8699**
C.V.	22.92553		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	**0.639414**	2.97363964	0.215	**0.8331**
T	1	**19.324873**	3.43620256	5.624	**0.0001**
T^2	1	**-2.379087**	0.91407281	-2.603	**0.0219**

Tolerance to WCMV: Analysis of variance of C1 F_1 and C2 F_1 progenies.

General Linear Models Procedure

Dependent Variable: ARCSIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2299.550288	574.887572	35.75	**0.0001**
Error	41	659.260556	16.079526		
Corrected Total	45	2958.810843			

R-Square	C.V.	Root MSE	ARCS Mean
0.777187	18.81405	4.009928	21.3134783

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CATEGORY	4	2299.550288	574.887572	35.75	**0.0001**

Source	DF	Type III SS	Mean Square	F Value	Pr > F
CATEGORY	4	2299.550288	574.887572	35.75	**0.0001**

Regression analysis for tolerance to WCMV in the C1 F_1 progenies.

Model: MODEL1
Dependent Variable: ARCSIN (% Tol)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	1075.01171	1075.01171	50.859	**0.0001**
Error	24	507.29132	21.13714		
C Total	25	1582 30303			

Root MSE	4.59751	R-square	**0.6794**
Dep Mean	21.42577	Adj R-sq	**0.6660**
C.V.	21.45787		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	**9.040217**	1.95683207	4.620	**0.0001**
T	1	**10.063261**	1.41109167	7.132	**0.0001**

Regression analysis for tolerance to WCMV in the C2 F_1 progenies.

Model: MODEL 1
Dependent Variable: ARCSIN (% Tol.)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	1	1499.49950	1499.49950	68.065	**0.0001**
Error	25	550.76141	22.03046		
C Total	26	2050.26092			

Root MSE	4.69366	R-square	**0.7314**
Dep Mean	18.58741	Adj R-sq	**0.7206**
C.V.	25.25183		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	**5.373070**	1.83886348	2.922	**0.0073**
T	1	**6.861291**	0.83165730	8.250	**0.0001**