# VARIATION AMONG CUCUMBER MOSAIC VIRUS (CMV) ISOLATES AND THEIR INTERACTION WITH PLANTS

WIWIEK SRI WAHYUNI

MAgSc. (1985, Gadjah Mada University, Indonesia)

Department of Crop Protection

Waite Agricultural Research Institute

The University of Adelaide

South Australia

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## SUMMARY

Eighteen strains of cucumber mosaic virus (CMV), including fourteen from Australia, two from the USA, and two from Japan, were distinguished into two subgroups on the basis of nucleic acid (RNA) hybridization. These subgroupings correspond to the subgroups I and II of Owen and Palukaitis (1988). Strains $F_{NY}$ and $L_{NY}$ from the USA were used in this study as the representatives of subgroups I and II, respectively. By this technique, strains $Y_{Jpn}$ and $P_{Jpn}$ from Japan were placed in subgroups I and II, respectively.

Serological tests, using glutaraldehyde fixed antigens and either polyclonal antibodies prepared against fixed antigen or monoclonal antibodies ($\alpha$L1 from the USA and 34.2 from France) separated the strains into two serogroups. These serogroups corresponded with the subgroups obtained by nucleic acid hybridization. Strain $Y_{WA}$ was serologically distinct from the other strains, although it was clearly a member of subgroup II by nucleic acid hybridization. Conversely, gel immunodiffusion tests using the alternate placement of (1) fixed homologous and heterologous antigens and (2) either fixed or unfixed homologous and heterologous antigens, were not useful for serogrouping the strains.

Subgrouping of the strains of CMV could not be achieved by host range tests. Moreover, no plant species tested showed symptoms which could be used to distinguish the strains into subgroups.

Some strains of CMV were found to induce necrosis in lupin. Virus and symptom distribution in *Medicago* spp. was uneven. The time to show the first symptoms exceeded 6 weeks, and this was also dependent on the strains of CMV and the species or cultivar of legume. *Medicago scutellata* cv. Sava was found to be resistant to all strains of CMV by mechanical inoculation, aphid transmission, and both patch and approach grafting.

Reactions of plants at different times of the year were variable, and symptoms were sometimes not reproducible. No strains belonging to either subgroup I or II preferentially infected plants at any particular time of the year.

None of the CMV strains were related to peanut stunt virus (PSV) either by host range or nucleic acid hybridization tests. Serological tests showed that several strains of CMV were distantly related to PSV-E or PSV-2, with an SDI of 6 to 7.

Purified preparations of some strains of CMV, which had been fixed with 0.25% glutaraldehyde, precipitated after storage for more than one year at 4°. Coincidental with this was the detection of an additional component in analytical sucrose density gradient (SDG) centrifugation.

Agarose gel electrophoresis of virus particles showed differences in the patterns obtained for fixed and unfixed virus. Both fixed and unfixed virus separated into several clear bands, and the bands were stained with both ethidium bromide and coomassie blue. The fixed virus contained a more rapidly migrating component than unfixed virus.

In a study of the effect of *Rhizobium* (R) inoculation on the susceptibility of *Medicago truncatula* ssp. *truncatula* (barrel medic) to CMV, R reduced the susceptibility of this species, whereas treatment with a high concentration of nitrate (N) increased the susceptibility. In a study of the interaction between R and N, this species was more susceptible in the absence of R and added N (-R+N) than in the presence of R and N (+R+N).

*Lupinus angustifolius* (lupin) was used as a model for studying the interaction between *Rhizobium* and CMV, because symptoms were clear and showed earlier than in *Medicago* spp. However, the time for nodulation was longer than for barrel medic. Inoculation of lupin with CMV by patch grafting showed that the susceptibility of lupins declined during nodulation. The efficiency of nitrogen fixation of infected lupin was lower than that of the healthy. Variation in the conditions of temperature and day length did not affect the susceptibility of lupin and the efficiency of nitrogen fixation.

L-glutamine and L-asparagine are intermediates in $N_2$ fixation. Since *Rhizobium* provides $N_2$ for plant growth, these amino acids were used as sources of $N_2$ in a study

of virus content and *Rhizobium* infection. Virus content was measured by either double antibody sandwich (DAS) ELISA or plate trapped antigen (PTA) indirect-ELISA. Application of 10 mM of these amino acids by a wick to lupin cv. Gungurru (both in the +R and -R treatment) which showed severe symptoms, did not affect virus content. These amino acids increased the efficiency of nitrogen fixation in healthy lupins. However, application of these amino acids by various methods to *N. glutinosa* at the beginning of symptoms developed, increased virus content.

CMV particles were detected in nodules of infected plants following treatment of tissue with RNase. Particles and crystals of virus, and bacteroids were not located in the same cells. No differences in the structure of healthy and virus infected nodules were observed.

## STATEMENT

This thesis has not previously been submitted for an academic award at this or any other University, and to the best of my knowledge and belief, this is the original work of the author, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Signed,

Wiwiek Sri Wahyuni

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# CHAPTER 1. GENERAL INTRODUCTION

## I. THE CUCUMOVIRUSES

### 1. Relationships within the Cucumoviruses

The cucumovirus group comprises three major members, cucumber mosaic [CMV], tomato aspermy [TAV] and peanut stunt viruses [PSV] (Mink, 1969; Devergne and Cardin, 1975; 1975a). Other possible members of this group are chrysanthemum mild mottle virus [CCMV] (Hanada and Tochihara, 1980, Hanada, 1984), soybean mosaic virus [SMV] (Hanada, 1984; 1986; Hanada and Tochihara, 1982), cowpea ring spot virus [CRSV] (Phatak *et al.*, 1976), robinia mosaic virus [RMV] (Francki *et al.*, 1979), clover blotch virus [CBV] (Richter *et al.*, 1979) and black locust true mosaic virus [BLTMV] (Boswell and Gibbs, 1983).

The major members and strains of each member of the group differ in biological and serological properties (Kaper and Waterworh, 1981). For example, TAV does not infect cucurbits (Marrou *et al.*, 1975), and CMV and TAV do not induce symptoms on peanut (Marrou, *et al*, 1975; Kaper and Waterworth, 1981). Differences and similarities in serological and physicochemical properties among this group have also been reported. Serological differentiation indices (SDI) in gel immunodiffusion tests (van Regenmortel, 1982) and in direct and indirect ELISA (Devergne *et al.*, 1981) are used to measure the relatedness among viruses, where viruses with the SDI values of 1-2 are categorized as closely related. The relationships between CMV and PSV, CMV and TAV are shown by SDI values of 3 and 6, respectively (van Regenmortel, 1982). It is possible that particular strains of different members of the cucumoviruses are closely related. CCMV was described by Tochihara (1976 *cit.* Hanada, 1984) as being serologically closely related to TAV, and a strain of soybean stunt virus (SSV) from Japan was found to be closely related to one of the CMV strains (Hanada and Tochihara, 1980). RMV was not considered to be a separate member of the cucumoviruses, because it was serologically related to PSV and CMV (Richter *et al.*, 1975). RMV and CBV may be strains of PSV (Richter *et al.*, 1979). Identification of a

new strain of a virus by gel immunodiffusion using some antisera of the cucumoviruses could give different reactions which complicate the classification a strain. For example, a strain of PSV and a CMV from Morocco produced precipitin lines when tested against antisera to three TAV isolates and PSV-E, respectively (Fischer and Lockhart, 1978).

In fact, although the physicochemical properties of three members of the cucumoviruses have not been compared together at the same time, some strains of PSV, CMV and TAV showed small differences in the electrophoretic mobilities of their viral protein (Diaz-Ruiz *et al.*, 1979; Hanada, 1984) and RNAs (Rao and Francki, 1981; Hanada and Tochihara, 1980; Lot and Kaper, 1976). For example, the molecular weight of coat protein subunits (Mr : $x10^3$) for CMV, TAV and PSV are 26.2, 26.1 and 26, respectively (Francki, 1985a). The molecular weight of RNAs 1, 2, 3 and 4 (Mr : $x10^6$) for CMV is 1.35, 1.03, 0.75 and 0.35, for TAV is 1.26, 1.10, 0.90 and 0.43, and for PSV is 1.3, 1.0, 0.75 and 0.35, respectively (Symons, 1985).

Therefore, TAV, CMV and PSV are considered to be distinct members of the cucumoviruses (Mink *et al.*, 1969; Habili and Francki, 1974a; Devergne and Cardin, 1975). Moreover, in relation to the strains of the cucumoviruses, Devergne and Cardin (1975), and Haase and Rabenstein (1988) decided that on the basis of location of epitopes on the virus capsid, TAV, CMV and PSV are serologically related. Strains of each member have an epitope specific to each homologous antibody. TAV is more distantly related to CMV than is PSV to CMV.

## 2. Virion and genomic structures

The cucumoviruses have icosahedral particles with a diameter of 28 -30 nm, and a central hole which is penetrable by negative stain (Tolin, 1977, Francki *et al.*, 1979). Particles consist of 18.25% RNA and 180 coat protein subunits arrayed in a T=3 capsid (Kaper and Waterworth, 1981).

The cucumoviruses have a single stranded plus sense RNA genome, consisting of three RNA species, designated as RNA 1, 2 and 3 (Kaper and Waterworth, 1981). RNAs 1 and 2 carry the determinants for symptom induction (Rao and Francki, 1982;

Hanada, 1986; Roossink and Palukaitis, 1990) and RNA 3 carries the determinant for coat protein synthesis, aphid transmissibility and translocation of particle *in vivo* (Habili and Francki,1974; Mossop and Francki, 1977; Gera *et al.*, 1979; Rao and Francki, 1982). The RNA 4 is subgenomic to RNA 3 and is the monocistronic message for *in vitro* synthesis of coat protein (Schwinghamer and Symons, 1977). Some strains of PSV and CMV contain RNA 5 and 6. The RNA 5 contains sequences common to RNA 1 - 4 (Gould *et al.*, 1978), and RNA 6 contains host plant RNA's, predominantly t-RNA with some sequences common to the viral genomic RNAs (Palukaitis and Zaitlin, 1984). An additional RNA 5 which is a satellite RNA (CARNA 5 and PARNA 5 for CMV and PSV, respectively [Kaper *et al.*, 1981, 1978]) has been detected in some isolates of CMV-Q (Gould *et al.*, 1978), CMV-B (three isolates, B1, B2 and B3), G, WT, WL (two isolates) (Palukaitis and Zaitlin, 1984, Palukaitis, 1988), Y [Japan] (Zenbayashi *et al.*, 1983, Devic *et al.*, 1989), P and E (Japan, satellites of these strains are not always present, Hanada and Tochihara, 1980), in PSV isolates 74-23 [synonymous with strain V of Naidu *et al.*, 1991] (Lot and Kaper, 1976), PSV-E, WC and G (Naidu *et al.*, 1991).

## II. NUCLEIC ACID PROPERTIES OF CUCUMOVIRUSES

### 1. Nucleotide sequences of viral RNA

RNAs 1, 2 and 3 are essential for infection (Lot *et al.*, 1974). Pseudo-recombinants constructed from RNAs of different strains are useful for the study of gene structure, function and regulatory mechanisms (Lakshman and Gonsalves, 1980; Lakshman *et al.*, 1985). For example, pseudorecombinants constructed from RNA 1 of CMV-$F_{NY}$ produced more severe symptoms which were typical of $F_{NY}$ than when constructed from RNA 1 of CMV-$S_{NY}$ (Zitter and Gonsalves, 1991). The RNA 1 is required for initiating virus replication, but its specific function in pathogenicity is unknown as yet (Roossink and Palukaitis, 1990; Zitter and Gonsalves, 1991). The exchange of RNA 1 and 2 between CMV isolates showed that symptoms produced by pseudorecombinants of these isolates may be dominated by the RNA 2 of parental

isolates (Hanada, 1986). Pseudorecombinants of either strain CMV-Q or TAV-V (aphid transmitted) with CMV-M (non aphid-transmitted) demonstrated that RNA 3 is specific for coat protein synthesis and aphid transmissibility (Mossop and Francki, 1977; Chen and Francki, 1989).

Nucleotide sequences of the RNAs have been used to investigate differences in biological and physicochemical phenomena between strains (Kaper and Waterworth, 1981). Some strains of CMV have been partially sequenced but only strain Q has been completely sequenced. RNAs 1 and 2 of Q-CMV consist of 3389 and 3035 nucleotides (nt), and have open reading frames (ORF) of 2973 and 2517 nt, respectively (Rezaian *et al.*, 1985, 1984). RNA 3 consists of 2197 nt with two ORFs, one is the 3a protein gene (837 nt) and the other is the coat protein gene (654 nt) (Davies and Symons, 1988). RNA 4 which is transcribed from RNA 3 and identical to the 3' half of the RNA 3, consists of 1030 nt (Davies and Symons, 1988). Compared to Q-CMV, the RNAs 1, 2 and 3 of strain $F_{NY}$ consist of 3357, 3050 and 2216 nt with ORFs of 2979, 2571, and 840 nt (3a gene) and 657 nt (coat protein gene), respectively (Rizzo and Palukaitis, 1988; 1989; Owen *et al.*, 1990). The RNA 3 of strain O (Japan) has 2217 nt which consists of two ORFs, 841 nt encoding the 3a protein and 656 nt encoding the coat protein (Hayakawa *et al.*, 1989). The $Y_{Jpn}$-satellite contains 368 nt (Hidaka *et al.*, 1984), while E and OY2 satellites consist of 339 and 386 nt, respectively (Hidaka *et al.*, 1988).

Some of the RNAs of PSV and TAV strains have also been sequenced. RNAs 1 and 2 of PSV strain J consist of 3355 nt and 2946 nt with ORFs 3015 nt and 2505 nt, respectively (Karasawa *et al.*, 1992). The RNA 3 of PSV-J consists of 2186 nt with ORFs of 867 nt for the 3a protein gene, and 654 nt for the coat protein which is expressed from subgenomic RNA 4 of 1010 nt (Karasawa *et al.*, 1991). The satellite-RNA of PSV isolate 1976 is 393 nt, linear, and with no sequence homology to its respective subgenomic helper RNAs (Collmer *et al.*, 1985). RNA 1 of TAV-V consists of 3410 nt with an ORF of 2982 nt (Bernal *et al.*, 1991), while RNA 2 consists of 3074 nt with an ORF of 2487 nt (Moriones *et al.*, 1991). RNA 3 of TAV-C consists of 2214

nt with an ORF of 831 nt for the 3a protein gene and an ORF of 686 nt for the coat protein gene (O'Reilly *et al.*, 1991).

The sequencing data above shows that the number of nucleotides in the RNAs of strains of the same virus group differs slightly.

## 2. Symptoms induced by satellite RNA

Satellite RNA (RNA 5 or sat) can either modify symptoms in some hosts (Palukaitis and Zaitlin, 1984; Francki, 1985) or affect viral replication in particular host plants (Waterworth *et al.*, 1979; Kaper *et al.*, 1981). Some species of satellite RNA tend to attenuate symptom induction (Jacquemond and Lauquin, 1988) and others induce new symptoms which are quite distinct from symptoms induced by the virus itself (Takanami, 1981; Palukaitis, 1988; Sleat and Palukaitis, 1990; Moriones *et al.*, 1991a). Symptom modification also depends on the plant host species (Mossop and Francki, 1979; Waterworth *et al.*, 1979; Devic *et al.*, 1989), and the helper virus (Palukaitis, 1988; Sleat and Palukaitis, 1990a). The same strain of helper virus with different satellite RNAs can induce different symptoms, e.g. amelioration (Palukaitis, 1988; Sleat and Palukaitis, 1990a), lethal necrosis (Waterworth *et al.*, 1979), chlorosis (Garcia-Arenal *et al.*, 1987; Gonsalves *et al.*, 1982; Sleat and Palukaitis, 1990), or a combination of these effects (Kurath and Palukaitis,1989).

Typical symptoms induced by satellite-RNA of CMV on tobacco and tomato plants are (**1**) lethal necrosis e.g. by In-sat on tomato (Kaper *et al.*, 1988), $Y_{Jpn}$-sat on tomato (Takanami, 1981), n-CARNA 5 on tomato (Kaper *et al.*, 1988), and by Ix-sat on tomato (Moriones *et al.*, 1991a); (**2**) non necrosis, e.g. by B-sat on tomato (Palukaitis and Zaitlin, 1984), G-sat on tomato (Gould *et al.*, 1978), and by Ix-sat on squash and tobacco (Moriones *et al.*, 1991a); (**3**) white leaf by WL-sat (Gonsalves *et al.*, 1982), and (**4**) yellow mosaic by $Y_{Jpn}$-sat on tobacco (Jaegle *et al.*, 1990)

## III. Symptoms and Induction of Disease by CMV

### 1. Isolation of strains

CMV has more than 60 characterized strains (Kaper and Waterworth, 1981). Some isolates are designated with the same name, so this name may confuse if it is not described by its origin. In this thesis, a system for naming the strains has been adopted, in which each is referred to by a capital letter which indicates its characteristics and a subscript which indicates its geographical origin [see Table 2.1] (Wahyuni *et al*, 1992).

The nomenclature of strains has not yet been standardized. Names have been given on the basis of (1) their isolation from a particular host, (2) selection by a particular host, or (3) symptom production (Matthews, 1991). Examples of designation by the name of host plant of the isolate are, strain C (*Cucurbita pepo*; Edwards and Gonsalves, 1983), strain Y (isolated from *Lupinus angustifolius* cv. Yandee in WA; Jones, 1988) and several strains of T (tomato; Kaper and Waterworth, 1981). Examples of designation by selection on a particular host plant are 3 strains L or Ls (L1, L2 and L3) which were isolated from lettuce (*Lactuca sativa*) in New York. Sub-designations were based on their interaction with *Lactuca saligna* as LsS (susceptible) and LsR (resistant). Formerly, strains LsS and LsR were designated as L2 and L1, respectively (Provvidenti *et al.*, 1980). Strain L3 was also isolated from lettuce and distinguished from these two isolates by its ability to induce systemic necrosis on *L. saligna* and *L. serriola* (Edwards and Gonsalves, 1983). Some isolates have been considered as strains on the basis of symptom production. Examples are, strain WL (white leaf on tomato; Gonsalves *et al.*, 1982), Y (Wisconsin, yellow on tobacco; Scott, 1968) and Y (Japan, yellow mosaic; Hanada and Tochihara, 1980). Some isolates from a muskmelon plantation in New York (Banik and Zitter, 1990, Zitter and Gonsalves, 1991) were distinguished as strains Fny (fast and severe) and Sny (slow and mild) on the basis of inducing symptoms in zucchini squash (*Cucurbita pepo*).

## 2. Host range and transmission of CMV

### a. Host range

CMV has a wide host range of more than 800 plant species (Francki *et al.*, 1979; Douine *et al.*, 1979; Horvárth, 1980), including natural and artificial hosts (Horvárth, 1980). A differential host range has been selected from the host list (Horvárth, 1980) and is useful for identifying new isolates and distinguishing them from other viruses (Lovisolo and Conti, 1969; Hampton *et al.*, 1978). For example, BBWV (broad bean wilt virus) was distinguished from CMV on the basis of local lesions produced in different hosts (Bruckart and Lorbeer, 1975). Strain C was distinguished from strain B (New York) by symptoms produced on several *Phaseolus* and *Macroptilium* species (Provvidenti, 1976). On the other hand, Nelson and McKittrick (1969) stated that symptoms produced by LMV (lettuce mosaic virus) and CMV in lettuce could not be distinguished easily, while Bruckart and Lorbeer (1976) found that the symptoms induced by CMV, LMV and BBWV on differential hosts were distinctive.

### b. Transmission

Most CMV strains can be transmitted mechanically to artificial hosts, but natural virus spread is by aphids and infected seeds. More than 60 aphid species have been listed, but *Aphis gossypii* and *Myzus persicae* are the most frequently reported vectors of CMV (Kaper and Waterworth, 1981). Success of vectors to transmit CMV is influenced by host species, strain of CMV and aphid colonies. Whitefly *(Bemisia tabaci)* was a very efficient vector of CMV on sweet potato (Cohen and Lobenstein, 1991). *M. persicae, Acythosiphon kondoi, Aphis craccivora* and *Lipaphis erysimi* were found to be very efficient in transmitting CMV from lupin *(Lupinus angustifolius)* to lupin in Western Australia [W.A.] (Jones and Proudlove, 1991).

CMV is seedborne in several host species. Strain B was seedborne in *Phaseolus vulgaris* cv. Pioneer (Provvidenti, 1976) and other strains were transmitted at rates of up to 30% in bean seeds (Marchoux *et al.*, 1977). Seeds of several weed species also transmit CMV. For example, 2-40 % of chickweed *[Stellaria media]* seeds carried virus

(Tomlinson and Carter, 1970) and 9-95% of *Echinocystis* seeds were infected (Neergard, 1977). Lupin and subterranean clover have seed transmission rates for CMV of 0.5-34% and 0.5-8.8% respectively, but these rates also depend on the cultivar and virus strains (Jones, 1988; Jones and Mc Kirdy, 1990).

Weeds or intermediate crops grown between main crops are also sources for the spread of CMV in the field. *Vinca rosea* (periwinkle) had a CMV incidence of 70% and was a source of virus for lettuce plantations in Arizona (Nelson and McKittrick, 1969). Several weeds were also detected as sources of CMV for lettuce and celery in New York (Bruckart and Lorbeer, 1976) and lupin (Jones, 1988).

### 3. Ecology and epidemiology of CMV

The epidemiology of CMV in lettuce crops had been described for Britain (Tomlinson and Carter, 1970), Arizona (Nelson and McKittrick, 1969) and New York (Bruckart and Lorbeer, 1976). Epidemics of CMV in lupin in South and Western Australia [S.A. and W.A.] were probably caused by sowing infected seeds with subsequent spread by *Myzus persicae* (Alberts *et al.*, 1985; Jones, 1988).

In temperate regions, seasons apparently affect the occurrence and epidemics of CMV, in which the population of plants infected with particular virus strains is also affected (Quiot, 1980; Haack, 1986). The different thermosensitivity of virus strains in the field has been reported in some countries. A study of the ecology of CMV infection in France and Germany showed that the number of plants infected with strains B and N (Marrou *et al.*, 1975; Haase *et al.*, 1989) were higher in winter, and strains C and U were predominant in summer (Quiot, 1980; Quiot *et al.*, 1983; Haack, 1986). The virus might be retained in annual weeds (Quiot, 1980; Qiuot *et al.*, 1983) or ornamental garden plants (Nelson and McKittrick, 1969; Bruckart and Lorbeer, 1976) when the main crop is not grown in summer or winter (Nelson and McKittrick, 1969; Bruckart and Lorbeer, 1976). In experiments on the time to induce the first symptoms in zucchini squash cv. Black beauty, some strains were categorized as fast (Fny, Rmi, UH, VE-85) and slow (Cap 9A, LE, O, Sny). The fast strains were able to infect muskmelon

(*Cucumis melo*) when this plant was maintained at 37° and 27°, whereas the slow strains infected this plant more efficiently at 27° (Roossinck, 1991).

## IV. CLASSIFICATION OF CMV STRAINS

Some CMV strains have been classified into two major subgroups based on symptoms and host range, serology and nucleic hybridization tests, as described below.

### 1. Symptoms and host range

Several hosts of CMV have been tested for use in characterizing CMV strains. Only one, *N. tabacum* var. Xanthi n.c. distinguishes between strains belonging to different serogroups (Devergne and Cardin, 1975). Based on the symptoms produced in this plant, CMV strains can be divided into 2 subgroups (Marrou *et al.*, 1975). Subgroup B strains produce characteristic etching and subgroup C strains induce a systemic mosaic.

Very few plant species produce specific symptoms which can be used for characterizing strains (Lovisolo and Conti, 1969; Douine *et al.*, 1979; Wang *et al.*, 1988). For example, isolate B (from bean, New York) is only able to infect legumes, but it does not infect *L. saligna,* while isolates L2 and L3 from lettuce are unable to infect beans (Edwards and Gonsalves, 1983).

### 2. Serological tests

#### a. Gel immunodiffusion

CMV strains have been classified by double immunodiffusion tests into two serogroups based on spur formation and by SDI. These groups are serogroup ToRS and serogroup DTL (Devergne and Cardin,1970; 1973; Devergne *et al.*, 1981). In this classification, the relationships between members of the serogroups have also been described in detail on the basis of epitopes specific to each group. Isolates To, O, B, Car (sub-serogroup To), R (sub-serogroup R), S and Q (sub-serogroup S) belong to serogroup ToRS, whereas isolates TL, D, G and T belong to serogoup DTL.

The group B strains (thermosensitive) correspond to the ToRS serogroup and the group C strains (thermoresistant) correspond to the DTL serogroup (Marrou *et al.*, 1975; Quiot, 1980). CMV strains from Japan are also classified into 2 major serogroups (Takanami and Tomaru,1969). The serogroup P corresponds to the ToRS serogroup and serogroup Y corresponds to the DTL serogroup (Hanada and Tochihara, 1980). CMV strains from Germany have been distingushed as serogroup N and U. The N serogroup corresponds to the B subgroup of Marrou *et al.* (1975), whereas the U serogroup corresponds to the C subgroup (Richter, 1983).

**b. ELISA**

ELISA is a more sensitive method for distinguishing between virus strains belonging to the same group than the gel immunodiffusion test. ELISA with either polyclonal or monoclonal antibodies gives results similar to those of serogrouping with immunodiffusion tests. A comparison of two methods of ELISA (direct and indirect) with polyclonal antisera showed that strains belonging to different serogroups, ToRS, DTL, and Co were distantly separated (Devergne *et al.* , 1981). Identification of some Japanese CMV isolates by $F(ab')_2$ ELISA with polyclonal antibodies cross-absorbed with either strain Y or Z [strain Z belongs to the P serogroup] showed that these isolates could be distinguished into two serogroups, Y and P (Maeda and Inouye, 1987).

Monoclonal antibodies specific to serogoups ToRS, DTL and Co (Porta *et al.*, 1989) and to serogroups Y and Z (Maeda *et al.*, 1988) have been produced. With these monoclonal antibodies, strains belonging to either of these serogroups were distinguishable. Haase and Rabenstein (1988) selected five monoclonal antibodies to distinguish members of the cucumovirus group. From these monoclonal antibodies, only one (CMV-BD 9) was useful for separating eleven CMV isolates into different serogroups, N and U.

On the basis of ELISA, CMV strains can be divided into two major serogroups, DTL and ToRS, with a minor serogroup, Co.

### 3. Sequence homologies of RNA, peptide mapping and nucleic acid hybridization tests

CMV strains are also divided into subgroups on the basis of the properties of their RNAs. Using RNA-RNA hybridization, 18 CMV strains were distinguished into two subgroups (Piazzolla *et al.*, 1979). The two subgroups, of 14 and 3 strains, respectively, had extensive sequence homologies with strains within the same subgroup, but had no detectable homologies between strains of the other subgroup. Only one strain had partial homology with members of one subgroup and none with the other. Some strains investigated by Piazzolla *et al.* (1979) were the same as those used for serological and symptom studies (Devergne and Cardin, 1975; Marrou *et al.*, 1975). These strains fell into a similar grouping.

Subgrouping of CMV isolates was also done by peptide mapping of their coat proteins (Edwards and Gonsalves, 1983). Peptide maps of isolates F and B (both from *P. vulgaris*), and C1 and 2 were different from isolates L2, L3 and WL. Isolates B, C, and F were placed in the same serogroup as the DTL of Devergne and Cardin (1973), while isolates L2, L3 and WL were placed in the sub-serogroup S of the ToRS serogroup. However, it needs to be noted that isolate B used in their experiment was not the same as strain B of Devergne and Cardin (1975), in which strain B belonged to serogroup ToRS.

Another method for identifying or distinguishing CMV isolates into different subgroups (serogroups) is by comparing their ds-RNAs (Wang *et al.*, 1988). On the basis of the different mobilities of ds-RNAs 1, 2 and 3, four of the six isolates were shown to belong to serogroup Y and another two to serogroup P. Although minor differences were detected in the molecular weight of ds-RNA 1 and 2 within isolates, it was assumed that these were characteristic of particular strains (Wang *et al.*,1988).

Twelve strains of CMV were also classified into two different subgroups (subgroup I and II) on the basis of their ability to hybridize with complementary DNAs (cDNA) transcribed from RNAs of either strain Fny or WL (Owen and Palukaitis, 1988). Four strains hybridized well to WL-cDNA, another eight hybridized to Fny-

cDNA. No cross hybridization occurred under washing conditions of low stringency. Four of the strains used had been serogrouped by Devergne and Cardin (1975) and Edwards and Gonsalves (1983). Strains D and B which belong to the DTL serogroup were then classified into subgroup I, and strains Q and S which belong to the ToRS serogroup, into subgroup II.

Strains of CMV can be classified as shown in Table 1.1

Table 1.1. Subgroupings of CMV strains on the basis of the nucleic acid hybridization, serology and host range

| Property | Subgroup | | | References |
|---|---|---|---|---|
| **Nucleic acid hybridization** | I | II | | Piazolla *et al.*, 1979; Owen and Palukaitis, 1988. |
| **Serology** | | | | |
| France | DTL | ToRS | Co | Devergne and Cardine, 1973; 1975; Devergne *et al.*, 1981: Porta *et al.*, 1989. |
| Germany | U | N | | Richter, 1983; Haase and Rabenstein, 1988. |
| Japan | Y | P | | Takanami and Tomaru, 1969; Hanada and Tochihara, 1980 |
| **Host plant** | C (thermostable) | B (thermosensitive) | | Marrou *et al.*, 1975; Quiot, 1980; Quiot *et al.*, 1983. |

## V. Effect of Virus Infection on the Interaction of Legumes and *Rhizobium*

### 1. Effect of nitrogen supplement on plant growth and virus infection

In 1930, it was reported that tobacco plants were more sensitive to tobacco mosaic virus (TMV) infection when treated with nitrogen supplements provided as either $Ca(NO_3)_2$ or $NH_4NO_3$. Virus content was determined either by measuring the virus protein content in the plant juice or by the number of local lesions produced on local lesion hosts (Spencer, 1941[a], 1941[b], 1942). The susceptibility of tobacco leaves at different ages from the same plant was affected by the concentration of nitrogen ($NO_3$ + $NH_4$) added (Spencer, 1935). Volk (1931) *cit.* Spencer (1935) showed that when tobacco was grown in sand culture and inoculated at the pre-flowering stage, the percentage of diseased plants increased as the nitrogen supply was increased. A high concentration of nitrogen as $NH_4$ in the growth medium also increased the multiplication of PVX on potato and tobacco, TMV and tomato aucuba virus on *N. glutinosa* (Bawden and Kassanis, 1950) and CMV (Cheo *et al.*, 1952) in tobacco.

Conversely, Weathers and Pound (1954) found that supply with a very high concentration of nitrogen also increased the amount of inhibitor present in tobacco infected with TMV, and this could reduce virus replication. This reduction was considered to be due to the harmful effect of the excess nitrogen on metabolic processes in plant cells (Weathers and Pound, 1954).

### 2. Effect of nitrogen supplement on the interaction between legumes and *Rhizobium*

In the presence of $N_2$, nitrogenase catalyses the reduction of $N_2$ to $NH_3$ and $C_2H_2$ to $C_2H_4$. The nitrogenase activity (NA) is expressed in µmol of $C_2H_4$ (Sprent and Sprent, 1990). The efficiency of nitrogen fixation is inhibited by the presence of nitrogen in the growth medium (Munns, 1977). For example, NA was inhibited by 80% in lupin, chickpea, soybean, and barrel medic when treated with 4 mM nitrate (Harper and Gibson, 1984). Silsbury *et al.* (1986) also found that even a small amount of added

nitrate (0.5 mM) can reduce the NA in subterranean clover. This inhibition is caused by the accumulation of nitrite in the nodule cytosol which is produced by nitrate reductase activity (Kanayana *et al.*, 1990). The accumulation of nitrite is also caused by the inhibition of supply of oxygen in bacteroid cells which is needed for stimulating NA (Tajima *et al.*, 1985). However, NA inhibition is not related to the reduction of carbohydrate accumulation in nodules (Streeter, 1985; 1985a). The decrease of acetylene reduction activity (ARA) is correlated with an increase of nitrosylleg-hemoglobin content in the nodule of soybean when it is supplied continuously with nitrate (Kanayana and Yamamoto, 1990).

### 3. Effect of treatment with *Rhizobium* and nitrogen supplement on the interaction between legumes infected and mycoplasma or virus

#### a. Effect of mycoplasma or virus infection on nodulation and nitrogen fixation in legumes

Mycoplasma infection of legume plants reduces the efficiency of nitrogen fixation. Joshi *et al.* (1967) showed that clover phyllody mycoplasma (CPM) affected the activity of *Rhizobium* in nodules of white clover which was grown in sterilized soil. Nodules of infected plants were small and white, indicating that the nodules did not actively fix nitrogen, while nodules of healthy plants were larger and pink. In other experiments, Joshi and Carr (1967) found that small and white nodules were also produced by healthy subclover which were grown in soil containing *Rhizobium* from CPM-infected plants. It was assumed that the mycoplasma was transmitted by *Rhizobium* from infected nodules to the healthy plant.

With virus infection, the nitrogenase activity and leghemoglobin content of mungbean cv. Pusa Baisakhi infected with common bean mosaic virus (CBMV) was lower than in healthy nodules, but the total amino acids was higher in infected than in healthy nodules (Chowdhury *et al.*, 1987). Tu *et al.* (1970) also found that the total nitrogen content in soybean nodules infected with soybean mosaic virus (SMV) was

higher, but the nodule weight was lower than that of healthy nodules. Nodules of peas (*Pisum sativum* L.) infected with either sesbania mosaic virus [SeMV] (Rao and Shukla, 1988) or CMV (Rao *et al.*, 1987) have a higher protein content than healthy nodules.

In an experiment with maize dwarf mosaic virus (MDMV) strains A and B, and sugarcane mosaic viruses (SCMV) strain H, Ford and Tu (1969) suggested that the virus altered the amino acid concentration in the plant. The concentration of ammonium and asparagine-glutamine (amide) in the infected corn was higher, but the concentration of other amino acids was lower. Thus, limiting the supply of several essential amino acids in the free amino acid pool, may limit the virus multiplication. The limited supply of several essential amino acids for viral protein synthesis in infected plant (Diener, 1960; Ford and Tu, 1969) might correspond to a decrease in enzymatic activity such as nitrogenase in nodules (O'Hair and Miller, 1982; Wongkaew and Peterson, 1986; Orellana, 1978, 1987), and nitrite reductase in leaf tissues (Chowdhury and Srivastava, 1986). Nitrate reductase was shown to be higher in nodules infected with SeMV which caused the reduction of nitrogenase activity (Rao and Shukla, 1988). Virus infection in plants treated with *Rhizobium* also causes several changes to the plant metabolism, but the mechanism of these changes is unknown (Rao *et al.*, 1987). For example, alfalfa mosaic virus (AMV) reduced the crude protein content in infected alfalfa (*Medicago sativa* L.) plants (Tu and Holmes, 1980), CBMV reduced the nitrite reductase activity in leaf tissues of mungbean (Chowdhury and Srivastava, 1986).

### b. Effect of nitrogen supplement on the interaction between legumes infected with virus and *Rhizobium*

Singh *et al.* (1984) worked with CBMV on mungbean cv. Pusa Baisakhi and a range of nitrogen concentration added in Bollard's nutrient solution. They found that the size and number of nodules produced by mungbean infected with CBMV were smaller and fewer than that of healthy plants. The size and number of nodules increased with the increase of nitrogen concentration and time of assay. Ohki *et al.* (1986) found that the

fresh weight of plants, root, and nodules and ARA of alfalfa infected with AMV was lower than those of healthy plants. The fresh weights of infected plants treated with nitrogen were greater than without nitrogen supplements. Nitrogen supplement reduced the number and the weight of nodules and ARA of both infected and healthy plants.

In an experiment similar to those of Singh *et al.* (1984), Singh and Srivastava (1983) found that the number of local lesions produced from extracts of leaves infected with CBMV increased with the increase of nitrogen concentration. The infectivity assay was measured by the number of local lesion on *Chenopodium amaranticolor,* and infected plants inoculated with *Rhizobium* produced fewer local lesions. Plants supplied with nitrate concentration of 168 and 224 mg/l produced the most severe symptoms (Singh and Srivastava, 1983).

## VI. Scope of this Thesis

The main objective of this thesis was to investigate whether CMV strains from Australia can be classified into the subgroups I and II of Owen and Palukaitis (1988) using cDNA probes. CMV strains $F_{NY}$ and $L_{NY}$ were used in this work as representatives of subgroups I and II, respectively. Other comparative methods using serological tests, host range and symptoms were included to distinguish the subgroups of the strains used.

CMV antisera prepared against fixed and unfixed virus were used for distinguishing CMV strains and the physicochemical properties of fixed and unfixed virus after longterm storage were investigated. As previously shown for AMV, antiserum prepared against fixed virus was more reliable for detecting strain differences among isolates than antiserum prepared against unfixed virus, in both immunodiffusion and in ELISA tests (Hajimorad and Francki, 1991a).

The previous results showed that CMV-$Y_{WA}$ has properties distinguishing it from other strains, therefore it was compared with two strains of PSV by its serological and RNA properties.

It is known that viral infection reduces the ability of plants to fix nitrogen (Tu, 1970; Orellana, 1978;1987; Rao and Shukla, 1988, Dall *et al.*,1988). The converse of this situation was studied in experiments with the effect of *Rhizobium* treatment on the susceptibility of some legume plants to CMV being investigated.

Some work in this thesis has been published as follow :

1. Wahyuni, W.S., Dietzgen, R.G., Hanada, K. and Francki, R.I.B. (1992). Serological and biological variation between and within subgroup I and II strains of cucumber mosaic virus. *Plant Pathology* **41**, 282-297
2. Wahyuni, W.S. and Francki, R.I.B. (1992). Response of some grain and pasture legumes to 16 CMV strains. *Australian J. Agric. Res.* **43**, 465-477.

# CHAPTER 2. MATERIALS AND METHODS

## A. MATERIALS

### 1. Virus isolates

The eighteen CMV strains used in this study and their respective host sources are listed in Table 2.1. Their designation is referred to by a capital letter and a subscript, the latter indicating its geographical origin. Strains $F_{NY}$ and $L_{NY}$, representative of subgroups I and II, respectively (Owen and Palukaitis, 1988) were kindly supplied by Dr. P. Palukaitis (Cornell University, New York). Strains $Y_{Jpn}$ and $P_{Jpn}$, which are representative of the two Japanese serogroups, respectively (Hanada and Tochihara, 1980) were supplied by Dr. K. Hanada (Department of Agriculture Research Center, Ministry of Agriculture, Forestry and Fisheries,Tsukuba, Japan). The remaining strains were collected from six states of Australia, provided by Ms. E. Alberts (Department of Agriculture, South Australia), Dr. R.A.C. Jones (Department of Agriculture, Western Australia), and Dr. R.D. Pares (BCRI, New South Wales). All these strains were passaged three times through single local lesions in *Beta vulgaris* then maintained continuously in *Nicotiana glutinosa*. The tomato aspermy virus (TAV) strain V and tobacco mosaic virus (TMV) strain U1 were from the Waite Agricultural Reseach Institute Collection. Peanut stunt virus (PSV) isolates 2 and E were a gift from Dr. O.W. Barnett (Clemson University, South Carolina).

### 2. Polyclonal and monoclonal antibodies from other laboratories

Polyclonal antisera to fixed virus preparations of strains $F_{NY}$, $L_{NY}$, $T_{WA}$, $Q_{Qld}$, and $U_{Qld}$ were kindly provided by Dr. R.I.B. Francki. Antisera to strains S (South Africa), Price's Yellow, R, B (*Beta vulgaris*), W (Wisconsin Yellow), and To which were used in this study were donated by Dr. J.C. Devergne (Station de Pathologie Végetale,Centre de Recherches Agronomiques, I.N.R.A., Antibes) and used previously by Habili (1974). Antisera to PSV isolates 74-23 and B2R were from Dr. S. A. Tolin (Virginia Polytechnic Institute and State University, Blacksburg) and antiserum to the isolate PSV-Kentucky was from Dr. J.A. Tomlinson (National Vegetable Research

TABLE 2.1 THE ORIGIN OF CMV STRAINS USED IN THIS STUDY

| Virus strain | Source plant | Locality of isolation | Designation of previous isolate | References |
|---|---|---|---|---|
| $F_{NY}$ | Musk melon | New York | Fny | Rizzo and Palukaitis (1988; 1989); Owen and Palukaitis (1988). |
| $T_{WA}$ | Capsicum | Western Australia | T | Hatta and Francki (1981); Rao *et al.* (1982). |
| $H_{NSW}$ | Tomato | New South Wales | TF | Dr. R.D. Pares (personal communication). |
| $O_{Qld}$ | Lupin | Queensland | Lp | Dr. G.M. Behncken (personal communication). |
| $L_{Vic}$ | Subterranean clover cv. Larisa | Victoria | SL | Jones and McKirdy (1990). |
| $C_{NSW}$ | Capsicum | New South Wales | Cap 9a | Dr. R.D. Pares (personal communication). |
| $E_{WA}$ | Subterranean clover cv. Esperance | Western Australia | SE | Jones and McKirdy (1990). |
| $Y_{Jpn}$ | Tobacco | Japan | Y | Tomaru and Hidaka (1960); Takanami and Tomaru (1969); Devic *et al.* (1989). |
| $L_{NY}$ | Lettuce | New York | LsS; $L_2$ | Provvidenti *et al.* (1980); Rist and Lorbeer (1989); Owen and Palukaitis (1988). |
| $Q_{Qld}$ | Capsicum | Queensland | Q | Francki *et al.* (1966); Rezaian *et al.* (1984; 1985); Davies and Symons (1988). |
| $A_{NSW}$ | Capsicum | New South Wales | 2a | Dr. R.D. Pares (personal communication). |
| $U_{Qld}$ | Banana | Queensland | U | Francki and Hatta (1980); Rao and Francki (1981). |
| $B_{SA}$ | Lupin | South Australia | $B_{14}$ | Ms. E. Alberts (personal communication). |
| $S_{WA}$ | Subterranean clover cv. Nungarin | Western Australia | SN | Jones (1988). |
| $V_{Qld}$ | Tomato | Queensland | Tn | Dr. G.M. Behncken (personal communication). |
| $W_{Tas}$ | White clover | Tasmania | WC | Jones and McKirdy (1990). |
| $Y_{WA}$ | Lupin cv. Yandee | Western Australia | LY | Jones (1988). |
| $P_{Jpn}$ | *Pettasites japonicum* (Butterbur) | Japan | P | Tochihara and Tamura (1976); Hanada and Tochihara (1980); Maeda and Inouye (1987). |

Station, Wellesbourne). Monoclonal antibodies (MAbs) to CMV of αL1, αL2a, and αL2b used by Rist and Lorbeer (1989) were kindly donated by Dr. D.L. Rist (Cornell University, New York). These McAs were affinity purified from ascites fluid and the isotype was shown to be IgG1, IgG1, and IgG2b, respectively. MAbs 34.2, 21.4, 76.1, 42.3, and 3.4 were those of Porta *et al.* (1989), and were a gift from Dr. M.H.V. van Regenmortel (Institute de Biologie Moléculaire et Cellulaire, Strasbourg).

### 3. Chemicals

The names of chemicals and biochemicals used are listed in Table 2.2.

### 4. Instruments

Low speed centrifugation was done in a Sorvall RC-2B centrifuge and the high speed centrifugation was done in either a Beckman TL-100, L8-70 or L2-65 refrigerated ultra centrifuges. Sucrose density gradients were fractionated in an ISCO model 640 coupled to an ISCO model UA-5 to record the virus fraction at a wavelength of 254 nm ($Abs_{254}$). The concentration of virus or viral RNA was measured with an ultraviolet spectrophotometer (Beckman model DU-8B).

The colour reactions in enzyme-linked immunosorbent assays (ELISA) were measured at a wavelength of 405 nm ($Abs_{405}$), with a Biorad model 2550 EIA Reader. Electron micrographs presented in this thesis were taken on a JEOL JEM 100 CX electron microscope. RNA was freeze-dried in a Dynavac freezedrier unit. RNA electrophoresis was done with a horizontal slab gel apparatus from Bethesda Research Laboratories, USA. A filtration manifold unit from Schleicher & Schuell, Kenne, NH. USA was used for dot blot assay. Radioactivity of cDNA probe was measured with a Beckman LS 5000 TD Liquid Scintillation spectrometer.

**Table 2.2. Chemicals, biochemicals and miscellaneous**

| Chemicals | Source |
|---|---|
| Adjuvant, Freund's complete & incomplete | Beckton, Dickinson & Co. USA |
| Alkaline phosphatase-conjugate Type VII-S | Sigma Chemicals Co. USA |
| Bovin Serum Albumin (BSA) | Sigma Chemical Co. USA |
| Cellulose DE 22 | Whatman Biochemicals Ltd. England |
| d-TTP, d-GTP, d- CTP | Boehringer Mannheim, West Germany |
| Diethanolamine | Ajax Chemicals, Ltd. Sydney |
| DL-Dithiothreitol (Clelands reagent) | Sigma Chemical Co. USA |
| Ethylene diamine tetra acetic acid (EDTA) | Ajax Chemicals, Australia |
| Ficoll 400 | Pharmacia, Sweden |
| G50 Sephadex (fine) | Pharmacia, Sweden |
| Glutaraldehyde | TAAB Laboratories, England |
| Glycerol | Ajax Chemicals, Australia |
| Goat anti-rabbit IgG-alkaline phosphatase conjugate | Sigma Chemicals, USA |
| Goat anti-mouse IgG-alkaline phosphatase conjugate | Sigma Chemicals, USA |
| Goat anti-mouse IgM-alkaline phosphatase conjugate | Sigma Chemicals, USA |
| Heparin | Sigma Chemical Co. USA |
| Hybond-N membrane | Amersham |
| Polyethylene glycol (PEG 6000) | Union Carbide, USA |
| Polyvinylpyrrollidone (PVP 40.000) | General Biochemicals, USA |
| $\alpha$ $^{32}$P-labelled dCTP | Bresatec, Adelaide |
| p-nitrophenyl phosphate | Sigma Chemicals Co. USA |
| Purified agar, fine | Oxoid, England |
| M-MLV reverse transcriptase | Bethesda Research Laboratories,USA |
| Ribonucleic acid (Torula yeast) | Sigma Chemical Co. USA |
| Salmon sperm DNA (primer) | Sigma Chemical Co. USA |

| | |
|---|---|
| Sodium dodecyl sulphate (SDS) | Sigma Chemical Co. USA |
| Sodium azide | Sigma Chemical Co. USA |
| Thiogycollic acid (TGA) | Ajax Chemical Ltd. Australia |
| Triethanolamine | Ajax Chemical Ltd. Australia |
| Triton X-100 | Ajax Chemical Ltd. Australia |
| Tween-20 | Drug Houses of Australia Ltd. |
| Nitroblue tetrazolium grade III (NBT) | Sigma Chemical Co. USA |
| Phenazine methosulphate (PMS) | Sigma Chemical Co. USA |
| 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) | Sigma Chemical Co. USA |

## B. METHODS

### 1. Inoculation and virus propagation

All virus strains were maintained in *Nicotiana glutinosa* and propagated for purification in *N. clevelandii*. The inoculum was the crude extract of sap of infected *N. glutinosa* leaf which was diluted with tap water. Plants were inoculated mechanically, by dusting with carborundum and rubbing with inoculum. The excess inoculum was washed off immediately with tap water.

### 2. Virus purification

Infected *N. clevelandii* was harvested 10 days after inoculation. The virus purification method used was as described by Habili and Francki (1974) in which leaf tissue was homogenized in citrate buffer (0.5 M sodium citrate, 0.005 M EDTA pH 6.5 with 0.1% thioglycollic acid) and chloroform in the ratio of 1 g: 1 ml: 1ml. The slurry was centifuged at 10,000 rpm for 10 min. The supernatant was treated with PEG-6000 (1 g/10 ml supernatant), stirred for 40 min at 4°, and centrifuged at 10,000 rpm for 10 min. Pellets were resuspended in borate buffer (50 mM Na-borate, 5 mM EDTA pH 9.0) mixed with 2% Triton X-100, then clarified at 10,000 rpm for 10 min. Preparations were then subjected to two cycles of high speed centrifugation (220,000 g for 60 min). In the second high speed centrifugation, the virus was sedimented through a cushion of

15% sucrose. Pellets were resuspended in borate buffer (pH 9.0). Virus was further purified by layering the virus (3 mg/13 ml tube) over a linear sucrose gradient density (5% to 30% ) then centrifuging in a SW-41 rotor at 40,000 rpm for 120 min. The gradients were fractionated using an ISCO density gradient-fractionator, then the fraction containing virus was sedimented at 220,000 g for 80 min. This virus preparation was designated as highly purified virus. The virus concentration was determined using the value $E_{260}^{0.1\%} = 5$ (Francki *et al.*, 1966).

### 3. Storage of purified virus preparations

The purified virus was stored either as fixed or unfixed virus. Virus was fixed in 0.25% glutaraldehyde and dialysed 3x 8 h against borate buffer at 4° and stored at 4° (Rao *et al.*, 1982). Unfixed virus in borate buffer (pH 8.6) and 50% glycerol was stored at -20°C.

### 4. Preparation of viral ribonucleic acid

The viral RNA was extracted by mixing purified virus with an equal volume of water saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline and RNA extraction buffer (5% SDS, 0.1 M Tris-HCl, 10 mM EDTA, pH 7.0) for 5 min at room temperature. After centrifugation at 10,000 rpm for 10 min, the buffer phase was extracted twice with an equal volume of phenol. Nucleic acid was precipitated by adding 2 volumes of ethanol, incubating for 2 h at -20° and low speed centrifugation. Pellets were washed twice with ethanol. RNA was freezedried and resuspended in distilled water, and stored at -20°C until required. The concentration of RNA was determined using the value $E_{260}^{0.1\%} = 25$ (Peden and Symons, 1973)

### 5. Preparation of nucleic acid from *Nicotiana clevelandii* leaves

One g of leaf tissue was ground with a mortar and pestle in 3.6 ml TNE buffer (0.1 M Tris-HCl, 0.1 M NaCl, 10 mM EDTA, ph 7.0), 0.4 ml of 2% SDS and 4 ml

phenol. The extract was centrifuged at 10,000 rpm. for 5 min and the buffer phase was extracted twice with equal volumes of phenol (Dr.K. Hanada, personal communication). The next steps were the same as for the preparation of viral-RNA.

**6. Preparation of complementary DNA (cDNA) to the viral RNA**

The method for cDNA synthesis was as described by Palukaitis and Symons (1980), and Palukaitis (1986). Random primer which was prepared from salmon sperm DNA as described by Palukaitis (1986), was provided by Dr. R.I.B. Francki. cDNA to RNA's was synthesized by adding 2 µg of total viral RNA and 5 µl random primer to the mixture of 5 µl S-2 buffer (200 mM Tris HCl, pH 8.3, 700 mM KCl and 100mM $MgCl_2$), 5 µl reducing agent (200 mM dithiothreitol), 3 µl of nucleotide triphosphates (8.3 mM each of d-TTP, d-ATP, d-GTP), 2 µl of 10 µCi/µl $^{32}$P-labelled d-CTP. Five µl of 40 mM pyrophosphate was added last, prior to the enzyme (1 µl M-MLV reverse transcriptase at 200 units). Sterilized double distilled water (SDDW) was added to adjust the final volume to 50 µl.

This mixture was vortexed and incubated at 37° for 1 h and the reaction was stopped by the addition of 5 µl each of 5% SDS and 0.4 M EDTA, 15 µl of 4M NaOH, and 125 µl SDDW, vortexed and left for 2 h at 37° or overnight at room temperature.

The mixture was fractionated in a column of Sephadex G-50 buffered in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and prepared in a 1.5 ml disposable pasteur pipette. Twenty fractions (fractions no. 1 and 11-20 were 1 ml each and the fractions no. 2-10 were 0.5 ml each) were collected, and their radioactivity counted. cDNA was in the first peak and fractions containing the cDNA were each mixed with 5 µl triethylamine, frozen and freeze dried. The dried cDNA was resuspended in SDDW, fractions were pooled and stored at -20°C.

**7. Dot blot hybridization assay**

Nylon membrane (Hybond-N Amersham) and Whatman 3 MM chromatography paper were washed in SDDW then in 20x SSC (3 M NaCl, 0.3 M tri Na-citrate and 200 µl of 0.2 N HCl). The wet membrane was placed on 2 pieces of chromatography paper

in a dot blot apparatus. Using suction, 0.5 µg RNA samples (in 20x SSC) were spotted onto the membrane. The membrane was then dried and baked at 80°C in a vacuum oven, placed in a plastic bag containing Maule's buffer [3x SSC, 0.08% (w/v) BSA, 0.08% (w/v) Ficoll, 0.08% (w/v) PVP, 1 mM EDTA, 250 µg/ml of phenol extracted yeast RNA] as the pre-hybridization and hybridization buffers (Palukaitis, 1986). All air bubbles in the plastic bag were removed before it was sealed, then it was immersed in an oscillating waterbath for 24 h at 65°C. For the hybridization step, 50,000 cpm/ml of cDNA-probe was added to the blotted membrane with the hybridization buffer in the plastic bag. After 24 h, the blotted membranes were washed 2x 10 min in 2x SSC containing 0.5% SDS at room temperature, then for 2x 10 min at 55° in 2xSSC containing 0.5% SDS, and another 2x 15 min at 55° in 0.1xSSC containing 0.5% SDS (Owen and Palukaitis, 1988). Membranes were placed between sheets of plastic film, excess liquid and air was removed and membranes were then exposed to an X-ray film in a cassette with an intensifying screen, at -70°.

**8. Agarose gel electrophoresis of total viral RNA**

Analyses of viral RNAs were done in 1.5% -1.8% agarose gels in TAE buffer [4.84 g Tris-HCl, 1.64 g anhydrous Na-acetate, 0.745 g EDTA, 1.35 ml (v/v) glacial acetic acid per l]. A sample of 1 µg RNA was mixed with an equal volume of sample buffer [50% (v/v) glycerol in 0.1x TAE containing 0.05% (w/v) bromophenol blue] then heated for 3-5 min at 55° and chilled on ice. Samples were loaded into the wells and gels were submerged in TAE buffer. Electrophoresis was at 100V for 1 h (mini gel) or 2h (medium size gel) or until the dye reached 1 cm from the end of gel. Gels were then stained with 0.2 µg/ml ethidium bromide and viewed in the UV transluminator at a wavelength of 302 nm. For northern blots, gels were blotted to nylon membranes by capillarity (Palukaitis, 1986, Sambrook *et al.*, 1989). Prehybridization, hybridization and film exposure were done as described above.

## 9. Preparation of polyclonal antisera

Polyclonal antisera against the fixed and unfixed viruses were prepared in rabbits. Highly purified virus (0.5 mg/ml) was emulsified with an equal volume of Freund's incomplete adjuvant. Rabbits were injected three times subcutaneously at weekly intervals, then were injected intravenously at two week intervals, until the antisera reached titres of 1/256-1/512 in gel immunodiffusion tests.

To prepare antisera to unfixed virus, rabbits were injected subcutaneously. Subsequent injections were administered at weekly intervals until the antisera reached titres of between 1/128 and 1/512.

The first bleeding was done a week after the third injection, blood was clotted for 3 h at room temperature then left overnight at 4°. After centrifuging at 2,000 rpm for 10 min, the supernatant (e.g. antiserum) was titrated by gel immunodiffusion. All antisera were stored at 4° in the presence of 0.02% sodium azide for continuous use, while the rest were stored at -15° or freeze-dried.

## 10. Gel immunodiffusion tests

Gels containing 0.7% (w/v) purified agar in 10 mM phosphate buffer at pH 7.2 and 0.02% Na-azide, were poured into plastic petridishes (10 cm in diameter). Wells (3 mm in diameter) were cut with a gel template cutter to give eight peripheral wells. Tests were done by placing 10 µl of purified virus at 1 mg/ml as the homologous antigen to the antiserum in alternate wells around the central antiserum well. The test antigens were placed in the remaining wells. Gels were incubated at 25°C for 48 h to develop precipitin lines. Serological differentiation indices (SDI) were determined as described by van Regenmortel (1982).

## 11. Preparation of γ-globulin (IgG) and conjugation of IgG with alkaline phosphatase

The IgG from selected antisera was prepared and conjugated with alkaline phosphatase (AP-ase) as described by Clark *et al.* (1986). Antiserum of 0.5 ml was diluted with 4.5 ml SDDW and mixed with 5 ml of saturated ammonium sulphate and

left at 4°C for 1 h, then centrifuged at 5,000 rpm for 10 min. The precipitate was dissolved in 1 ml of 0.5x PBS (1x PBS = 8.0 g NaCl, 0.2 g $KH_2PO_4$, 1.15 g $Na_2PO_4$, 0.2 g KCl, pH 9.6) then dialysed at 4°C against 500 ml of 0.5x PBS with changes each 8 h for 3 times. The IgG was fractionated in a column of cellulose DEAE-22. IgG fractions (1 ml each) were collected in 1.5 ml centrifuge tubes and each fraction was adjusted with 0.5 x PBS to an OD 280 nm of 1.4, e.g. as 1 mg/ml.

To conjugate IgG, 2.5 mg enzyme was dissolved directly in the solution of 1 mg/ml purified IgG in 1x PBS. If $(NH_4)_2SO_4$ was presence in enzyme, this mixture was dialysed 3x 8 h against 0.5x PBS at 4°, then fixed with 0.06% (v/v) glutaraldehyde (electron microscope grade). The excess glutaraldehyde was removed by dialysing 3x 8 h against 0.5x PBS. Bovine serum albumin (BSA) was added to the final concentration 0.5 mg/ml, then stored at 4°.

## 12. Double Antibody Sandwich Enzyme-linked Immunosorbent Assay ELISA (DAS-ELISA)

### a. General procedure

DAS-ELISA was done as described by Clark and Adam (1977) and Clark *et al.* (1986). Microtitre wells were coated with 100 µl of 1µg/ml IgG of polyclonal antisera to either strain $F_{NY}$ or $T_{WA}$ for detecting viruses belonging to subgroup I, and strain $L_{NY}$ or $Q_{Qld}$ for detecting viruses belonging to subgroup II (Wahyuni *et al.*, 1992). The coating buffer used to dilute IgG contained 1.59 g $Na_2CO_3$, 2.93 g $NaHCO_3$, 0.02% (w/v) $NaN_3$ per litre. After 3 h incubation at 25°C, plates were washed 3x 5 min in PBS-Tween (1 l of 1x PBS + 0.5 ml Tween-20), then blocked with 100 µl of BSA in 0.14 M NaCl for 1 h at 25°C. Virus antigens or extracts of leaf samples were loaded in the wells and incubated overnight at 4°. The antigens present bound the appropriate anti-rabbit IgG AP-ase conjugate, in the conjugate buffer [1x PBS-Tween pH 7.4, 2% (w/v) PVP, 0.5% (w/v) BSA, 0.02% $NaN_3$] for 3 h, at 25°C. The plate was washed 3x 5 min with PBS-Tween between each step. The conjugated antigen-antibody was hydrolysed with 100 µl substrate of 1 mg/ml solution of the p-nitrophenyl phosphate in

diethanolamine buffer [97 ml diethanolamine, 0.02% $NaN_3$, made up to 1l with SDDW, and the pH was adjusted to 9.6 with 11.5 N HCl].

**b. Distinguishing between and within subgroup I and II strains**

Tests were done by the procedure described in section B.12. Antibodies used were from polyclonal antisera prepared to fixed antigens of strains $F_{NY}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$ (subgroup I), and $L_{NY}$, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$ and $Y_{WA}$ (subgroup II). The ELISA plate wells were coated with 100 µl of 1 µg/ml of each IgG, blocked. Then each well was loaded with 100 µl of 1µg/ml of purified fixed virus in 10 mM borate buffer, and incubated for 16 h at 4°. After washing, the wells were filled with 100 µl of the appropriate anti-rabbit IgG AP-ase conjugate. Twenty min after the addition of 100 µl of 1 mg/ml substrate of the p-nitrophenyl phosphate, the plate was read at Abs $_{405}$. TAV strain V which is known to be distantly related to CMV, and TMV strain U1, were used in this study as the negative controls.

**13. Indirect ELISA**

**a. Triple antibody sandwich ELISA (TAS-ELISA) with monoclonal antibody (MAb)**

TAS-ELISA with MAbs αL1, αL2a and αL2b, and MAbs 34.2, 21.4, 76.1, 42.3 and 3.4 was done as recommended by Rist and Lorbeer (1989) and by Porta *et al.* (1989), respectively.

Wells were coated with 100 µl of mixed IgG's each at 1µg/ml from polyclonal antisera to strains of $F_{NY}$, $L_{NY}$ and $Y_{WA}$, and incubated for 3 h at 25°. After washing, wells were blocked with 100 µl of 1% BSA in 0.14 M NaCl for 1 h, 25°C. Wells were then loaded with 100 µl of 1µg/ml of fixed virus in 10 mM borate buffer and incubated for 16 h at 4°. The 100 µl of MAb at a dilution recommended by the donors was added to each well and incubated for 3 h, 25°, then the conjugate (third antibodies) of either goat anti-mouse IgG (the whole IgG) or IgM (specific to µ-chain) AP-ase was added at a dilution as recommended by the supplier. Plates were washed 3x 5 min between each

step with PBS-Tween. The substrate was then added and hydrolysed for a specified time. The reaction was read at a wavelength of 405 nm (Abs $_{405}$).

**b. Plate trapped antigen ELISA (PTA-ELISA)**

The solutions used in PTA- indirect ELISA were the same as in section B.12.a. The microtitre plate was precoated directly with the virus antigens for 3 h at 25°, blocked with BSA for 1h, then incubated with antiserum overnight at 4°. The complex of antigen-antibody was then conjugated with goat anti-rabbit AP-ase for 3 h then hydrolysed with the substrate solution for a specified time.

A diagram of ELISA procedures used in this thesis is shown in Fig. 2.1.

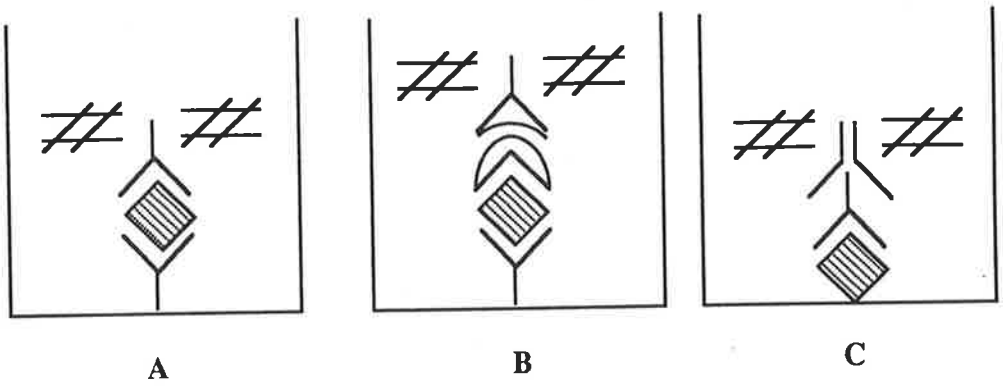
**14. Host range and symptomatological tests**

**a. Host range and inoculation at different times of the year**

Plants used in host range tests are listed in Table 5.1 (see Chapter 5). All plants were inoculated mechanically at the cotyledon stage or primary leaf. Experiments were done in the glasshouse in three different seasons. The first was done during summer to autumn (16-16.5 h daylight), the second from spring to early summer (12-14 h daylight), and the third from autumn to winter (12 h daylight). Descriptions of symptoms were according to Bos (1971) and designated by a number (Table 3.1). Whether plants were symptomatic, symtomless or not infected, was determined either by direct double antibody sandwich enzyme immunosorbent assays (DAS-ELISA) or by back inoculation to *N. glutinosa*.

**b. Sampling for host range and symptomatology tests**

Leaf of *Lycopersicon esculentum, Capsicum frutescens, Lactuca sativa, Zea mays,* and *Lupinus angustifolius* were sampled at 5-6 weeks after inoculation (a.i.), *Nicotiana* spp., *Gomphrena globosa, Datura stramonium, Cucumis sativus, Vigna sinensis* were sampled at 7-14 days a.i., *Phaseolus vulgaris, Vicia vaba, Lens esculenta, Arachis*





virus specific rabbit antibody


virus antigen


virus specific mouse antibody


enzyme conjugated virus specific antibody


goat anti-mouse IgG or IgM enzyme conjugate


goat anti-rabbit IgG enzyme conjugate


substrate p-nitrophenyl phosphate

Figure 2.1. Diagrams showing the different enzyme-linked immunosorbent assays (ELISA) used in this thesis. **A**. Double antibody sandwich ELISA (DAS-ELISA), **B**. Triple antibody sandwich (TAS) indirect -ELISA, **C**. Plate trapped antigen (PTA) indirect-ELISA.

*hypogea, Glycine max* were sampled at 6-8 weeks a.i., *Medicago* spp., *Trifolium subterraneum, T. repens,* and *T. resupinatum* were sampled at 6-10 weeks a.i.

About 1 g of leaf sample to be tested was homogenized between two metal rollers. Two ml of CMV ELISA sample buffer (0.4 M trisodium citrate, 0.005 M EDTA, 0.5% thioglycollic acid, 0.05% tween-20, 2% polyvinyl pyrollidone, 5 units/ml heparin, the pH adjusted to 6.5 with 3 M NaOH; A.D.W. Geering, personal communication) was added. The DAS-ELISA was as described in section B.12.a.

Samples with an Abs $_{405}$ equal to or less than 0.20 (three times the value of healthy sap) were subsequently tested by back inoculation to *N. glutinosa* to determine whether these plants were infected but symptomless or if some non-specific reactions were involved during ELISA test. The infected plants were categorised as "infected" [Abs $_{405} > 0.20$], "infected without symptoms, or symptomless" [Abs $_{405} \leq 0.20$ and positive by indexing to *N. glutinosa*] and "not-infected" [Abs $_{405} < 0.20$ and negative by indexing to *N. glutinosa*].

**15. Sampling to detect virus distribution in** *Medicago* spp.

Plants of various *Medicago* spp. with shoots bearing leaves showing an obvious symptom were sampled and diagrams of leaf positions were drawn. The eight terminal leaves were removed from each tip, weighed then extracted in 5 vol (w/v) of the CMV ELISA sample buffer. DAS-ELISA steps were done as described above. The polyclonal antibody used was from CMV strain $H_{NSW}$ (1/1000 dilution) and the presence of bound antigens was assayed by measuring $Abs_{405}$, 20 min after the addition of the p-nitrophenyl phosphate substrate.

**16. Inoculation of lupin (*Lupinus angustifolius* cv. Illyarrie) with purified virus under sterile conditions**

Lupin seeds were sterilized wih sodium hypochlorite (containing 0.6% available chlorine) for 5 min and rinsed in absolute alcohol for 3 min, then rinsed 3x 5 min with sterile water (Neergard, 1977). The sterilised seed was sown in a plastic container containing the MS-growth medium (Murashige and Skoog, 1962; Kassanis, 1967, see

appendix 1). Inoculum of purified virus (0.1 mg/ml) which had been prepared by filtering through sterile Millipore filter (0.45 µm filter unit, MILLEX-Ha) was mixed with autoclaved carborundum. One week after sowing, cotyledons were inoculated with inoculum of a range of strains of CMV. Plants were maintained at 25°C under 16 h light and symptom development was recorded every 2 days.

### 17. Grafting and aphid transmission

To determine whether *M. scutellata* cv. Sava was resistant to CMV, plants were inoculated mechanically, by grafting and aphid transmission. For graft inoculation, three week old plants were grafted by either approach or patch grafting with the stem tissue of *L. angustifolius* cv. Illyarrie infected with strains $F_{NY}$ or $B_{SA}$. Aphid transmission was done by feeding starved *Myzus persicae* or *Aphis craccivora* on *N. glutinosa* infected with strains $F_{NY}$, $H_{NSW}$, $E_{WA}$, $T_{WA}$, $Q_{Qld}$, $B_{SA}$, or $Y_{WA}$ for 1 min. Each test plant was inoculated by placing 3 aphids on a leaf for a 16 h inoculation access period. Plants were tested by DAS-ELISA, between 1 and 10 weeks later.

### 18. Inoculation of *Medicago truncatula* var. *truncatula* cv. Jemalong (barrel medic) with *Rhizobium* and CMV

*M. truncatula* seeds free of CMV were used in this experiment. They were surface sterilized as described in section 2.B.16 and sown in a coarse sand medium, 16 seeds/pot. The*Rhizobium*, Group A (Nitrogerm, available for medic and lucerne) was inoculated directly to the sterilized seeds, as recommended by the supplier. At 10 days after emergence (a. e.), plants were inoculated on the cotyledons with crude sap of *N. glutinosa* infected with either CMV-$C_{NSW}$ or $T_{WA}$. At 14 days a.e., plants were watered with 0.5x Hoaglands solution containing either no additional $NO_3$, or 7.5 mM $NO_3$ (Gates and Silsbury, 1986, see appendix 2). Each pot received 250 ml solution every 3 days and watering with this solution was stopped at 2 days before assay. DAS-ELISA was done at 2 days prior to the ARA assay at 33 days and 66 days a.i.

## 19. Estimation of nitrogenase activity

The efficiency of nitrogen fixation of plants was estimated as nitrogenase activity and measured by acetylene reduction activity (ARA), using a closed system (Hardy *et al.*, 1968). Plants were removed from sand medium and roots were washed gently in tap water at room temperature. The excess water was blotted off then 16 plants from each pot were placed in a 1.035 l glass jar which was then sealed tightly. 110 ml acetylene (Commonwealth Industrial Gases Ltd. Australia) representing 10% of the jar volume, was injected into the jar. An escape needle was also inserted to allow excess gas to escape and to prevent the build up of pressure in the jar. One ml gas samples were taken in duplicate with a 1 ml syringe at 10 and 40 min after acetylene was added. Samples were injected into a gas chromatograph (Varian Aerograph model 3400 GC) equipped with a flame ionisation detector and an 80-100 mesh Porapak R column.

The rate of acetylene accumulation was calculated as :

$$\mu\text{mol } C_2H_2 \text{ /pot/h} = \frac{s \,.\, u \,.\, y \,.\, 2}{24.06 \,.\, v \,.\, x}$$ where

s = known amount standard of $C_2H_2$ (500 µl)

u = volume of gas in jar containing plants measured by displacement with water or by weighing volume of the jar and subtracting the plant weight

v = volume standard of jar which is measured by displacement with water

x = gas chromatograph value (in area units) of standard

y = gas chromatograph value (in area units) of samples obtained by subtracting the average value of two samples at 10 and 40 min

24.06 = a correction factor for temperature during assay

## 20. Electron micrographs

Roots and nodules of barrel medic cv. Jemalong from the experiment above were taken from plants before being assayed for the efficiency of nitrogen fixation. Preparation of electron microscopy was done according to Hatta and Francki (1979; 1981). Roots and nodules (both from healthy plants and infected with virus) were fixed in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in Millonig's phosphate

buffer (2.26 % $NaH_2PO_4.H_2O$, 2.52% NaOH and 5.4 % glucose) for 16 h, 4°. The tissues were then washed in several changes of 2x SSC (0.15 M NaCl, 0.015 M Na-citrate, pH 7) for 6 h at room temperature. Difficulties in distinguishing viral RNA from ribosomes during the EM examination was avoided by treating the tissues in 2x SSC containing 2 µg/ml pancreatic RNase (Type IIIA, Sigma Chemical Co.) for 16 h at 25°. The tissues were post-fixed with 1% osmic acid and dehydrated in ethanol, then were stained with uranyl acetate and embedded in Epon.

Ultrathin sections were collected on grids coated with formvar-carbon films and stained with uranyl acetate.

# CHAPTER 3. SUBGROUPING STRAINS OF CMV BY SEQUENCE HOMOLOGY

## INTRODUCTION

Complementary DNA (cDNA) transcribed from total viral RNA has been used as a probe to detect subgenomic viral RNA (Gonda and Symons, 1978; Gould and Symons, 1982; Palukaitis, 1986; Hayakawa *et al.*,1989) and to separate strains into distinct subgroups (Owen and Palukaitis, 1988). Dot blot hybridization assay was used in this study to determine whether CMV strains from Australia can be subgrouped on the basis of sequence homology in a manner similar to that described by Owen and Palukaitis (1988).

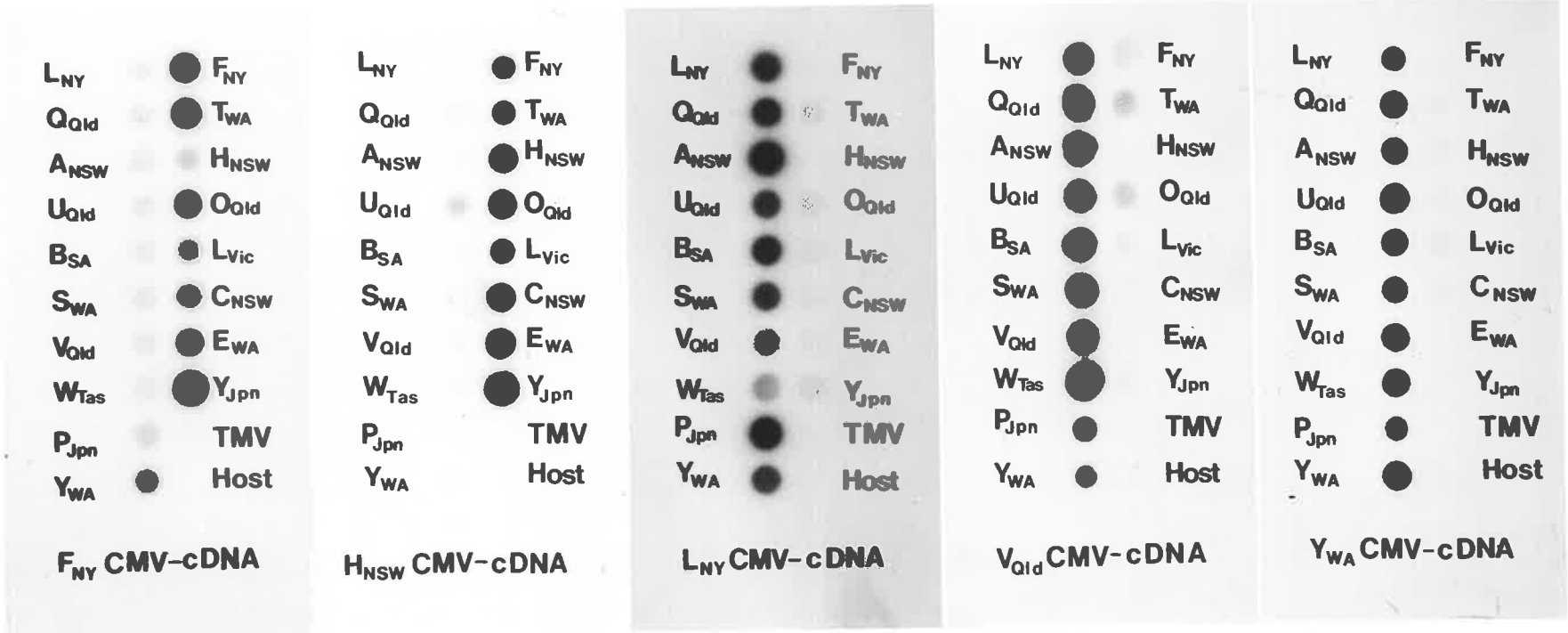
## EXPERIMENTAL

### 1. Dot-blot hybridization assay

The preparation of viral RNA and nucleic acid from uninfected *N. clevelandii* leaves was as described in Chapter 2.B.4 and 5. cDNA to the total RNA of $F_{NY}$ and $L_{NY}$, the type strains of subgroup I and II respectively, was synthesized as described by Palukaitis (1986) [Chapter 2.B. 6]. Other cDNAs were made to the total RNA of strains $H_{NSW}$, $V_{Qld}$ and $Y_{WA}$ as representative strains of CMV from Australia. Dot blot hybridization assays were done as described in Chapter 2.B.7.

The results of dot blotting with cDNA's transcribed from the total RNA of five of the eighteen CMV strains are summarized in Fig 3.1. When $F_{NY}$-cDNA was used to probe RNAs of strains $F_{NY}$, $Y_{Jpn}$, $T_{WA}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, and $E_{WA}$, they produced strong signals, whereas the $H_{NSW}$-RNA hybridized relatively weakly. The reaction with $H_{NSW}$-RNA is considered to be positive because very strong signals were obtained when RNA's from strains $F_{NY}$, $T_{WA}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, EWA were hybridized to $H_{NSW}$-cDNA. Moreover, the signals were much stronger in other experiments (data not shown). On the other hand, the cDNA from $F_{NY}$ and $H_{NSW}$ failed to hybridize with RNA from strains $L_{NY}$, $P_{Jpn}$, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{Qld}$, and $W_{Tas}$.

Figure 3.1. Dot-blot hybridization assay using cDNAs reverse transcribed from RNAs of strains $F_{NY}$, $H_{NSW}$, $L_{NY}$, $V_{Qld}$, $Y_{WA}$. Strains $L_{NY}$, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{Qld}$, $W_{Tas}$, $P_{Jpn}$, and $Y_{WA}$ (left hand side) are shown to belong to subgroup II. Strains $F_{NY}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, $E_{WA}$ and $Y_{Jpn}$ (right hand side) belong to subgroup I. The RNAs of TMV strain U1 and healthy *N. clevelandii* (Host) were used as negative controls.


$L_{NY}$
$Q_{Qld}$
$A_{NSW}$
$U_{Qld}$
$B_{SA}$
$S_{WA}$
$V_{Qld}$
$W_{Tas}$
$P_{Jpn}$
$Y_{WA}$
$F_{NY}$
$T_{WA}$
$H_{NSW}$
$O_{Qld}$
$L_{Vic}$
$C_{NSW}$
$E_{WA}$
$Y_{Jpn}$
TMV
Host
$F_{NY}$ CMV-cDNA
$H_{NSW}$ CMV-cDNA
$L_{NY}$ CMV-cDNA
$V_{Qld}$ CMV-cDNA
$Y_{WA}$ CMV-cDNA

Although $Y_{WA}$-RNA hybridized relatively strongly to $F_{NY}$-cDNA, this RNA failed to produce a detectable signal when hybridized to $H_{NSW}$-cDNA (Fig. 3.1).

In contrast, RNAs of strains $F_{NY}$, $Y_{Jpn}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$ and $E_{WA}$ were undetectable when probed with cDNA to $L_{NY}$, $V_{Qld}$ and $Y_{WA}$ RNA's, while RNAs of strains $L_{NY}$, $P_{Jpn}$, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{Qld}$, $W_{Tas}$, and $Y_{WA}$ produced strong signals. No signals were detected when any of the probes were hybridized to RNAs of TMV strain $U_1$ and uninfected *N. clevelandii* leaves (Fig. 3.1).

These results show that the eighteen CMV strains used in this experiment can be separated into two subgroups designated as subgroup I and subgroup II as defined by Owen and Palukaitis (1988). The six Australian strains, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, and $E_{WA}$ belong to subgroup I, and the other eight strains, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{Qld}$, $W_{Tas}$ and $Y_{WA}$ belong to subgroup II. The type strains $F_{NY}$ and $L_{NY}$ clearly fell into subgroups I and II respectively. $Y_{Jpn}$ was shown to belong to subgroup I and $P_{Jpn}$ was shown to belong to subgroup II.

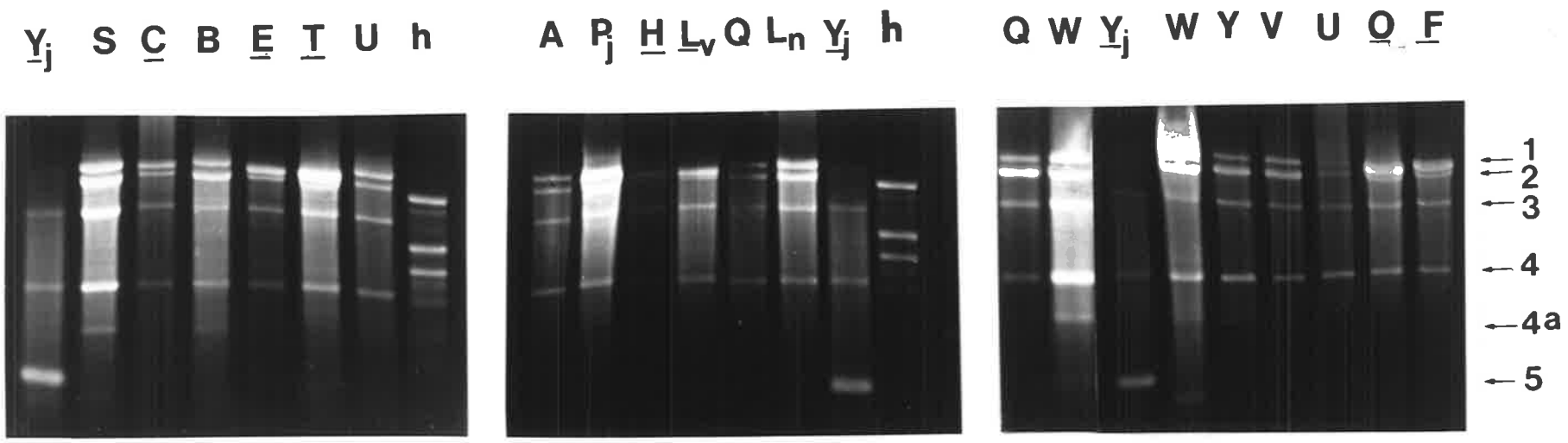
## 2. Viral-RNAs

### a. Pattern of the electrophoretic mobilities of viral-RNA of the subgroup I and II strains

Strains of CMV were distinguished by analysing their RNA patterns in 1.8% agarose gels and electrophoresing in TAE-buffer (see Chapter 2.B.8). One µg of RNA from the same preparation as used in section 3.1 above, was loaded into the wells of a submerged minigel.

The RNAs of all CMV strains separated into 4 major bands, designated as RNAs 1, 2, 3 and 4 (Fig. 3.1). Strains $S_{WA}$, $B_{SA}$, $W_{Tas}$ and $Y_{WA}$ contained RNA 4a. From an analysis of the RNA patterns of 18 strains, only strain $Y_{Jpn}$ was observed to contain a satellite RNA as shown by Zenbayashi *et al.* (1983). Another band with a molecular weight slightly lower than $Y_{Jpn}$ satellite-RNA was shown by strain $W_{Tas}$ and $Y_{WA}$. This band was not considered to be a satellite RNA, because it was only present in such preparation. RNAs 1 and 2 of strains belonging to subgroup II showed a wider

Figure 3.2. The mobilities of RNAs of CMV strains in agarose mini gels with TAE running buffer. The eighteen CMV strains are designated as follows: $\underline{Y_j}$ = $Y_{Jpn}$, S = $S_{WA}$, $\underline{C}$ = $C_{NSW}$, B = $B_{SA}$, $\underline{E}$ = $E_{WA}$, $\underline{T}$ = $T_{WA}$, U = $U_{Qld}$, A = $A_{NSW}$, $\underline{H}$ = $H_{NSW}$, $\underline{Lv}$ = $L_{Vic}$, Q = $Q_{Qld}$, $L_n$ = $L_{NY}$, $P_j$ = $P_{Jpn}$, Y = $Y_{WA}$, W = $W_{Tas}$, V = $V_{NSW}$, $\underline{O}$ = $O_{NSW}$, $\underline{F}$ = $F_{NY}$, h = healthy. Strains from subgroup I are underlined.


Yj S C B E T U h
A Pj H Lv Q Ln Yj h
Q W Yj W Y V U Q F
1
2
3
4
4a
5

separation than RNAs of strains belonging to subgroup I (Fig. 3.2). The repetition of loading RNAs of the same strains in different gels such as $Q_{Qld}$ or $Y_{Jpn}$ was for a marker, and these RNAs had been sequenced. In another experiment using a medium gel apparatus with TBE as the running buffer, the RNAs 1 and 2 from both these subgroups separated at about the same distance. The separation between RNA 1+2, 3 and 4 was wider with TBE buffer than with TAE buffer (data not shown).

**b. Northern blot hybridization**

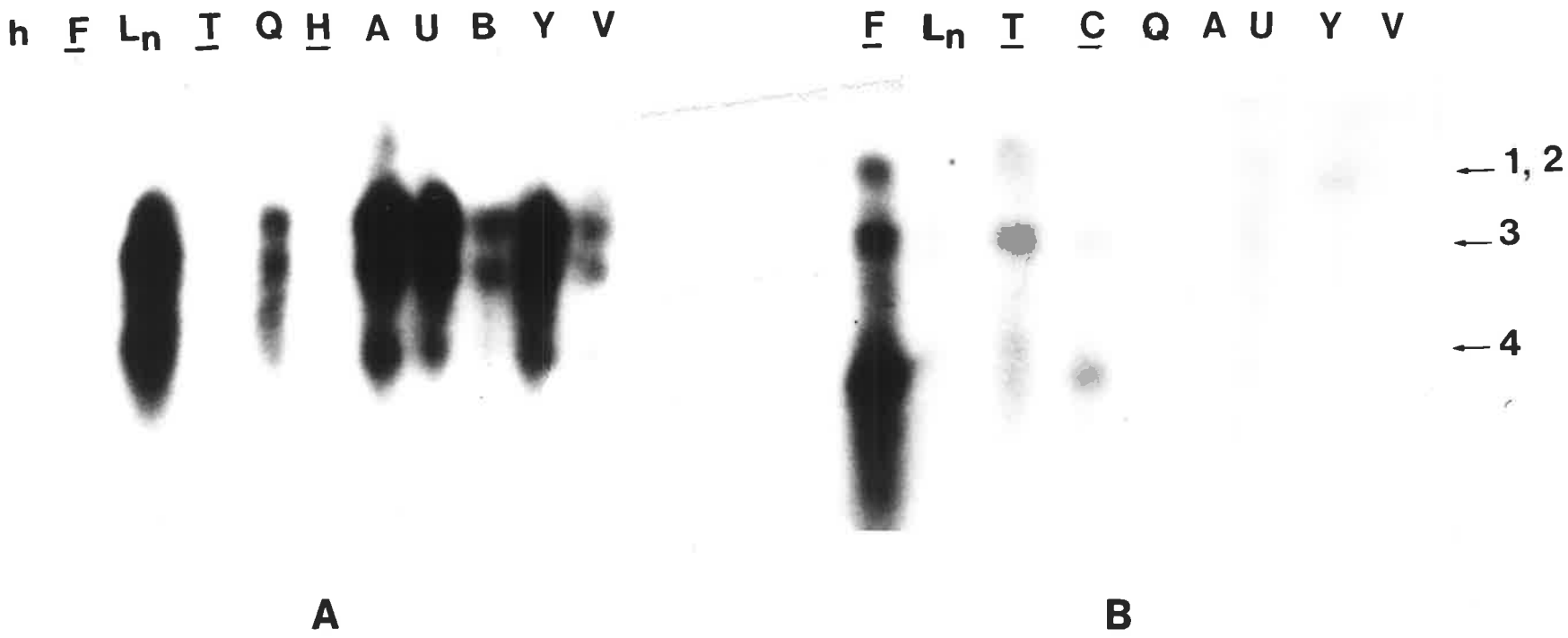
Northern blot hybridization was done as described in Chapter 2.B.7 and 8, using cDNAs to strains $F_{NY}$ and $L_{NY}$.

The results of northern blot hybridization of gels run with either TAE or TBE buffers, are shown in Fig. 3.3 A and B, respectively. The RNAs of strains belonging to subgroup II produced a strong signal when hybridized to $L_{NY}$-cDNA probe (Fig. 3.3 A) whereas strains belonging to subgroup I were not detectable. Conversely, immobilized RNAs of the subgroup I strains produced strong signals with cDNA to $F_{NY}$ but this cDNA did not hybridize with RNAs of the subgroup II strains (Fig. 3.3 B).

## CONCLUSION

1. The CMV strains from Australia, USA and Japan can be separated into two subgroups. Strains $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, $E_{WA}$ fall into subgroup I together with $F_{NY}$, $Y_{Jpn}$, on the basis of hybridization to cDNA of $F_{NY}$ and $H_{NSW}$. Strains $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{NSW}$, $W_{Tas}$ fall into subgroup II together with and $L_{NY}$, $P_{Jpn}$, on the basis of hybridization to cDNA of $L_{NY}$, $V_{Qld}$ and $Y_{WA}$.
2. The RNAs 1 and 2 of subgroup I strains were less well separated than RNAs 1 and 2 of subgroup II strains when subjected to agarose gel electrophoresis using TAE buffer.
3. None of the CMV strains from Australia contained a satellite RNA.

Figure 3.3. Northern blot hybridization patterns from RNAs of CMV strains, F = $F_{NY}$, Ln = $L_{NY}$, T = $T_{WA}$, Q = $Q_{Qld}$, H = $H_{NSW}$, A = $A_{NSW}$, U = $U_{Qld}$, Y = $Y_{WA}$, B = $B_{SA}$, C = $C_{NSW}$, h = control. The RNAs were transblotted by capillarity from agarose gels run with either TAE buffer (blot A) or TBE buffer (blot B) to Hybond-N membranes. Probes used were $L_{NY}$-cDNA (A) and $F_{NY}$-cDNA (B). Strains from subgroup I are underlined.


h F Ln T Q H A U B Y V
F Ln T C Q A U Y V
1, 2
3
4
A
B

# CHAPTER 4. SEROLOGICAL VARIANTS

## INTRODUCTION

Purified particles of CMV tend to be degraded rapidly (Kaper and Gallen, 1971) when prepared without any treatment to stabilize virus conformation (Devergne, 1975; Rao *et al.*, 1982). CMV is known to be poorly immunogenic (Devergne, 1975), but its immunogenicity can be enhanced by fixation of virus particles with either 0.2% formaldehyde (Francki and Habili, 1972) or 0.25 % glutaraldehyde (Rao *et al.*, 1982).

The antigenic specificity of some strains of CMV was investigated in this study using both polyclonal antisera prepared against glutaraldehyde-fixed antigens, and monoclonal antibodies. The objective was to determine whether the strains could be grouped serologically and to compare these groupings with those established by RNA-cDNA hybridization. All virus preparations used as antigens in this work were fixed with 0.25% glutaraldehyde.

## EXPERIMENTAL

### A. Double immunodiffusion tests using virus and antisera prepared against glutaraldehyde-fixed virus.

#### 1. Homologous and heterologous titres of antisera

The titres of antisera to five subgroup I and six subgroup II strains were determined by immunodiffusion against purified preparations of eight subgroup I and ten subgroup II viruses (Table 4.1). The results show that the homologous and heterologous titres of virus strains belonging to the two serogroups are very similar. The titres which differed by SDI=1 cannot be considered as significantly different and only eight of the 187 heterologous titres determined differed by SDI=2. Moreover, three of the heterologous titres with SDI=2 were between strains belonging to the same subgroup. Thus the CMV strains which had been separated into two subgroups by RNA-cDNA hybridization (Fig. 3.1), are antigenically very similar. In spite of the close antigenic relationships,

TABLE 4.1. SEROLOGICAL RELATIONSHIPS AMONG EIGHTEEN CMV STRAINS

| | Test antigen:[a] | Reciprocal of precipitin titre in immunodiffusion tests with antisera to CMV strains: $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $P_{Jpn}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $F_{NY}$ | *256*[b] | 256 | 128 | 512 | 512 | 256*[c] | 256 | 128 | 128 | 64 | 256* |
| | $T_{WA}$ | 256 | *256* | 128 | 512 | 256* | 256* | 256 | 128 | 128 | 64 | 256* |
| | $H_{NSW}$ | 256 | 256 | *128* | 512 | 512 | 128** | 256 | 128 | 128 | 16** | 256* |
| | $O_{Qld}$ | 256 | 256 | 128 | *512* | 512 | 256* | 256 | 128 | 128 | 32* | 256* |
| Subgroup I | $L_{Vic}$ | 256 | 256 | 128 | 512 | 256* | 512 | 256 | 128 | 128 | 32* | 256* |
| | $C_{NSW}$ | 128* | 256 | 64* | 128** | 256* | 256* | 256 | 128 | 128 | 16** | 256* |
| | $E_{WA}$ | 256 | 256 | 128 | 512 | 256* | 512 | 256 | 128 | 128 | 16** | 256* |
| | $Y_{Jpn}$ | 256 | 128* | 128 | 512 | *512* | 512 | 256 | 128 | 128 | 64 | 512 |
| | $Y_{WA}$ | 256 | 256 | 128 | 256* | 512 | *512* | 256 | 128 | 128 | 16** | 256* |
| | $L_{NY}$ | 256 | 256 | 128 | 512 | 512 | 256* | *256* | 128 | 128 | 64 | 512 |
| | $Q_{Qld}$ | 256 | 256 | 128 | 512 | 512 | 256* | 256 | *128* | 128 | 64 | 512 |
| | $A_{NSW}$ | 128* | 64** | 64* | 512 | 512 | 256* | 256 | 128 | *128* | 64 | 512 |
| | $U_{Qld}$ | 256 | 128* | 128 | 512 | 512 | 256* | 256 | 128 | 128 | *64* | 512 |
| Subroup II | $B_{SA}$ | 256 | 256 | 128 | 512 | 256* | 128** | 256 | 128 | 128 | 32* | 256* |
| | $S_{WA}$ | 256 | 256 | 128 | 512 | 256* | 256* | 256 | 128 | 128 | 32* | 256* |
| | $V_{Qld}$ | 256 | 256 | 64* | 512 | 256* | 256* | 256 | 128 | 128 | 32* | 256* |
| | $W_{Tas}$ | 256 | 256 | 128 | 512 | 256* | 256* | 256 | 128 | 128 | 32* | 256* |
| | $P_{Jpn}$ | 256 | 128* | 128 | 512 | 512 | 512 | 256 | 128 | 128 | 64 | *512* |

a All antigen preparations consisted of purified, glutaraldehyde-fixed virus adjusted to a concentration of 500 µg/ml.
b Reciprocals of maximum antiserum dilution producing visible immunoprecipitin lines (figures in italics and underlined, refer to homologous reactions).
c One asterisk indicates a difference of one and two asterisks of two SDIs from the homologous titre.

differences were detected at the junction between homologous and heterologous antigens as spurs on the precipitin lines formed in immunodiffusion tests.

## 2. Relationships between and within CMV strains

The relationships between and within CMV strains were investigated by placing the homologous antigen to the antiserum, designated as a standard antigen, in alternate peripheral wells and placing the heterologous antigens in adjacent wells (Fig. 4.1). For example, when antiserum to $F_{NY}$ was placed in the central well and heterologous antigens were placed in the wells between the homologous antigen wells, the precipitin lines were all confluent when the heterologous strains were $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, $E_{WA}$, and $Y_{WA}$. On the other hand, spurs were detected when the heterologous strains were $L_{NY}$, $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{SA}$, $S_{WA}$, $V_{Qld}$, $W_{Tas}$, and $P_{Jpn}$ (Fig. 4.1). In similar tests with the antiserum to $L_{NY}$, the lines were confluent with virus strains $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, $B_{WA}$, $S_{WA}$, $V_{Qld}$, $W_{Tas}$, $P_{Jpn}$ and $Y_{WA}$, whereas spurs were produced with strains $F_{NY}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{Vic}$, $C_{NSW}$, and $E_{WA}$ (Fig. 4.1). In contrast, antiserum to $Y_{WA}$ showed precipitin lines which were confluent in all tests with the 16 heterologous virus strains (Fig. 4.1). In other tests, antisera to strains $Q_{Qld}$, $A_{NSW}$, $U_{Qld}$, and $P_{Jpn}$ reacted similarly to the antiserum to $L_{NY}$, and antisera to $T_{WA}$, $H_{NSW}$, $O_{Qld}$, and $Y_{Jpn}$ reacted as with the antiserum to $F_{NY}$ (Table 4.2).

These results show that all the subgroup I strains can be distinguished serologically by the use of spurs from those of subgroup II, except $Y_{WA}$ which could not be differentiated from either of the two subgroups.

## 3. Reactivity of viral strains to different standard antigens

In a test similar to that in experiment A.2, antisera from other laboratories were used to investigate whether the $Y_{WA}$ strain belongs to one of the CMV serogroups as described by Devergne and Cardin (1973). Antisera used were to strains S (South Africa), Price's Yellow, R, B, W and To (Habili, 1974), and $Y_{Jpn}$, $P_{Jpn}$ and SA [= $J_{SA}$ from soybean] from Japan. The latter three antisera were prepared against unfixed virus

Figure 4.1. Immunodiffusion tests using antisera to three strains of CMV (centre wells: $f_{ny}$ antiserum to strain $F_{NY}$, $y_{wa}$ to strain $Y_{WA}$ and $l_{ny}$ to strain $L_{NY}$) prepared against preparations of fixed antigens. The standard antigen homologous to the antiserum was placed in the selected wells and the heterologous antigens in the remaining wells.

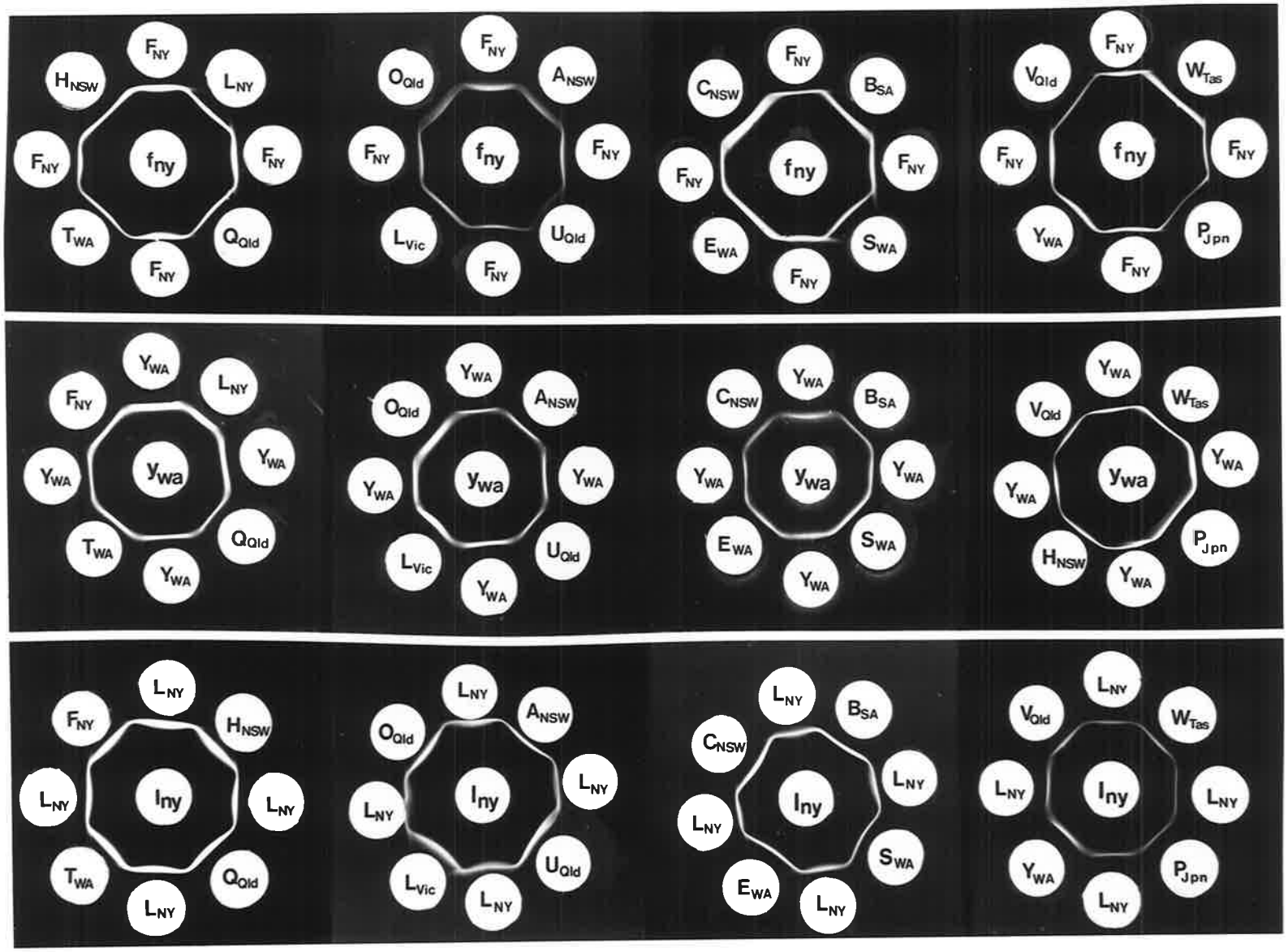

fny
FNY
HNSW
LNY
TWA
QQld
LVic
OQld
ANSW
UQld
CNSW
BSA
EWA
SWA
VQld
WTas
YWA
PJpn
ywa
lny

Table 4.2. Reactivity of seventeen strains of CMV with standard antigen homologous to the antiserum

| Antiserum [a] | Standard antigens [b] | Reaction to antigens of :[b]: | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Subgroup I | | | | | | | | Subgroup II | | | | | | | | |
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ | $P_{Jpn}$ |
| $F_{NY}$ | $F_{NY}$ | -[c] | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + |
| $T_{WA}$ | $T_{WA}$ | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + |
| $H_{NSW}$ | $H_{NSW}$ | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + |
| $L_{NY}$ | $L_{NY}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $Q_{Qld}$ | $Q_{Qld}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $A_{NSW}$ | $A_{NSW}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $U_{Qld}$ | $U_{Qld}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $P_{Jpn}$ | $P_{Jpn}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $Y_{WA}$ | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

[a] Antisera were prepared to the fixed antigens.

[b] All antigens used were prepared by glutaraldehyde fixation.

[c] Confluent lines are indicated by - and spur formation by +.

and only used in this experiment. Antisera to $Y_{Jpn}$ and $P_{Jpn}$ from both fixed (Table 4.2) and unfixed viruses used in the other experiments, were prepared in our laboratory.

The antigens to CMV strains S, Price's Yellow, R, B, W, and To were not available in our laboratory. Therefore, either strain $Q_{Qld}$ or $H_{NSW}$ or $F_{NY}$ or $L_{NY}$, were used as the standard antigens and placed in the selected wells and other heterologous antigens in the remaining wells. As shown in Table 4.3, the heterologous antigens reacted differently to a range of standard antigens. For example, using antisera to strains Price's Yellow, B, W and R, antigen $Y_{WA}$ produced confluent lines to the standard antigen of $Q_{Qld}$. However, spurs were formed between $Y_{WA}$ and $Q_{Qld}$ when antisera to strains To and S were used. When strain $F_{NY}$ was used as the standard antigen against antisera to R and $P_{Jpn}$, the confluent lines were produced against all adjacent antigens. $F_{NY}$ formed spurs to a few antigens belonging to subgroup II in the adjacent wells, using antisera to strains Price's Yellow, B, W, To, and $Y_{Jpn}$ (Table 4.3). Different reactions were also observed between the standard antigen of $F_{NY}$ or $L_{NY}$ with antiserum to $J_{SA}$ (Fig. 4.2). With this antiserum, $F_{NY}$ (standard antigen) produced confluent lines against antigens $T_{WA}$ and $E_{WA}$ in the adjacent wells. $L_{NY}$ (standard antigen) and $Q_{Qld}$ (both antigens belong to subgroup II) did not react with antiserum to $J_{SA}$, while antigens belonging to subgroup I ($T_{WA}$, $E_{WA}$, $H_{NSW}$) produced confluent lines (Fig. 4.2). Using standard antigens homologous to the antisera $Y_{Jpn}$ and $P_{Jpn}$, the CMV strains from Australia and the USA can be serogrouped as shown in Table 4.3.

The above experiment was repeated to further investigate whether CMV strains can be serogrouped by the alternate placement of heterologous antigens to the antiserum. The distinct strains were distinguished by the formation of either spurs or crossing-over of precipitin lines. Results of the experiments with 11 antisera against 11 different virus strains as the standard antigen showed that some antisera readily differentiated many of the strains and some were not able to do so (Table 4.4). For example, antisera to strains $H_{NSW}$ and $T_{WA}$ detected antigenic differences between the majority of 14 strains from Australia and 2 from the USA (Table 4.4). On the other hand, antiserum to $Q_{Qld}$ failed

TABLE 4.3. REACTIVITY OF GLUTARALDEHYDE-FIXED VIRUS PREPARATIONS TO ANTISERA FROM OTHER LABORATORIES

| Antiserum (dilution) | Standard Antigen | Reaction to strains of CMV : Subgroup I | | | | | | | | Subgroup II | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
| *Price's* | $Q_{Qld}$ | +[a] | * | + | + | + | + | + | + | - | * | - | + | - | - | - | - | - | - |
| *Yellow* | $H_{NSW}$ | - | ≠ | - | ≠ | - | - | - | - | - | + | + | - | - | - | + | + | + | + |
| (1/2) | $F_{NY}$ | - | - | - | - | - | - | - | - | + | + | - | - | - | + | + | + | + | + |
| (France) | $L_{NY}$ | - | - | + | - | + | + | + | - | - | - | + | + | + | + | - | - | - | - |
| *B* (1/2) | $Q_{Qld}$ | + | + | + | + | * | + | + | + | - | - | - | - | * | - | - | - | - | - |
| (France) | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | + | + | + | - | + | + | + | + | + |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | + | + | - | - | - | + | + | - | - | - |
| | $L_{NY}$ | + | + | + | + | + | + | + | + | + | - | + | + | + | - | + | + | + | + |
| *W* (1/2) | $Q_{Qld}$ | - | - | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| (France) | $H_{NSW}$ | + | + | + | - | - | + | + | + | + | - | - | - | - | - | - | - | - | - |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | + | - | - | - | + | + | - | - | + | - |
| | $L_{NY}$ | - | - | + | - | + | + | - | - | - | - | - | - | + | - | - | - | + | - |
| *R* (1/2) | $Q_{Qld}$ | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| (France) | $H_{NSW}$ | - | - | - | - | - | ≠ | ≠ | ≠ | - | - | - | - | - | - | - | - | - | - |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |

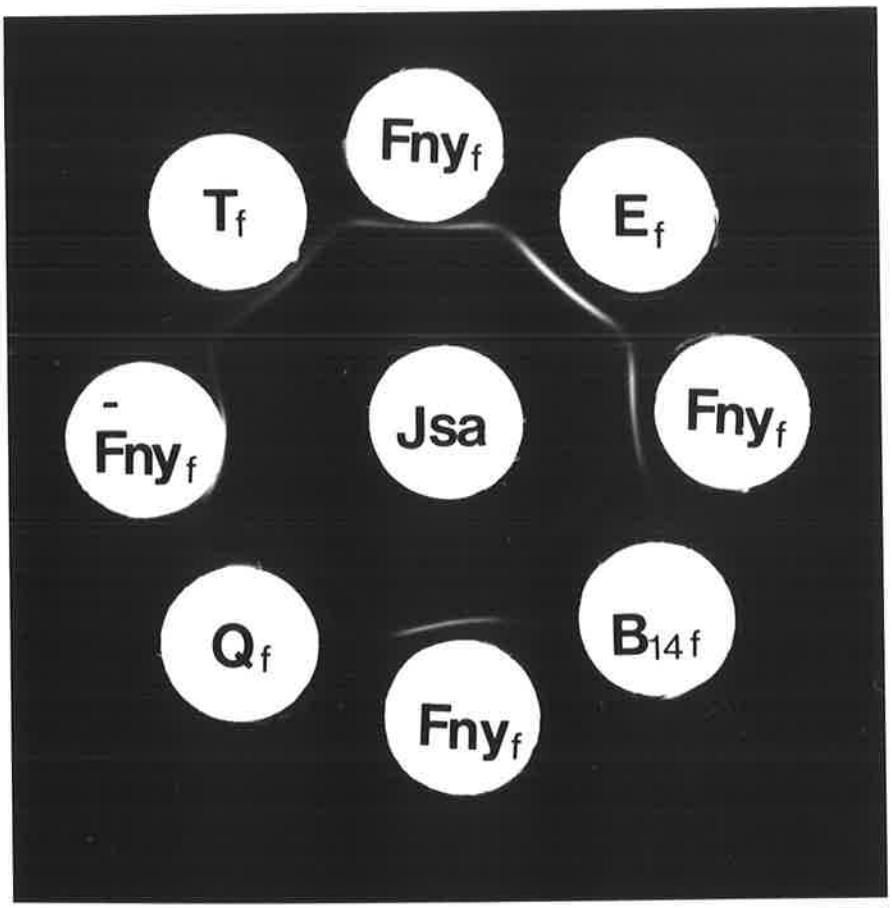
[a] - indicates confluent precipitin lines, + spur formation produced by tested antigens, ≠ spur formation produced by standard antigens

* crossing-over lines

Table 4.3 (continued, p. 2)

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *To* (1/2) (France) | $Q_{Qld}$ | - | - | - | - | - | - | - | - | + | - | - | + | - | + | + | + | - | + |
| | $H_{NSW}$ | + | + | - | - | + | - | - | + | + | - | - | - | - | - | + | - | ≠ | - |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | + | + | - | - | ≠ | ≠ | ≠ | ≠ | - | - |
| | $L_{NY}$ | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| *S* (1/2) (South Africa) | $Q_{Qld}$ | + | + | + | + | + | + | + | + | + | + | - | + | - | - | - | - | + | + |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | + | + | + | - | + | - | - | - | - |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | + | + | + | + | - | - | + | + | + | + |
| | $L_{NY}$ | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | + | - | - |
| *$Y_{Jpn}$*(1/2) (Japan) | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | + | + | + | + | + | + | + | + | + |
| | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + |
| *$P_{Jpn}$*(1/4) | $P_{Jpn}$ | + | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *$SA_{Jpn}$*(1/4) | $F_{NY}$ | - | - | - | - | - | - | - | - | - | + | + | - | - | + | + | + | + | + |
| | $L_{NY}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | - | - | - | - | - | - | - | - | - |

Figure 4.2. Immunodiffusion test using antiserum to CMV-SA (Japan). Two different heterologous standard antigens of either strains $F_{NY}$ or $L_{NY}$ were used instead of strain $J_{SA}$ which was not available in our laboratory. The standard antigen was placed in the selected wells and other heterologous antigens in the remaining wells.


Fny$_f$
T$_f$
E$_f$
Jsa
Fny$_f$
-
Fny$_f$
Q$_f$
B$_{14f}$
Fny$_f$

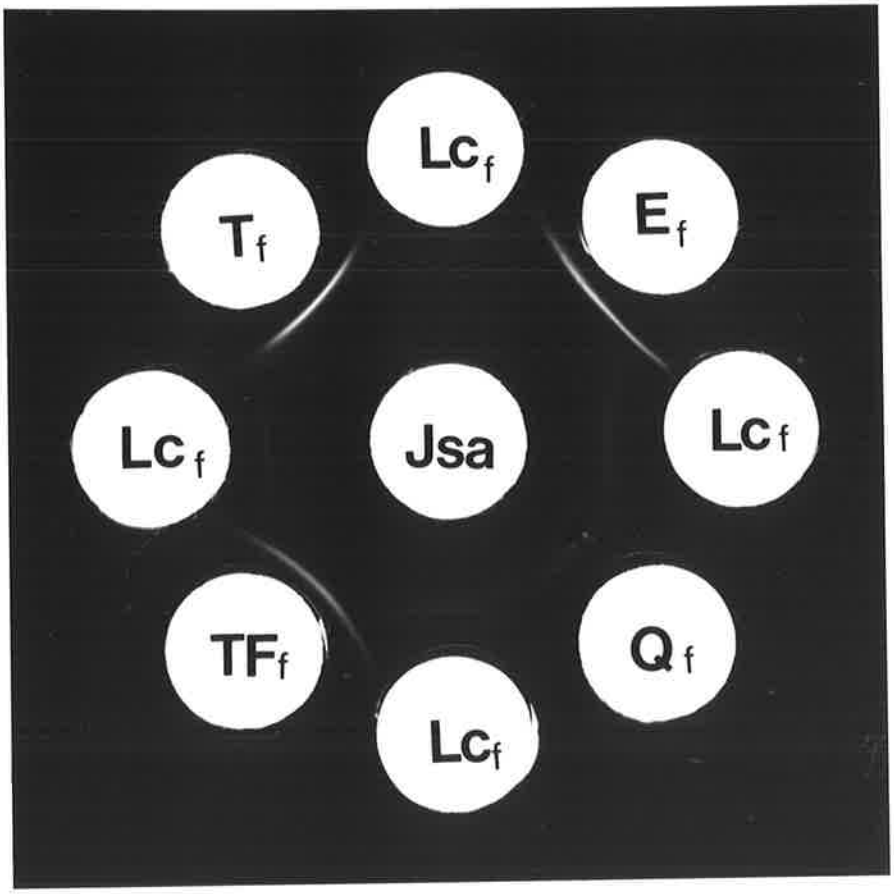

Lc$_f$
T$_f$
E$_f$
Lc$_f$
Jsa
Lc$_f$
TF$_f$
Q$_f$
Lc$_f$

TABLE 4 4. HETEROGENEITY REACTIONS BETWEEN STANDARD FIXED ANTIGENS OF ELEVEN CMV STRAINS

| Antiserum to strain:[b] | Standard Antigen used: [c] | Test antigen used:[a] Subgroup I $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | Subgroup II $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $F_{NY}$ | $H_{NSW}$ | -[d] | - | - | - | - | - | - | ≠ | ≠ | ≠ | - | ≠ | ≠ | ≠ | ≠ | ≠ |
| | $T_{WA}$ | - | - | - | - | - | - | - | ≠ | ≠ | ≠ | ≠ | - | ≠ | ≠ | - | ≠ |
| | $O_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $A_{NSW}$ | ≠ | ≠ | - | - | ≠ | - | - | ≠ | - | - | - | ≠ | - | ≠ | ≠ | ≠ |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | ≠ | - | - | - | - | + | + | + | + |
| | $U_{Qld}$ | - | - | - | ≠ | - | - | - | - | - | + | + | - | - | - | + | + |
| | $P_{Jpn}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | + | + | + | + | + | + | + | + |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $H_{NSW}$ | $F_{NY}$ | - | * | * | - | - | - | - | ≠ | ≠ | ≠ | - | ≠ | ≠ | ≠ | - | ≠ |
| | $T_{WA}$ | * | - | * | - | * | - | - | ≠ | ≠ | ≠ | - | - | ≠ | ≠ | ≠ | ≠ |
| | $O_{Qld}$ | - | - | - | - | - | - | - | - | + | - | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | ≠ | ≠ | ≠ | - | ≠ | ≠ | ≠ | - | ≠ |
| | $L_{NY}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | - | * | * | * | - | - | - | - |
| | $A_{NSW}$ | - | - | * | * | - | - | - | ≠ | + | + | - | + | + | + | - | + |
| | $Q_{Qld}$ | * | * | * | * | * | * | * | ≠ | ≠ | - | * | + | + | + | + | + |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | - | - | ≠ | ≠ | ≠ | ≠ | * | - | + | + | + | - | - | - | + | + |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - |

a The test antigens (see [c] above) were placed in the peripheral wells between those filled with the "standard antigens".

b All antisera were diluted so as to have a homologous titre of 1/16.

c Purified virus preparations of glutaraldehyde-fixed, heterologous CMV strains adjusted to 500 µg/ml were placed in every peripheral well of agar gels as shown in Fig. 2.

d Production of confluent precipitin lines is indicated by -, spurs produced by tested antigens by +, spur produced by standard antigens by≠, and crossing over of the precipitin lines by *.

Table 4.4 (continued, p. 2)

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_{WA}$ | $F_{NY}$ | - | * | - | - | - | - | - | ≠ | ≠ | - | - | - | + | + | + | + | |
| | $H_{NSW}$ | - | * | - | - | - | - | - | ≠ | ≠ | - | + | + | + | + | + | + | |
| | $O_{Qld}$ | * | - | * | - | - | * | * | * | * | ≠ | * | * | + | * | * | * | |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | * | ≠ | ≠ | + | + | * | * | * | * | |
| | $L_{NY}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | - | ≠ | * | * | - | - | - | |
| | $A_{NSW}$ | - | - | - | - | - | - | - | + | + | - | - | + | + | + | + | + | |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | + | + | - | - | - | + | + | + | + | |
| | $U_{Qld}$ | - | - | - | - | - | - | - | + | + | + | - | - | - | - | + | + | |
| | $P_{Jpn}$ | ≠ | - | ≠ | ≠ | - | ≠ | ≠ | ≠ | - | - | * | + | - | + | - | - | |
| | $Y_{WA}$ | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | |
| $O_{Qld}$ | $F_{NY}$ | - | - | - | - | - | - | - | + | + | - | - | - | + | + | - | + | |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| | $T_{WA}$ | - | - | - | - | - | - | - | + | + | - | - | - | + | + | - | + | |
| | $Y_{Jpn}$ | * | * | * | - | * | - | - | - | * | * | * | * | - | - | - | - | |
| | $L_{NY}$ | - | - | - | - | - | - | - | + | - | + | + | + | + | + | * | + | |
| | $A_{NSW}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | - | - | - | - | - | - | - | - | - | |
| | $Q_{Qld}$ | - | - | - | - | - | * | * | + | + | - | - | - | + | + | + | + | |
| | $U_{Qld}$ | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | |
| | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| $L_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | - | |
| | $H_{NSW}$ | - | - | - | - | - | - | ≠ | ≠ | ≠ | ≠ | - | - | - | - | - | - | |
| | $T_{WA}$ | * | - | * | * | * | * | * | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | |
| | $O_{Qld}$ | - | - | - | - | - | - | - | - | + | ≠ | ≠ | ≠ | - | - | - | - | |
| | $Y_{Jpn}$ | * | ≠ | * | ≠ | ≠ | ≠ | ≠ | - | ≠ | * | * | - | ≠ | ≠ | * | * | |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | |
| | $P_{Jpn}$ | - | - | ≠ | - | - | ≠ | ≠ | ≠ | - | - | - | - | - | - | + | - | |
| | $Y_{WA}$ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | ≠ | ≠ | ≠ | - | * | - | - | ≠ | |

Table 4.4 (continued, p. 3)

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $A_{NSW}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $T_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $O_{Qld}$ | - | * | - | - | + | - | - | - | * | ≠ | - | * | - | - | - | - | - |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | * | * | * | - | + | * | ≠ | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Y_{WA}$ | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + |
| $Q_{Qld}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $T_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $O_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $U_{Qld}$ | $F_{NY}$ | - | - | - | - | - | - | - | + | - | + | + | + | + | + | + | + | + |
| | $T_{WA}$ | ≠ | ≠ | - | - | ≠ | - | - | - | - | + | - | + | - | - | - | - | - |
| | $H_{NSW}$ | ≠ | ≠ | - | ≠ | ≠ | ≠ | ≠ | ≠ | + | - | + | + | + | + | + | + | + |
| | $O_{Qld}$ | - | + | - | - | - | - | - | + | - | - | + | + | - | - | - | - | + |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | + | - | ≠ | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Q_{Qld}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Table 4.4 (continued, p. 4)

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $Y_{WA}$ | $F_{NY}$ | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | $T_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $H_{NSW}$ | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | $O_{Qld}$ | + | - | - | - | - | + | - | - | * | - | - | - | + | - | + | + |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | ≠ | ≠ | ≠ | - | - | - | - | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | ≠ | - | ≠ |
| $Y_{Jpn}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + |
| | $T_{WA}$ | - | - | - | - | - | - | - | + | - | - | - | - | + | + | + | + |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $O_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - |
| | $L_{NY}$ | + | - | + | + | - | + | + | + | - | - | + | - | - | - | - | - |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | + | + | - | - | + | - | - | - |
| | $Q_{Qld}$ | - | + | + | - | * | + | * | + | * | - | - | - | + | + | + | + |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $P_{Jpn}$ | - | - | - | - | - | - | - | - | + | + | + | + | + | + | - | - |
| | $Y_{WA}$ | * | * | + | * | * | + | + | - | + | * | + | * | * | + | + | + |
| $P_{Jpn}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $T_{WA}$ | - | - | ≠ | - | - | - | - | - | - | - | - | + | - | - | - | - |
| | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $O_{Qld}$ | - | ≠ | - | ≠ | - | ≠ | - | ≠ | - | - | - | - | + | - | - | - |
| | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $U_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

to detect any differences between any of strains studied, irrespective of whether they belonged to subgroups I or II as determined by RNA-cDNA hybridization (Fig. 3.1). When strain $Y_{WA}$ was used as a heterologous standard antigen against antisera to strains $L_{NY}$, $A_{NSW}$, $T_{WA}$, and $H_{NSW}$, each strain showed different reactivity to the adjacent antigens. The pattern of precipitin lines in Table 4.4 shows that serogrouping of CMV strains could not be achieved by this method.

## B. Enzyme-linked Immunosorbent Assay (ELISA)

### 1. Double antibody sandwich direct-ELISA (DAS-ELISA) using polyclonal antibodies

The previous results with immunodiffusion tests were re-evaluated in this study in which the same CMV strains were serotyped by the DAS-ELISA technique. Antibodies (IgG) prepared against glutaraldehyde-fixed preparations of $F_{NY}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{NY}$, $U_{Qld}$, $Q_{Qld}$, $A_{NSW}$, and $Y_{WA}$ were used in this experiment. Direct DAS-ELISA was done as described in Chapter 2.B.12.b. The degree of cross-reactivity was measured by the strength of the reaction. The closely related strains were expected to have a strong reaction to an antibody of the same subgroup, whereas the distantly related strains were expected to react weakly (Table 4.5).

IgG from $F_{NY}$ (subgroup I) reacted more strongly with its homologous virus than with a preparation of $L_{NY}$ (subgroup II), whereas preparations of $Y_{WA}$ (subgroup II) were shown to have intermediate reactivity (Fig 4.3 A). In similar experiments with IgG from $L_{NY}$, strain $F_{NY}$ reacted poorly, but the reaction with $Y_{WA}$ was similar to that with the homologous antigen (Fig 4.3 B). In contrast, IgG from $Y_{WA}$ (Fig. 4.3 C) failed to distinguish among them, all strains tending to fall into one serogroup. Other IgG's from subgroup I ($T_{WA}$, $H_{NSW}$, $O_{Qld}$ ) showed results similar to the IgG from $F_{NY}$. IgG's from subgroup II ($L_{NY}$, $U_{Qld}$, $Q_{Qld}$, $A_{NSW}$) behaved similarly to the IgG from $L_{NY}$ (Table 4.5). The heterologous antigens from subgroup I reacted weakly with IgGs from subgroup II, whereas the stronger reaction from subgroup I showed that those two subgroup strains could be distinguished. Similar results were obtained when using IgGs

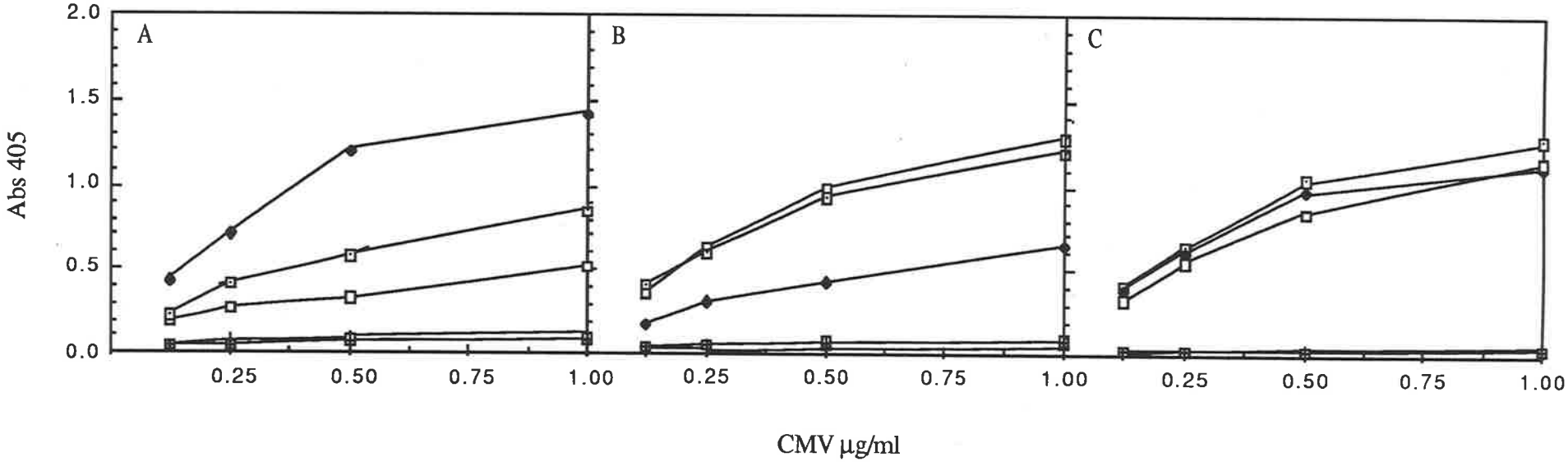



Figure 4.3. Serogrouping of CMV strains in DAS-ELISA with polyclonal IgG to strains $F_{NY}$ (A), $L_{NY}$ (B) and $Y_{WA}$ (C). Plates were coated with a mixture of 1µg/ml of each IgG, then incubated with a twofold dilution series of purified virus from fixed preparations of $F_{NY}$ (-◆--), $L_{NY}$ (-□--) and $Y_{WA}$ (-⊡--). Preparations of tobacco masaic virus (--+--) and tomato aspermy virus (--■--) were used as negative control antigens.

TABLE 4.5. REACTIVITY OF SOME CMV ANTISERA WITH SOME CMV STRAINS IN DAS ELISA

| | Virus strain [a] | Absorbance at 405 nm with IgG from antisera to: [b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ |
| Subgroup I CMVs | $F_{NY}$ | +++ | +++ | +++ | ++++ | +++ | ++ | ++ | ++ | + |
| | $T_{WA}$ | +++ | +++ | +++ | ++++ | +++ | ++ | ++ | + | + |
| | $H_{NSW}$ | +++ | +++ | +++ | ++++ | +++ | ++ | ++ | ++ | + |
| | $O_{Qld}$ | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ | + |
| Subgroup II CMVs | $Y_{WA}$ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ |
| | $L_{NY}$ | ++ | + | ++ | ++ | +++ | +++ | +++ | +++ | ++ |
| | $Q_{Qld}$ | ++ | + | + | + | +++ | +++ | +++ | ++ | ++ |
| | $A_{NSW}$ | ++ | + | ++ | + | +++ | +++ | +++ | +++ | ++ |
| | $U_{Qld}$ | ++ | + | + | + | +++ | ++ | +++ | ++ | ++ |
| Control antigens | TAV | - | - | - | - | - | - | - | - | - |
| | TMV | - | - | - | - | - | - | - | - | - |

a Purified virus preparations adjusted to a concentration of 1 µlg/ml were used.

b - indicates absorbance <0.1, + absorbance <0.5, ++ absorbance 0.5-1.0, +++ absorbance 1.0-1.5, ++++ absorbance > 1.5 after 30 min hydrolysis

from subgroup I, in which the subgroup II strains reacted more weakly than the subgroup II strains.

## 2. Triple Antibody Sandwich Indirect-ELISA (TAS-ELISA)

Experiments were done to determine whether any of the monoclonal antibodies could be used to separate the CMV strains into the same serogroups as the results in the section 4.A.2.

### 2.a.TAS-ELISA using monoclonal antibodies αL2a, αL2b, αL1,

The monoclonal antibodies αL2a, αL2b, αL1 have been used to detect viruses in lettuce in New York, where isolate $L_{NY}$ (syn. LsS or $L_2$) originated (Provvidenti *et al.*, 1980; Edwards *et al.*, 1983). Isolate $L_{NY}$ differs from isolate LsR (=$L_1$) by its ability to infect *Lactuca saligna* (Provvidenti *et al.*, 1980). MAb αL1 is specific to isolate $L_1$, whereas MAb's αL2a and αL2b only recognise isolate $L_2$ (Rist and Lorbeer, 1989).

Microtitre plates were coated with 100 µl of a mixture containing 1 µg/ml of IgG of each of the polyclonal antisera to $F_{NY}$, $L_{NY}$ and $Y_{WA}$. The ELISA procedure was done as described in Chapter 2.B.13 a. In this experiment, the polyclonal antibodies (PAb's) from $F_{NY}$ and $L_{NY}$ were used for comparison with the MAb's. After 20 min of hydrolysis with the substrate solution, all antigens reacted with both PAb's, but not with MAb's (Table 4.6). PAb's distinguished all strains mainly to 2 serogroups, where the strain $Y_{WA}$ fell in between these serogroups. After a hydrolysis time of 120 min, both MAb's αL2b and αL2a reacted with some strains. Only the MAb αL1 separated strains belonging subgroup I from subgroup II (Table 4.6).

### 2.b. TAS-ELISA using monoclonal antibodies specific for strains in serogroups DTL, ToRS, and Co

Three MAb's (76.1, 42.3, 3.4) which were identified as IgM and another two (34.2, 21.4) were identified as IgG 2a (Porta *et al.*, 1989). These were used to confirm the results in sections 4.A 3 and 4.B.1. MAb's 34.2 and 42.3 were found by Porta *et*

TABLE 4.6. REACTIONS OF 16 CMV STRAINS TO MONOCLONAL ANTIBODIES αL2a, αL2b AND αL1 IN TAS-ELISA

| Test antigen[a] (1/400) | MAb αL2b[b] (1/200) | | MAb αL2a (1/200) | | MAb αL1 ($6x10^3$) | | PAb $F_{NY}$ [c] ($2x10^3$) | | PAb $L_{NY}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | -[d] | + | - | + | - | + | - | + | - | + |
| $F_{NY}$ | - | - | - | - | + | +++ | +++ | +++ | - | - |
| $T_{WA}$ | - | - | - | - | + | +++ | +++ | +++ | - | - |
| $H_{NSW}$ | - | - | - | - | + | ++ | +++ | +++ | - | - |
| $O_{Qld}$ | - | - | - | - | + | +++ | ++ | +++ | - | - |
| $L_{Vic}$ | - | - | - | - | ++ | + | ++ | +++ | - | - |
| $C_{NSW}$ | - | - | - | - | +++ | +++ | ++ | +++ | - | - |
| $E_{WA}$ | - | - | - | - | +++ | +++ | ++ | +++ | - | - |
| $Y_{WA}$ | ++ | + | ++ | - | + | - | ++ | ++ | ++ | +++ |
| $L_{NY}$ | + | - | + | - | - | - | - | - | +++ | +++ |
| $Q_{Qld}$ | ++ | ++ | ++ | ++ | - | - | - | - | +++ | +++ |
| $A_{NSW}$ | - | - | - | - | - | - | - | - | ++ | +++ |
| $U_{Qld}$ | + | - | - | - | - | - | - | - | ++ | +++ |
| $B_{SA}$ | + | - | ++ | - | - | - | - | - | ++ | +++ |
| $S_{WA}$ | ++ | ++ | ++ | ++ | - | - | - | - | +++ | +++ |
| $V_{Qld}$ | - | - | - | - | - | - | - | - | ++ | +++ |
| $W_{Tas}$ | ++ | + | +++ | ++ | - | - | - | - | ++ | +++ |
| VTAV | - | - | - | - | - | - | - | - | - | - |
| TMV | - | - | - | - | - | - | - | - | - | - |

a Test antigens were prepared against glutaraldehyde and adjusted to a concentration of 1µg/ml

b With MAb's; - indicates an $Abs_{405} < 0.25$, + was 0.25-0.50, ++ was 0.50 - 7.5, and +++ > 0.75 after 120 min hydrolysis of substrate

c PAb's were used as the positive control to the MAb's with - indicates an $Abs_{405} < 0.25$, + was 0.25-0.75, ++ was 0.75-1.50 and +++ > 1.50 after 20 min hydrolysis of substrate

d - plate uncoated and + plate pre-coated with the antibody mixture of 1 µg/ml of each of the polyclonal antibodies to $F_{NY}$, $L_{NY}$ and $Y_{WA}$.

*al.* (1989) to be specific to CMVs belonging to the DTL serogroup (subgroup I) and MAb 21.4 to the ToRS serogroup (subgroup II). MAb's 3.4 and 76.1 have very similar specificity, and both recognise strain Co. This strain is antigenically distinct from members of both the DTL and ToRS serogroups (Devergne and Cardin, 1970).

The indirect TAS-ELISA procedure was as described in the section 4.B.2.a. The mixture of polyclonal IgG from $F_{NY}$, $L_{NY}$ and $Y_{WA}$ each at 1 µg/ml was used for coating. Dilution of MAb's was as recommended by Porta *et al.* (1989). Two conjugates were used with concentrations as recommended by the supplier. One plate was treated with goat anti-mouse IgG AP-ase conjugate, and the other plate was treated with goat anti- mouse IgM AP-ase conjugate (Sigma, Co). The antigen-antibody complex was hydrolysed for 30 min.

Table 4.7 shows that with the appropriate AP-ase conjugate, MAb 34.2 reacted more strongly to subgroup I strains whereas it reacted weakly with strains $V_{Qld}$. MAb 21.4 reacted to subgroup II strains, and MAb 3.4 recognised all strains belonging to subgroup I and II. MAb 76.1 which is more specific for strain Co, failed to react with subgroup I and II strains. MAb 21.4 was found to be useful in distinguishing strains of the two subgroups as it reacted strongly with all subgroup II but not with the subgroup I strains. No strains of subgroup I and II were recognized by any of MAb's from class IgG when incubated with anti-mouse IgM AP-ase conjugate. In contrast, MAb 3.4 (IgM) reacted with both subgroup I and II strains using either anti-mouse IgM or IgG AP-ase conjugate. The MAb 42.3 only reacted with the subgroup I strains using either anti-mouse IgM or IgG AP-ase conjugates.

### 2.c. Plate trapped antigen (PTA) indirect-ELISA with MAbs used in the section 4.B.2.a and b above

Tables 4.6 and 4.7 show that not all strains of CMV reacted with MAbs. This experiment was done to test whether the polyclonal IgG's which was used for coating

TABLE 4.7. REACTIONS OF 16 CMV STRAINS TO MONOCLONAL ANTIBODIES 34.2, 21.4, 76.1, 42.3, 3.4 IN TAS-ELISA

| Test antigen[a] | Monoclonal antibodies : 34.2 (IgG2a) ($10^{-4}$)[b] | | 21.4 (IgG2a) ($10^{-5}$) | | 76.1 (IgM) ($10^{-4}$) | | 42.3 (IgM) (1/50) | | 3.4 (IgM) (1/30) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | IgG [c] | IgM | IgG | IgM | IgG | IgM | IgG | IgM | IgG | IgM |
| $F_{NY}$ | ++[d] | - | - | - | - | - | - | - | + | +++ |
| $T_{WA}$ | ++ | - | - | - | - | - | + | - | - | +++ |
| $H_{NSW}$ | ++ | - | - | - | - | - | ++ | + | + | +++ |
| $O_{Qld}$ | ++ | - | - | - | - | - | + | - | + | +++ |
| $L_{Vic}$ | ++ | - | - | - | - | - | + | + | ++ | +++ |
| $C_{NSW}$ | ++ | - | - | - | - | - | ++ | ++ | ++ | +++ |
| $E_{WA}$ | +++ | - | - | - | - | - | ++ | + | ++ | +++ |
| $Y_{WA}$ | - | - | +++ | - | - | - | - | - | ++ | +++ |
| $L_{NY}$ | - | - | +++ | - | - | - | - | - | + | +++ |
| $Q_{Qld}$ | - | - | +++ | - | - | - | - | - | + | ++ |
| $A_{NSW}$ | - | - | +++ | - | - | - | - | - | - | ++ |
| $U_{Qld}$ | - | - | ++ | - | - | - | - | - | + | ++ |
| $B_{SA}$ | - | - | ++ | - | - | - | - | - | + | +++ |
| $S_{WA}$ | - | - | ++ | - | - | - | - | - | + | +++ |
| $V_{Qld}$ | + | - | +++ | - | - | - | - | - | ++ | + |
| $W_{Tas}$ | - | - | ++ | - | - | - | - | - | + | +++ |
| VTAV | - | - | - | - | - | - | - | - | - | - |
| TMV | - | - | - | - | - | - | - | - | - | - |

a All antigen preparations consisted of glutaraldehyde fixed virus which had been adjusted to a concentration of 1 µg/ml

b MAb's as the second antibodies were diluted as recommended by the supplier

c IgG and IgM goat-anti mouse AP-ase conjugates used as the third antibodies

d + indicates absorbance ≥ 0.500, ++ was 0.500-1.500 and +++ ≥ 1.500

the plate produced non-specific reactions to those MAb's. The method used in this experiment was as described in Chapter 2.B.13.b.

### 2.c.1. Reactions with MAbs αL2a, αL2b and αL1

With this method, MAb's αL2b and αL2a failed to react with strains belonging to subgroup I, and not all strains belonging to subgroup II can be recognized by these two MAb's (Table 4.6 shown by [d]). MAb α L1 was more specific to the subgroup I strains than those two MAbs.

Strains $A_{NSW}$ and $V_{Qld}$ were not recognized by all MAb's, but these strains reacted strongly with both PAb's. Both MAb's αL2b and αL2a reacted positively with $L_{NY}$, $U_{Qld}$ and $B_{SA}$ (subgroup II), whereas these strains reacted negatively in the plate coated with polyclonal IgG's (Table 4.6). None of the viruses used as negative control antigens (TMV-U1 and TAV-V) reacted to all MAb's and PAb's. MAb's aL1 which were expected to react only with isolate L1 [subgroup II] (Rist and Lorbeer, 1989), reacted only with subgroup I strains in the experiments 4.B.2.a and 4.B.2.c.1 (Table 4.6).

### 2.c.2. Reactions with MAbs 76.1, 42.3, 3.4

The fixed antigens tested were $T_{WA}$ and $L_{Vic}$ (subgroup I), $B_{SA}$ and $V_{Qld}$ (subgroup II). Incubation with MAb 76.1 (1/1000 dilution), MAb 42.3 (1/50 dilution) and MAb 3.4 (1/30 dilution) was for 3h. The dilution of goat anti-mouse IgG or IgM AP-ase conjugates were according to the supplier. $Abs_{405}$ was determined at 25 min after adding the substrate solution.

Both MAb 42.3 and MAb 3.4 reacted with all CMV strains tested, but MAb 76.1 did not recognise these strains although its concentration was increased to 1/1000 (Table 4.8). MAb 42.3 and MAb 3.4 reacted to 4 strains of CMV tested with either anti-IgG or IgM AP-ase conjugates, while the 76.1 did not react with these strains. With MAb 3.4, these strains reacted more strongly with the anti-IgM AP-ase conjugate than with anti-

IgG enzyme conjugate. With MAb 42.3, these strains reacted weakly with both anti-IgG and IgM AP-ase conjugates (Table 4.8).

Another experiment was done to further investigate whether the non-specific reaction was produced by MAbs from class IgM (see Chapter 4.B.2.b) when using anti-IgG AP-ase conjugate (Table 4.6).The plate wells were coated directly with the MAbs for 3 h, then conjugated with either anti-mouse IgG or IgM AP-ase. After 25 min hydrolysis with the substrate solution, MAb 76.1 did not react with these conjugates. MAb 42.3 reacted very weakly and MAb 3.4 reacted less strongly with anti-IgG AP-ase conjugate than with anti-IgM AP-ase conjugate.

TABLE 4.8. REACTION OF BOUND VIRUS WITH MONOCLONAL ANTIBODIES FROM CLASS IGM IN PTA INDIRECT-ELISA

| | MAbs from class IgM | | | | | |
|---|---|---|---|---|---|---|
| | 76.1 (1/1000 dilution) | | 42.3 (1/50 dilution) | | 3.4 (1/30 dilution) | |
| Virus strain | IgG * | IgM | IgG | IgM. | IgG | IgM |
| $T_{WA}$ | - ** | - | + | + | ++ | ++++ |
| $L_{Vic}$ | - | - | + | + | ++ | ++++ |
| $B_{SA}$ | - | - | + | + | ++ | ++++ |
| $V_{Qld}$ | - | - | + | + | ++ | ++++ |

* Anti-mouse IgG or IgM AP-ase conjugate

** $Abs_{405}$ was determined at 25 min after adding the substrate solution, - was < 0.100, + was 0.100- 0.500, ++ was 0.500-1.000 and ++++ was 1.500 -2.000

The results of experiments in section 4.B.2 b and 4.B.2.c.2 show that except for MAb 76.1, MAb's from class IgM did cross-react with anti-mouse IgG AP-ase conjugate.

## CONCLUSION

1. When standard antigen homologous to antisera was used in immunodiffusion tests, all strains of CMV, except $Y_{WA}$, were assigned to subgroups I or II. This correlated with the subgrouping from RNA-cDNA hybridization. Strain $Y_{WA}$ fell between these two subgroups. Results similar to this were also obtained by direct DAS-ELISA with polyclonal IgG to strains $F_{NY}$, $T_{WA}$, $H_{NSW}$, $O_{Qld}$, $L_{NY}$, $U_{Qld}$, $Q_{Qld}$, $A_{NSW}$, and $Y_{WA}$.
2. The combined placement of heterologous and homologous antigens in gel immunodiffusion tests could not be used for serogrouping CMV strains.
3. Monoclonal antibodies αL1 from the USA and 21.4 from France separate CMV strains into two serogroups and strain $Y_{WA}$ is more closely related to the strains belonging to subgroup II.
4. None of the CMV strains from Australia and the USA reacted with MAb 76.1, indicating that none belonged to the Co serotype.

# CHAPTER 5. BIOLOGICAL VARIATION IN CMV

## INTRODUCTION

The host range and symptoms of CMV have been studied for purposes such as virus epidemiology (Quiot, 1980, Quiot *et al.*, 1983; Jones and Kirdy, 1990), the economic effects of CMV on commercially important hosts (Provvidenti, 1976; Hack, 1986; Davis and Hampton, 1986; Rist and Lorbeer, 1989) and for distinguishing virus strains (Lovisolo and Conti, 1969; Marrou, *et al.*, 1975).

The experiments described in this chapter were done to determine the plant species which would be the most useful for distinguishing and characterizing strains of CMV, and for the study of variation in the reaction of plants at different times of year. The study of distribution of symptoms in relation to virus distribution in *Medicago* spp., and whether some strains of CMV were able to induce necrosis in lupin, are also described in this chapter.

## EXPERIMENTAL

### 1. Host range and variation in symptoms

The symptoms described in this study were recorded after they had developed fully (Table 5.1). The terminology according to Bos (1970) was used.

Peanut and soy bean were immune to CMV. These species were used to determine whether the $Y_{WA}$ strain was related to CMV as its serological reaction had suggested that it belongs to other member of the cucumoviruses, e.g. PSV (see Chapter 4, Table.4.2).

Although the symptoms produced were variable, *N. tabacum* cv. Xanthi n.c., *N. edwardsonii*, and *Capsicum frutescens* cv. Giant Bell could be useful as indicator plants for separating CMV of subgroup I from subgroup II (Table 5.1). In *N. tabacum* Xanthi n.c., subgroup I strains induced severe greening mosaic, crinkle, leaf distortion (Fig.5.1 C,D), and subgroup II strains induced mild mosaic (Fig. 5.1 A,B). Symptoms produced on *N. glutinosa* was less useful for this purpose. For example, only some strains of subgroup I induced a greening mosaic [i.e. shown in Fig. 5.1 G,H,K,L] and subgroup

Figure 5.1. Symptoms produced on two indicator species which were suitable for separating CMV strains into subgroups I and II. *Nicotiana tabacum* cv. Xanthi n.c. showed a mild chlorotic mosaic with strains $Q_{Qld}$ and $L_{NY}$ (A, B); strain $O_{Qld}$ induced greening mosaic and leaf distortion (C); strain $F_{NY}$ induced a greening mosaic, wrinkle, leaf distortion and stunting (D). *Nicotiana glutinosa* showed a chlorotic mosaic and mild interveinal necrosis with strains $V_{Qld}$ (E) and $Y_{WA}$ (F); strains $T_{WA}$ (G), $F_{NY}$ produced a fernleaf symptom (H), $L_{Vic}$ (K) and $H_{NSW}$ (L) induced a greening mosaic; a tip necrosis induced by strain $A_{NSW}$ (I) which then recovered by producing new leaf tips (J).

Subgroup II

Subgroup I

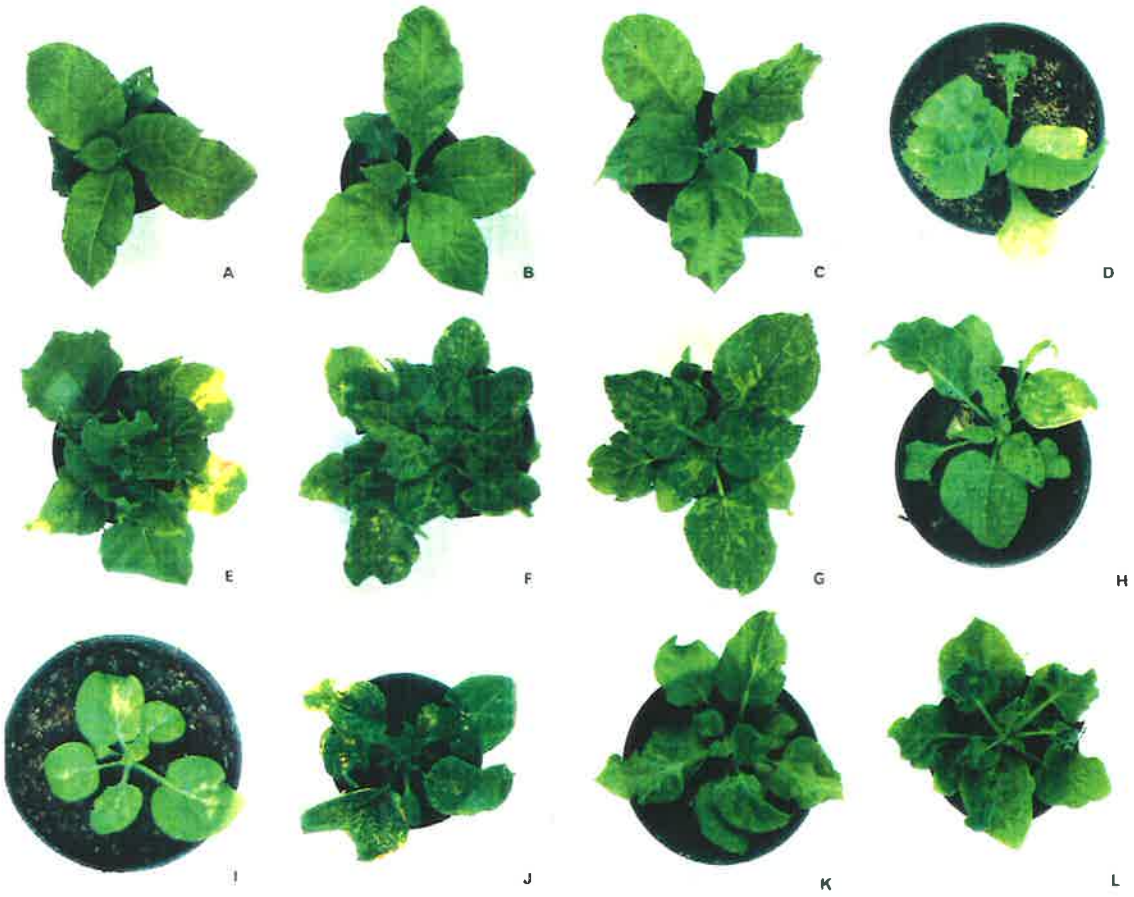


TABLE 5.1. REACTIONS OF A SELECTED HOST RANGE TO STRAINS OF CMV

| Isolate / Host | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Solanaceae** | | | | | | | | | | | | | | | | |
| *Nicotiana glutinosa* | 1.5 | 1 | 1.5 | 2 | 1.5 | 1.5 | 1 | 2.3 | 2.3 | 1.2 | 2.4 | 2.3 | 2.3 | 2.3 | 2 | 2 |
| *N. clevelandii* | 1 | 1 | 1 | 1.6 | 1 | 1 | 1 | 1 | 1.6 | 2 | 2 | 3 | 3 | 1 | 1.6 | 1 |
| *N. edwardsonii* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 | 3.4 |
| *N. tabacum* cv. White Burley | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *N. sylvestris* | 1 | 2*.3 | § | 2*.3 | § | § | § | § | § | 6 | 1 | § | 1 | 6 | § | 6 |
| *N. rustica* | 1*.10 | 1 | 1 | 1.2 | 1.2 | 1 | 1 | 2 | 2 | 2 | 2 | 1.2 | 2 | 2 | 2 | 2 |
| *N. tabacum cv.* Xanthi *nc* | 1*.10 | 1* | 1 | 1 | 1 | 1*.10 | 1* | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| *Lycopersicon esculentum* cv. Rutgers | 1.5 | 1.5 | 1.5 | 1.5* | 1.5* | 1.5 | 1.5 | 1.5* | 1.5 | 5 | 1.5 | 1.5 | 1.5* | 1.5 | 1.5 | 1.5 |
| *Capsicum frutescens* cv. Giant Bell | 2.3* | 2.3 | 2.3 | 2.3 | 2.3 | 2.3* | 2.3 | 2.3* | 1.3 | 1.3 | 1.3 | 1.3 | 1.3* | 1.3 | 1.3 | 1.3 |
| *Datura stramonium* | 1.5* | 6 | 1 | § | § | 1* | 1 | 6 | 1 | 1* | 1.5 | 2 | 1 | 1.4 | 1 | 1 |
| **Amaranthaceae** | | | | | | | | | | | | | | | | |
| *Gomphrena globosa* | 1 | 6.2 | 6.2 | - | - | 6.1 | 1* | 6.2 | 6 | - | 6.1 | 6.1 | 6.1 | 6 | 6.2 | - |
| **Asteraceae** | | | | | | | | | | | | | | | | |
| *Lactuca sativa* cv. Salinas | 1* | 1 | 1 | § | § | 1* | 1 | § | 2* | - | 2.8 | - | 2*.8 | 2*.8 | - | - |
| **Poaceae** | | | | | | | | | | | | | | | | |
| *Zea mays* cv. Iochief | 2*.7 | - | 2* | - | - | 2* | - | - | - | - | 2*.7 | - | - | - | - | - |
| **Cucurbitaceae** | | | | | | | | | | | | | | | | |
| *Cucumis sativus* cv. Supermarket | 6.1 | 8 | 6.1 | 8. | 1.§ | 1.8 | 8 | 1.§ | 1.§ | 8 | 2 | 8 | 6 | 6.8 | 1 | 1 |

Symptoms produced : - Immune; § Symptomless infection; * Severe stunting; 1 Green mosaic & leaf distortion; 2 Chlorotic mosaic & leaf distortion; 2a Chlorosis and leaf distortion; 3 Vein chlorosis; 4 Vein or apical necrosis; 5 Fernleaf symptom; 6 Local lesion without systemic infection; 7 Plant died and positive by back inoculation to *N. glutinosa*. 8 Yellow spotting; 9 Leaf curling ; 10 Epinasty; 11 Rosetting; 12 Interveinal chlorosis and leaf distortion.

Table 5.1 (ccontinued, p.2)

| Isolate / Host | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fabaceae** (Leguminosae) | | | | | | | | | | | | | | | | |
| *Arachis hypogea* cv. Early bunch | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Glycine max* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Lens esculenta* cv. Laird | 2a | 2a | 2a.9 | 2a.9 | 2a.9* | 2a | - | - | 2a.9* | 2a | 2a | - | - | - | - | 2.9 |
| *Vicia faba* cv. Aquadulce | 6 | 6 | 6 | 6 | - | 6 | 2 | - | - | - | - | - | 6 | - | - | - |
| *Vigna sinensis* cv. Black eye | 1* | 1 | 1 | 1 | 3 | 3 | 12 | 6 | 3 | 6 | 3.6 | 3 | 3.6 | 3.6 | 3 | § |
| *Phaseolus vulgaris* cv. Hawkesbury Wonder | 1 | §.1 | 1 | §.12 | § | 1 | 3 | - | 1 | §.1 | 12 | - | §.1 | 1 | §.1 | - |
| *Lupinus angustifolius* cv. | | | | | | | | | | | | | | | | |
| Illyarrie | 7.10.11* | 2a.11* | 2a.10.11* | 2a.8.7* | 2a.10 | 2a.10.11* | 2a.11* | 2a*.10.11 | 2a.7.8.11 | - | 2a.8.10.11 | - | 2a*.7.8.10.11 | 2a.10 | - | - |
| Gungurru | 2a.9 | 2a.9 | 9.7 | 2a.11* | - | 2a.9* | 2a.9* | 9.11* | 2a.7 | § | 7 | - | 2a.7 | 2a.7 | 2a.7 | 2a.9 |
| Warrah 408 | 7.2a.9* | 2a.9.11* | 2a.11 | 2a.9 | - | 2a.9.* | 2a.8.9 | 2a.8.9. | 2a* | - | 2a.8*.7 | - | 2a.8.11 | 2a.7 | - | - |
| Warrah 437 | 7.9 | - | 9.11 | 2a.9.11* | - | 2a.9.11* | 2a.9* | 9.11* | 2a.11 | 7 | 2a.7 | - | 2a.11.7 | 2a.11 | - | 2a.7 |
| Wandoo | 2a.1.9* | 2a.7 | 7 | 2a.9* | 9.7 | 2a.9* | 2a.9* | 9.11* | 2a.7 | § | 8.9.7 | 2a.7 | 2a.11 | 2a.11* | 8* | 2a.8 |
| Yandee | 2a.9.11* | 2a.8.11* | 9.7 | 2a.9 | - | 2a.8.11* | - | 2a.8.9.11* | 2a.*8.7 | - | 9.7 | 2a.7 | 2a.8* | 2a.9* | - | - |
| Yorrel | 9.11* | 9.7 | 7 | 2a.9* | 2a.9 | 2a.8.9.11 | 2a* | 2a* | 2a* | 2a* | 8*.11.7 | - | 2a.11*7 | 2a.11* | - | 2a.8 |
| Danja 432 | 2a.11*.7 | 7 | §.8.11*.7 | 2a.9.11* | - | 2a.8.11.7 | 2a.8.11* | - | - | - | - | - | - | - | - | - |
| Danja | 9.7 | 8.7 | 2a.7 | 2a.7.11 | 9 | 2a.8.9.7 | 8.9.7 | 8.11.7 | 2a.11* | - | 9.7 | 9 | 2a.7 | 9* | 9.7 | - |
| *Trifolium subterraneum* ssp. *subterraneum* cv. Mt. Barker | 12.*4.7 | 4.14* | 12*.4.7 | 12.4 | 12.4.7 | 12*4.7 | 12*.4.7 | 12*.4.7 | 12*.4.7 | - | 12*.4.7 | 2a | 12*.4.7 | 12*.4.7 | 12.4.7 | - |
| Geraldton | 12*.4.7 | 12* | 12.*4 | 12*.4.7 | 12*.4 | 12* | 12*.4 | 12*.4 | 12*.4.7 | - | 12*.4 | - | 12* | 12*.4 | - | 12* |
| Dinninup | 12* | 12* | 12* | 12* | 12 | 12 | 12* | 12* | 12* | 12 | 12 | - | 12 | 12* | 2a | - |
| Enfield | 12*.4 | - | 12 | - | 12* | - | 12* | - | 12* | 12 | - | 12 | 12* | 12* | 12 | - |
| Daliak | 12*.4.7 | 12*4 | 12* | 12 | 12*.4 | - | 12* | 12*.4 | 12*.4 | - | 12* | - | 12*.4 | 12* | 12*.4.7 | - |
| *T. subterraneum* ssp. *jannicum* cv. Trikkala | 12* | 12*.4 | 12* | 12 | 12*.4 | - | 12* | - | 12*.4 | 12 | 12* | 2a | 12 | 12* | 12*.4.7 | - |

Table 5.1 (continued, p.3)

| Isolate / Host | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. subterraneum* ssp. *brachy-calycinum* cv. Clare | 12*.4.7 | 12* | 12* | 12* | 12* | 12* | 12*.4 | 12*.4 | 12*.4 | 2a | 12*.4 | 2a | 12 | 12*.4 | 12*.4 | 12 |
| *Medicago truncatula* var. *truncatula* cv. Jemalong | 12* | 12* | 12* | 12* | 12* | 12* | 12* | 12* | 12* | 12 | 12 | 12 | 12* | 12* | 12 | 12 |
| Sephi | 12* | 12* | 12* | 12* | 12* | 12 | 12* | 12* | 12 | 12* | 12 | 12* | 12 | 12* | 12* | 12 |
| Paraggio | 12 | 12 | 121* | 12 | 12* | 12* | 12* | 12 | 12 | 12 | 12 | 12 | 12 | 12* | - | 12 |
| Cyprus | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12 | - | 12 | 12 | 12 | 12 |
| Parabinga | 12* | 12* | 12 | 12 | 12 | 12* | 12 | 12 | 12* | - | 12 | - | 12 | 12 | 12* | 12 |
| *M. rugosa* cv. Paragosa | 12 | 12 | - | 12 | - | 12 | 12 | 12 | - | - | 12 | - | 12 | 12 | - | 12 |
| *M. scutellata* cv. Sava | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *M. littoralis* cv. Harbinger | 12 | - | - | - | - | 12 | 12* | 12 | 12 | 12 | 12* | 12 | 12 | 12* | 12 | - |
| *M. polymorpha* cv. Serena | 12 | - | 12 | 12 | 12* | 12* | 12 | 12 | 12 | - | 12 | 12 | 12* | 12 | - | - |
| *Trifolium repens* cv. Haifa | §.12 | §.- | §.- | §.- | §.- | §.- | §.- | §.- | §.- | §.- | 2a | - | - | § | - | 2a |
| Ladino | 2a | - | 2a | - | 2a | 2a | - | - | - | - | 2a | - | - | 2a | - | 2a |
| *T. resupinatum* spp. *majus* cv. Maral (Shaftal) | 12 | 12* | 12 | 12* | 12* | 12 | 12 | 12 | 12 | 12 | 12 | 12 | 12* | 12* | 12 | 12 |

II induced a chlorotic mosaic with mild to severe interveinal necrosis [Fig. E,I,J]. Strain $Y_{WA}$ tended more towards a chlorotic mosaic (Fig. 5.1 F) than to a greening mosaic. Strain $A_{NSW}$ induced top necrosis (Fig.5.1 I) at 5-8 days after inoculation, then recovered by producing new tips (Fig. 5.1 J).

Strain $H_{NSW}$ induced chlorotic mosaic, leaf scrolling, and severe stunting in sweet corn (*Zea mays* cv. Iochief), while strains $F_{NY}$ and $C_{NSW}$ induced a severe chlorotic streak and stunting with anther malformation (Table 5.1). Necrotic local lesions were produced on *Datura stramonium, Gomphrena globosa*, cowpea (*Vigna sinensis* cv. Black eye), and broad bean (*Vicia faba* cv. Aquadulce) [Fig.5.2 A,B,C] but this symptom was not always reproducible. None of the CMV strains produced visible symptoms on French bean (*Phaseolus vulgaris cv.* Hawkesbury Wonder) as observed by Francki and Hatta (1980). However, when tested by ELISA, some of the plants were infected with strains $O_{Qld}$, $L_{Vic}$, $E_{WA}$, and $A_{NSW}$.

The most common symptoms on cultivars of *Trifolium* spp. and *Medicago* spp. were interveinal chlorosis (Fig. 5.2 D,E,F), leaf distortion and severe stunting, several weeks after inoculation.

Strains $F_{NY}$, $O_{Qld}$, $C_{NSW}$, $L_{NY}$ and $B_{SA}$, caused the death of lupin cv. Illyarrie (Fig.5.4 A). Strains $T_{WA}$, $E_{WA}$, $H_{NSW}$, $L_{Vic}$, $Y_{WA}$, $A_{NSW}$ and $S_{WA}$ induced symptoms without showing stem necrosis, and the remaining 4 strains, $Q_{Qld}$, $U_{Qld}$, $V_{NSW}$ and $W_{Tas}$ were symptomless (Table 5.1). The virulence of particular strains to different seed-lots of the same lupin cultivar was variable. In one trial (Table 5.1), cultivar Danja 432 was susceptible to all CMV strains belonging to subgroup I but it was immune to strains belonging to subgroup II, while another seed-lot of Danja was susceptible to all strains from both these subgroups. The most common symptoms on lupin cultivars were epinasty, chlorosis, rosetting, stunting, distortion , stem necrosis, and premature death (Fig. 5.5 A).

*M. scutellata* (snail medic) cv. Sava was not infected by any of the CMV strains following mechanical inoculation (Table 5.1). In other experiments, this species was inoculated either by aphid transmission or by grafting (see Chapter 2.B.17). *Myzus*

Figure 5.2. CMV symptoms on several host species. Local lesions on *Gomphrena globosa* produced by strain $E_{WA}$ (A), *Vigna sinensis* cv. Black eye by strain $Y_{WA}$ (B), *Vicia faba* cv. Aquadulce by strain by strain $T_{WA}$ (C); systemic symptoms of interveinal chlorosis on *Trifolium subterraneum ssp. brachycalycinum* cv. Clare produced by strain $E_{WA}$ (D), *T. resupinatum* ssp. majus cv. Maral by strain $T_{WA}$ (E) and *Vigna sinensis* cv. Black eye by strain $L_{Vic}$ (F).

A

D

B

E

C

F

*persicae* and *Aphis craccivora* failed to transmit strains $F_{NY}$, $H_{NSW}$, $E_{WA}$, $T_{WA}$, $Q_{Qld}$, $B_{SA}$ and $Y_{WA}$ to this species. Both patch and approach grafting with *L. angustifolius* (lupin) cv. Illyarrie infected with strains $F_{NY}$ and $B_{SA}$ failed to cause infection of this species.

**2. Effect of time of the year on susceptibility of legumes**

The susceptibility of many particular species of legumes to particular strains of CMV varied with the time of year. Symptoms were frequently not reproducible (Table 5.2) when plants were inoculated at different times of the year. Some strains belonging to either subgroup I ($F_{NY}$, $T_{WA}$, $O_{Qld}$) or subgroup II ($L_{NY}$, $B_{SA}$, $S_{WA}$) infected certain cultivars of *L. angustifolius, M. truncatula, T. subterraneum,* and *T. resupinatum* at all times of the year [Table 3.2 shown by +++], but others were only able to infect them once [+].

**3. Distribution of virus in *Medicago* spp.**

*Medicago* spp. generally developed symptoms more than 6 weeks a.i. For example, symptoms appeared when plants produced seedpods (Fig. 5.3 A and B). To further investigate whether virus could be detected before symptoms appeared, the distribution of virus and symptoms in this species was determined as described in chapter 2.B.15.

Nine species and cultivars of *Medicago* infected with different strains of CMV differed in the rate of symptom appearance and the distribution of symptoms (Fig. 5.3 A-C). For example, some strains induced mild or no symptoms on the tips of *M. truncatula* cv. Parabinga and cv. Sephi, and *M. littoralis* cv. Harbinger at 10 weeks a.i., but severe interveinal chlorosis was found on leaves in the middle shoots (data not shown). The tips of *M. truncatula* cv. Paraggio infected with strains $F_{NY}$, $L_{Vic}$ and $T_{WA}$ showed the first symptoms at 6 weeks a.i. On the other hand, strains $Q_{Qld}$ and $U_{Qld}$ induced first symptoms at 10 weeks a.i. and no virus was detected on the leaves below the symptomatic tips by DAS-ELISA (data not shown).

TABLE 5.2. SUSCEPTIBILITY OF A RANGE OF LEGUME SPECIES TO 16 CMV STRAINS INOCULATED AT DIFFERENT TIME OF THE YEAR

| Isolate / Host | Subgroup I | | | | | | | Subgroup II | | | | | | | | | No. of infectious strains[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $B_{SA}$ | $S_{WA}$ | $V_{Qld}$ | $W_{Tas}$ | $Y_{WA}$ | |
| *Arachis hypogea* cv. Early bunch | --[a] | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 0 |
| *Glycine max* | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- | 0 |
| *Lens esculenta* cv. Laird | +++ | --+ | +-+ | +-- | ++- | ++- | --- | --+ | --- | --+ | --- | --- | --- | --- | --- | --- | 8 |
| *Vicia faba* cv. Aquadulce | ++- | +++ | -+- | +++ | --- | -++ | +-- | -- | -- | -- | -- | +-- | -- | -- | --- | --- | 7 |
| *Phaseolus vulgaris* cv. Hawkesbury Wonder | --- | --- | --- | §§- | §-§ | --- | --§ | --- | --- | §-§ | --- | --- | --- | --- | --- | ---- | 4 |
| *Vigna sinensis* cv. Black eye | +++ | +-+ | +-+ | --+ | +++ | +++ | +++ | ++- | --+ | ++- | ++- | +++ | -++ | +-- | §-- | +++ | 16 |
| *Lupinus angustifolius* cv. Illyarrie | +*+*+ | ++*+ | ++*+ | ++*+ | +*+*+ | +*+*+ | +++ | +++ | --- | +++ | --- | +++ | ++- | --- | --- | ++- | 12 |
| Warrah 408 | +++* | +++* | +++ | +++* | --- | +++* | -+- | --+ | --- | ++- | --+ | ++- | --+* | --- | --- | -+- | 12 |
| Warrah 437 | -+ | -- | -+ | ++ | -- | ++ | ++ | +- | -*- | +*+ | -- | +*+ | +*+ | -- | -+* | ++ | 11 |
| Gungurru | +++* | -++* | --+ | +++* | --- | ++- | +-- | -+*+ | -§- | --+* | --- | -*+- | +*++* | +*-- | +-- | ++- | 14 |
| Wandoo | ++- | +§+ | -+ | ++- | ++- | +-- | -+- | +*+- | -+- | --+ | +*-- | +++* | +++ | +-+* | -+- | ++- | 16 |
| Yandee | +++* | +-- | -++* | --+ | --- | ++- | --- | +++ | --- | +§+* | +-- | ++- | +++* | --- | --- | +++ | 10 |
| Yorrel | ++- | -++* | -++* | -++* | -+- | +++* | +-- | ++- | ++- | -+- | --- | +++* | ++- | --- | -+- | ++- | 14 |
| Danja 432 | §*+ | -*- | §+ | §*+ | -- | +*+ | +*+ | -*- | -- | -*- | -- | -*- | -*- | -- | -- | -- | 5 |
| Danja | -*-+ | +*++ | -+*+ | -++* | +-- | +*+*+ | +++* | -++-- | --- | --+* | +-- | ++*- | --+ | --+* | --- | -++* | 14 |
| *Medicago truncatula* var. *truncatula* cv. Jemalong | -++ | +++ | ++- | +++ | +++ | +++ | +++ | --+ | +-+ | --+ | --- | ++- | +++ | -++ | +++ | +-+ | 15 |
| Sephi | +++ | +++ | +-+ | ++- | +-+ | +++ | +++ | ++- | +-+ | +++ | +++ | +++ | +++ | -++ | +++ | +-+ | 16 |
| Paraggio | +++ | -+++ | +-++ | --+ | +--+ | +++ | §++ | +-+ | +-- | -++ | -++ | -+- | +++ | --- | +-+ | ++- | 15 |
| Cyprus | +++ | -++ | -+- | ++- | -++ | +++ | ++- | +++ | --- | ++- | --- | +++ | +-- | +-+ | +-- | ++- | 14 |
| Parabinga | +++ | +-+ | +++ | +-+ | +-- | +-- | ++- | +++ | --- | ++- | --- | ++- | +-+ | +-- | +-+ | ++-+ | 14 |

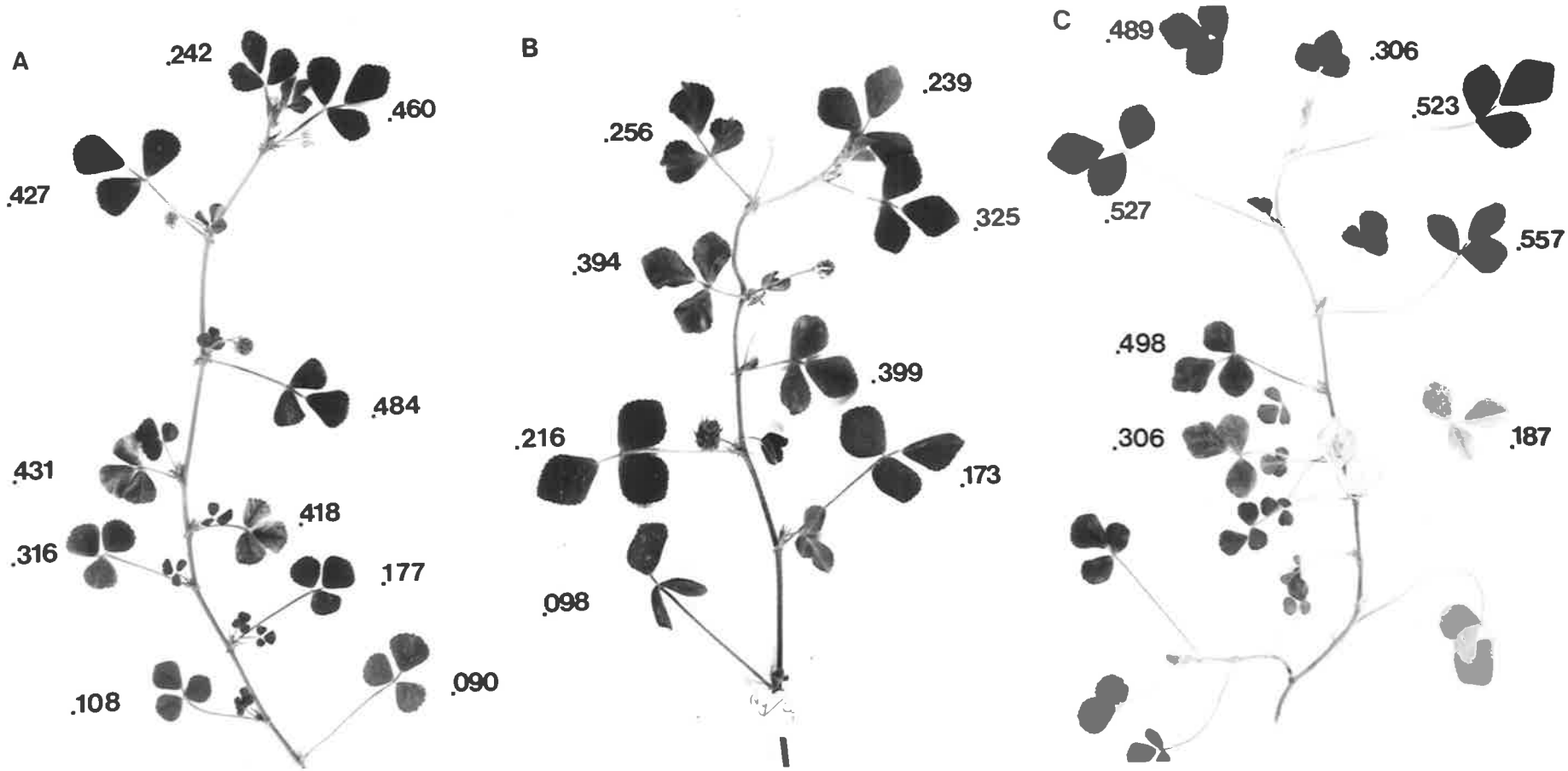
Table 5.2 (continued, p.2)

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *M. rugosa* cv. Paragosa | --+ | --+ | --- | --+ | --- | --+ | --+ | --+ | --- | --+ | --- | --+ | --+ | --- | --+ | --- | 10 |
| *M. scutellata* cv. Sava | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | 0 |
| *M. littoralis* cv. Harbinger | -++ | --+ | --- | -++ | -++ | --+ | +++ | +-+ | +-- | -++ | --+ | ++- | -++ | +-- | +-- | --+ | 15 |
| *M. polymorpha* cv. Serena | +++ | --- | ++- | +++ | +++ | --+ | +++ | ++- | --- | -++ | +-- | +++ | +++ | --- | --- | -+- | 12 |
| *Trifolium subterraneum* ssp. *subterraneum* cv. Mt. Barker | +-+ | +++ | +++ | ++- | +-+ | ++*+ | +++ | +-+ | --- | +++ | -+- | ++- | ++- | -+- | --- | +++ | 14 |
| Geraldton | -++ | -++ | +++ | --+ | +++ | -++ | ++- | +-+ | --- | -++ | --- | -++ | ++- | --- | +-+ | -++ | 13 |
| Dinninup | +++ | +++ | +-+ | ++- | +-+ | ++- | ++- | +++ | +-+ | +-+ | --- | ++- | +++ | -+- | --- | ++- | 14 |
| Enfield | -++ | --- | +-- | --- | +-- | --- | +-+ | +++ | +-+ | --- | +-- | -+- | +-- | ++- | --- | --- | 10 |
| Daliak | +-++ | -++ | +-+ | --+ | ++- | --- | ++- | +-+ | --- | +-+ | --- | --+ | --+ | -++ | --- | -++ | 12 |
| *T. subterraneum* ssp. *jannicum* cv. Trikkala | +-+ | -+- | +-- | --+ | +-+ | --- | +-+ | ++- | -+- | --+ | +-- | -+- | ++- | +-+ | --+ | --- | 14 |
| *T. subterraneum* ssp. *brachycalycinum* cv. Clare | +++ | --+ | +-+ | +-+ | +++ | +++ | +-+ | +-+ | +-- | +-+ | +-+ | +++ | +-+ | --+ | +-+ | -+ | 16 |
| *Trifolium repens* cv. Haifa | §-- | §-- | --- | --- | --- | §+- | --- | --- | --- | ++- | --- | --- | §-- | --- | ++- | --- | 6 |
| Ladino | -++ | --- | --+ | --- | --+ | --- | --- | --- | --- | +-+ | --- | --- | --+ | --- | +-- | --- | 6 |
| *T. resupinatum* ssp. *majus* cv. Maral (Shaftal) | +++ | +-+ | +++ | +++ | +++ | +++ | +++ | +++ | +-+ | +-+ | ++- | +++ | +++ | +-+ | +++ | +++ | 16 |

a + indicates infected with symptoms; § infected without symptoms (symptomless); - not infected; * death of plant (+/- when assayed). The number of +/- indicates the repetition of experiments at different time of year

b number of strains which induced + or §.

Figure 5.3. Variation in the distribution of symptoms and virus (A 405nm) in shoots of *Medicago* spp. (A) cv. Paraggio infected with strain $F_{NY}$, (B) cv. Cyprus infected with strain $F_{NY}$, and (C) cv. Jemalong infected with strain $L_{NY}$.


A
.242
.460
.427
.484
.431
.418
.316
.177
.108
.090
B
.239
.256
.325
.394
.399
.216
.173
.098
C
.489
.306
.523
.527
.557
.498
.306
.187

With DAS-ELISA, the virus concentration in each leaf (shown by Abs $_{405}$) was not correlated with the intensity of symptoms. In most samples, although leaf tips were symptomless and small because of distortion, they could have a concentration of virus higher than leaves with severe symptoms which were of equal weight (Fig. 5.3 A,B,C).

**4. Delay in symptom appearance in *M. rugosa* cv. Paragosa**

*M. rugosa* (Gamma medic) cv. Paragosa was first thought to be immune to all the strains tested because at 10 weeks a.i., no symptoms were observed and no virus was detected either by DAS-ELISA or by indexing on *N. glutinosa*. Symptoms appeared 14 weeks a.i. on the new shoots emerging from the crown of the plant. Fig. 5.4 shows the diagram of symptoms and virus distribution at this time. Virus was also detected in the symptomless parts of the infected shoots (Fig. 5.4 shown by the dots).

**5. Necrosis induced on lupins cv. Illyarrie in sterile cultures**

As described in Table 5.1 some inoculated lupins in the glasshouse died either with or without showing typical CMV symptoms. Another trial was done under the same conditions to observe whether CMV induced stem necrosis on lupin and caused premature death. Lupin cv. Illyarrie was inoculated with a range of strains, some of which were able to cause necrosis (Table 5.1). Plants were observed for induction of necrosis at 6 weeks a.i.(Table 5.3). Strains $A_{NSW}$, $S_{WA}$, $B_{SA}$ did not induce necrosis on the stem above the soil surface but a necrosis was produced in the crown (Fig. 5.5 A). Necrotic parts of the lupins contained infectious virus.

In a trial done under sterile conditions (see Chapter 2.B.16), plants showed stem browning just above the cotyledons at 8-9 days a.i. and this was followed by wilting of the tip. Necrosis initially occurred on the third nodes and progressed up to the tip or down to the crown. Two types of necrosis were observed. Dry necrosis where some plants either survived or became completely necrotic, and wet necrosis which resulted in complete tissue breakdown and necrosis (Fig 5.5 B-D). Some infected lupins recovered by producing new shoots at the nodes below the necrotic zone.

Figure 5.4. Distribution of symptoms in *Medicago rugosa* (Gamma medic) cv. Paragosa infected with strain $F_{NY}$ [the solid dark areas indicate the symptom distribution and the dots indicate the virus distribution. The arrows indicate the position of the leaf sampled taken at 10 weeks after inoculation].

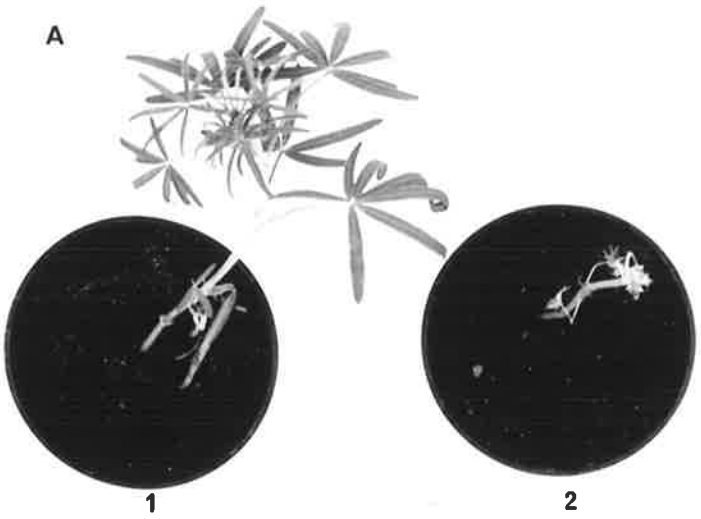
TABLE 5.3. INCIDENCE OF NECROSIS ON LUPIN CV. ILLYARRIE INOCULATED WITH A RANGE OF CMV STRAINS

| Virus strain | Glasshouse conditions [a] | | Sterile conditions [a] | | |
|---|---|---|---|---|---|
| | No. of plants infected / No. of plants inoculated | No. of necrotic plants / No. of plants inoculated | No. of plants infected / No. of plants inoculated | No. of necrotic plants / No. of plants inoculated | Days for necrosis to develop |
| $F_{NY}$ | 4/8 | 4/8 | 4/4 | 4/4 | 8-24 |
| $T_{WA}$ | 7/8 | 6/8 | 4/4 | 4/4 | 8-22 |
| $C_{NSW}$ | 5/8 | 5/8 | 3/4 | 3/4 | 12-28 |
| $E_{WA}$ | 8/8 | 4/8 | not tested | - | - |
| $H_{NSW}$ | 8/8 | 4/8 | not tested | - | - |
| $A_{NSW}$ | 4/8 | 4/8 | 4/4 | 2/4 | 12-30 |
| $B_{SA}$ | 5/8 | 4/8 | 4/4 | 2/4 | 10-28 |
| $S_{WA}$ | 1/8 | 0/8 | 3/4 | 2/4 | 10-30 |
| $Y_{WA}$ | 2/8 | 0/8 | 4/4 | 2/4 | 6-16 |
| $W_{Tas}$ | 0/8 | 0/8 | 1/4 | 1/4 | 7-32 |
| $V_{Qld}$ | 0/8 | 0/8 | 1/4 | 1/4 | 20-28 |
| $Q_{Qld}$ | 0/8 | 0/8 | 3/4 | 3/4 | 20-28 |
| Control | 0/8 | 0/8 | 0/4 | 0/4 | - |

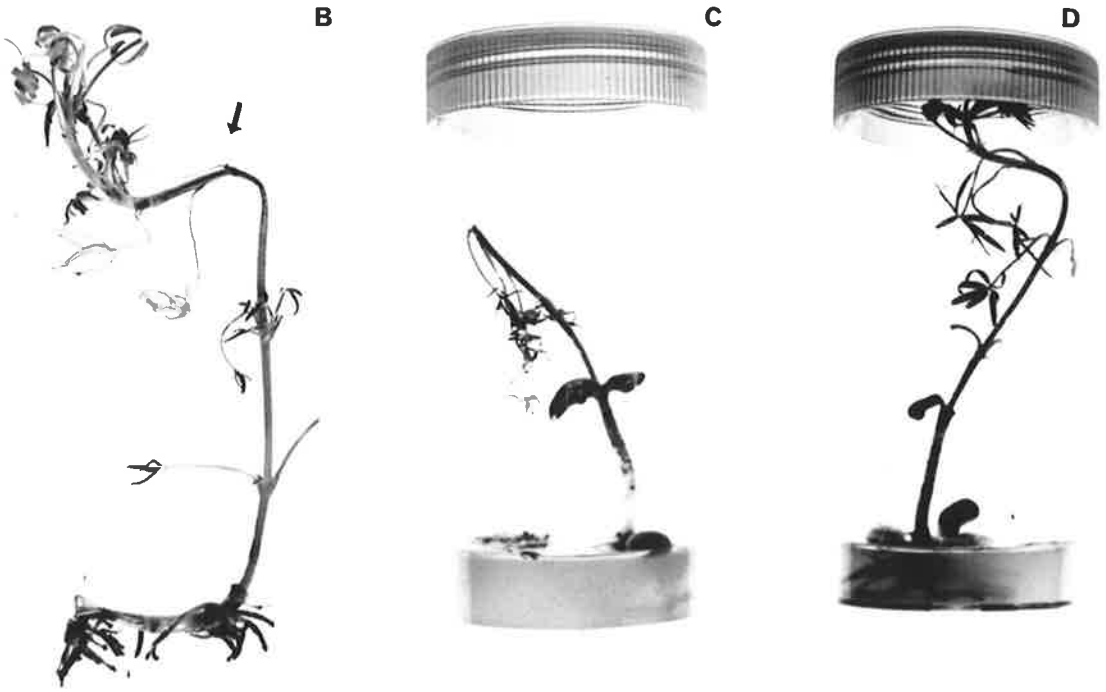
a) Data were recorded at 6 weeks after inoculation

Figure 5.5. Type of necrosis on *Lupinus angustifolius*. cv. Danja infected by strain $C_{NSW}$ in the glasshouse [left, infected with symptom; right, infected and subsequently dying] (A); cv. Illyarrie in MS-medium showing dry necrosis (arrow, B), wet necrosis (C) and control (D).

# Glasshouse




# Sterile cultures



Lupins inoculated with strain $Y_{WA}$ showed severely chlorotic leaves, 2/4 became stunted and another 2 showed stem necrosis. Under glasshouse conditions, strains $Q_{Qld}$, $W_{Tas}$ and $V_{Qld}$ did not induce any symptoms on cv. Illyarrie, but they induced necrosis in sterile culture.

## CONCLUSION

Results from this study showed that :

1. The symptoms of CMV infection on a wide host range showed no systematic pattern, particularly with respect to which species might be useful for distingushing strains into subgroups. Only *N. edwardsonii, N. tabacum* cv. Xanthi n.c. and *C. frutescens* cv. Giant Bell seemed to be useful for this purpose.
2. CMV-$Y_{WA}$ which is serologically different from other strains, does not belong to the PSV subgroup (Mink, 1972) on the basis of its inability to infect peanut and soybean.
3. *M. scutellata* cv. Sava is resistant to CMV.
4. The symptoms caused by individual strains on given host species vary with different times of year. No strains of either subgroup I or II preferentially infected plants at any particular time of the year.
5. The distribution of symptoms and virus in *Medicago* spp. is uneven. The delay of symptom appearance indicates that the virus can have long latency and that tests for resistance should take this into account.
6. CMV is able to cause the death of a number of cultivars of lupin.

# CHAPTER 6. ANTIGENIC PROPERTIES OF FIXED AND UNFIXED VIRUS

## INTRODUCTION

ELISA is one of the most sensitive serological methods, and the specificity of indirect and direct ELISA to detect plant viruses has been compared (Devergne *et al.*, 1981; Lommel *et al.*, 1982; Rocha-Pena and Lee, 1991). Direct DAS-ELISA has a high sensitivity for detecting strains belonging to the same virus (Barbara *et al.*, 1978; Lister and Rochow, 1979; Bar-Joseph and Salomon, 1980), but it is less sensitive for detecting a broad range of serologically related viruses (Koenig, 1981). Furthermore, Richter *et al.* (1989) and Mowat and Dawson (1987) found that DAS-ELISA gave unsatisfactory results when detecting CMV from crude extracts of leaf sap. They then developed indirect ELISA by coating plates with crude extracts of leaf sap, and allowing the adsorbed virus antigen to react with antiviral IgG or unfractionated antiserum. The antigen-antibody complex was detected with commercial goat anti-rabbit IgG-conjugate and enzyme substrate. Mowat and Dawson (1987) recommended use of this conjugate, because protein A-horseradish peroxidase conjugate gave a high background due to the nonspecific reactions of uninfected or infected leaf extracts. This method was designated as plate trapped antigen (PTA) indirect ELISA. It is more sensitive for detecting distantly related viruses when using purified virus than when using crude extracts of leaf sap (Lommel *et al.*, 1982; Jaegle and van Regenmortel, 1985; Richer *et al.*, 1989).

The ELISA system has been optimised to eliminate high background readings due to nonspecific antigen-antibody reactions (Bar-Joseph and Salomon, 1980). Several methods have been used for this purpose, such as by blocking with proteins (Vogt *et al.*, 1987) or by adding heparin to the conjugate buffer (Dietzgen and Francki, 1987). Blocking with BSA (see Chapter 2.9 and 13) is commonly used to saturate unoccupied binding sites on microtitre plates (Clark *et al.*, 1986). Furthermore, Zimmermann and van Regenmortel (1989) found that blocking with milk protein gave a better result than

BSA or other proteins.

The virus particle of CMV is unstable, and degrades during or after virus preparation (Kaper and Waterworth, 1981). For long term storage, either 0.25% glutaraldehyde (Korn *et al.*, 1972; Rao *et al.*, 1982) or 0.2% formaldehyde (Francki and Habili, 1972) are used as protein cross-linking reagents for stabilising the antigenic binding sites of the viral protein. The use of fixed antigens as the immunogen increases the titre of the antiserum, as demonstrated with CMV (Francki and Habili, 1972) and AMV (Hajimorad and Francki, 1991). During study of the serological reactions of CMV strains (Chapter 4), it was observed that some strains of purified virus preparation which had been fixed with glutaraldehyde were likely to degrade. Such preparationss showed an accumulation of a precipitate in the storage containers.

Fixed and unfixed purified virus preparations (Chapter 2.B.3) were used in this study. The preparation of antisera to fixed and unfixed virus was done as described in Chapter 2.B.9. These are designated as fixed and unfixed antisera, respectively, in the following text.

Serological reactions between unfixed and fixed antigens, and fixed and unfixed antisera, were investigated by gel immunodiffusion tests or PTA indirect ELISA (see Chapter 2.B.10 and 13.b). These experiments were done to further investigate whether unfixed antisera could be used to distinguish between unfixed antigens of strains of CMV, as shown by the results obtained with fixed antisera (see Chapter 4.A.2).

The effect of glutaraldehyde on the stability of virus preparations after long term storage was also investigated in this chapter, by analysing the virus particles in fixed and unfixed preparations, either by sucrose density gradient centrifugation and ISCO density gradient fractionation (see Chapter 2.B.2), by gel electrophoresis or by electron microscopy.

# EXPERIMENTAL

## A. REACTIONS OF FIXED AND UNFIXED ANTIGENS TO ANTISERA PREPARED AGAINST FIXED AND UNFIXED ANTIGENS

### 1. Gel immunodiffusion tests

#### 1.a. Homologous titre of antisera prepared against fixed and unfixed antigens

Both fixed and unfixed antisera were titrated to either homologous fixed or unfixed antigens (Table 6.1). The titres of fixed antisera with their homologous fixed antigens differed by no more than one twofold dilution from those obtained with unfixed antigens. Similarly, the titres of unfixed antisera to homologous fixed and unfixed antigens differed by no more than one twofold dilution (Table 6.1).

Bleed number was varied to allow the use of antisera with maximum titres. No host reaction was detected in any of the antisera tested.

Different rabbits reacted differently to the same virus strain. For example, when two rabbits were injected with the same preparation of unfixed $Q_{Qld}$ antigen, after 3 injections, their respective titres were 1/16 and 1/64.

#### 1.b. Reactivity of homologous fixed and unfixed antigens with fixed or unfixed antiserum

A test was done as shown in Figure 6.1. The results showed that spurs formed between homologous fixed and unfixed antigens when using antisera to fixed $H_{NSW}$ ($h_1$), fixed $O_{NSW}$ ($o_1$), fixed $L_{NY}$ ($l_1$), unfixed $Q_{Qld}$ ($q_2$), and unfixed $P_{Jpn}$ ($p_{J2}$).

In some tests with antiserum to unfixed $F_{NY}$ from the second, third, and fourth bleedings, a precipitin line resembling an extension of the spur developed between the homologous antigen wells. This, however, was not seen with antiserum from the next bleeds (Fig. 6.1). So, this is regarded as being anomalous and does not indicate a true spur.

TABLE 6.1. TITRES OF CMV ANTISERA PREPARED AGAINST FIXED AND UNFIXED ANTIGENS TO THE HOMOLOGOUS FIXED OR UNFIXED ANTIGENS IN GEL IMMUNODIFFUSION TESTS

| Antiserum to | Bleed number | Titre to the homologous fixed antigens * | unfixed antigens* |
|---|---|---|---|
| **Fixed antigens** | | | |
| $F_{NY}$ | 3th | 1/512 | 1/512 |
| $T_{WA}$ | 2nd | 1/256 | 1/256 |
| $H_{NSW}$ | 4th | 1/512 | 1/256 |
| $O_{Qld}$ | 3rd | 1/512 | 1/256 |
| $Y_{Jpn}$ | 7th | 1/512 | 1/512 |
| $L_{NY}$ | 4th | 1/1024 | 1/1024 |
| $Q_{Qld}$ | 3rd | 1/128 | 1/64 |
| $A_{NSW}$ | 4th | 1/512 | 1/256 |
| $U_{Qld}$ | 6th | 1/256 | 1/128 |
| $Y_{WA}$ | 7th | 1/512 | 1/512 |
| $P_{Jpn}$ | 7th | 1/512 | 1/256 |
| **Unfixed antigens** | | | |
| $F_{NY}$ | 6th | 1/512 | 1/512 |
| $T_{WA}$ | 4th | 1/2048 | 1/2048 |
| $H_{NSW}$ | 4th | 1/512 | 1/512 |
| $Y_{Jpn}$ | 3rd | 1/512 | 1/512 |
| $L_{NY}$ | 6th | 1/1024 | 1/1024 |
| $Q_{Qld}$ | 9th | 1/128 | 1/256 |
| $A_{NSW}$ | 3rd | 1/256 | 1/256 |
| $Y_{WA}$ | 9th | 1/512 | 1/512 |
| $P_{Jpn}$ | 3rd | 1/256 | 1/256 |

* Both fixed and unfixed antigen concentrations were adjusted to 0.5 µg/ml. 10 µl of either antigen or antiserum was placed in each well.

In these tests, some antisera produced patterns with double precipitin lines. For example, double precipitin lines were produced by homologous fixed and unfixed $H_{NSW}$ and $A_{NSW}$ antigens to their respective unfixed antiserum, where the second lines were located close to the antigen wells. However, with fixed $A_{NSW}$ antiserum, these second lines were produced close to the antiserum well (Fig 6.1).

### 1.c. Comparison between homologous and heterologous unfixed antigens in reactions with unfixed antiserum

The aim of this experiment was to determine whether unfixed antisera can be used to distinguish strains of CMV into distinct serogroups. Unfixed antigens and antiserum were placed in wells as described in experiment 4.A.2 (see Chapter 4).

This experiment showed that on the basis of spur formation, most strains belonging to subgroup I could be distinguished from strains belonging to subgroup II (Table 6.2). Strain $Y_{WA}$ was distinct from all other antigens tested in that it produced a spur in comparison against heterologous antigens (Table 6.2). The other exceptions were $T_{WA}$ and $H_{NSW}$ (subgroup I) which did not form spurs to $V_{Qld}$ (serogroup II) using antisera to strains $T_{WA}$ and $H_{NSW}$ (subgroup I). $V_{Qld}$ fell into subgroup II when tested with other antisera (Table 6.2).

### 1.d. Combined placement between homologous and heterologous fixed and unfixed antigen in reactions with either fixed or unfixed antiserum.

Some antisera provided by other laboratories (see Chapter 4, Table 4.3) were made from unfixed antigens, e.g. antisera to strains $Y_{Jpn}$, $P_{Jpn}$ and SA from Japan (Dr. K. Hanada, personal communication). Using antiserum to SA and heterologous standard antigen (the homologous antigen was not available in our laboratory), some strains from different subgroups did not react with this antiserum (Fig. 4.2). A test similar to that of described above (see Chapter 4.A.3 ) was done to investigate the antigenic differences between fixed and unfixed antigens.

**Figure 6.1.** Reactions of homologous fixed and unfixed antigens with antiserum prepared against either fixed or unfixed antigens in gel immunodiffusion. The reaction was recorded after 40 h incubation. The subscript 1 refers to fixed virus and 2 refers to unfixed virus. **F** or **f** = $F_{NY}$, **T** or **t** = $T_{WA}$, **H** or **h** = $H_{NSW}$, $\mathbf{Y_J}$ or $\mathbf{y_j}$ = $Y_{Jpn}$, **O** or **o** = $O_{Qld}$, **L** or **l** = $L_{NY}$, **Q** or **q** = $Q_{Qld}$, **A** or **a** = $A_{NSW}$, $\mathbf{Y_A}$ or $\mathbf{y_A}$ = $Y_{WA}$, and $\mathbf{P_J}$ or $\mathbf{p_J}$ = $P_{Jpn}$. The antiserum wells are labelled with lower case letters.

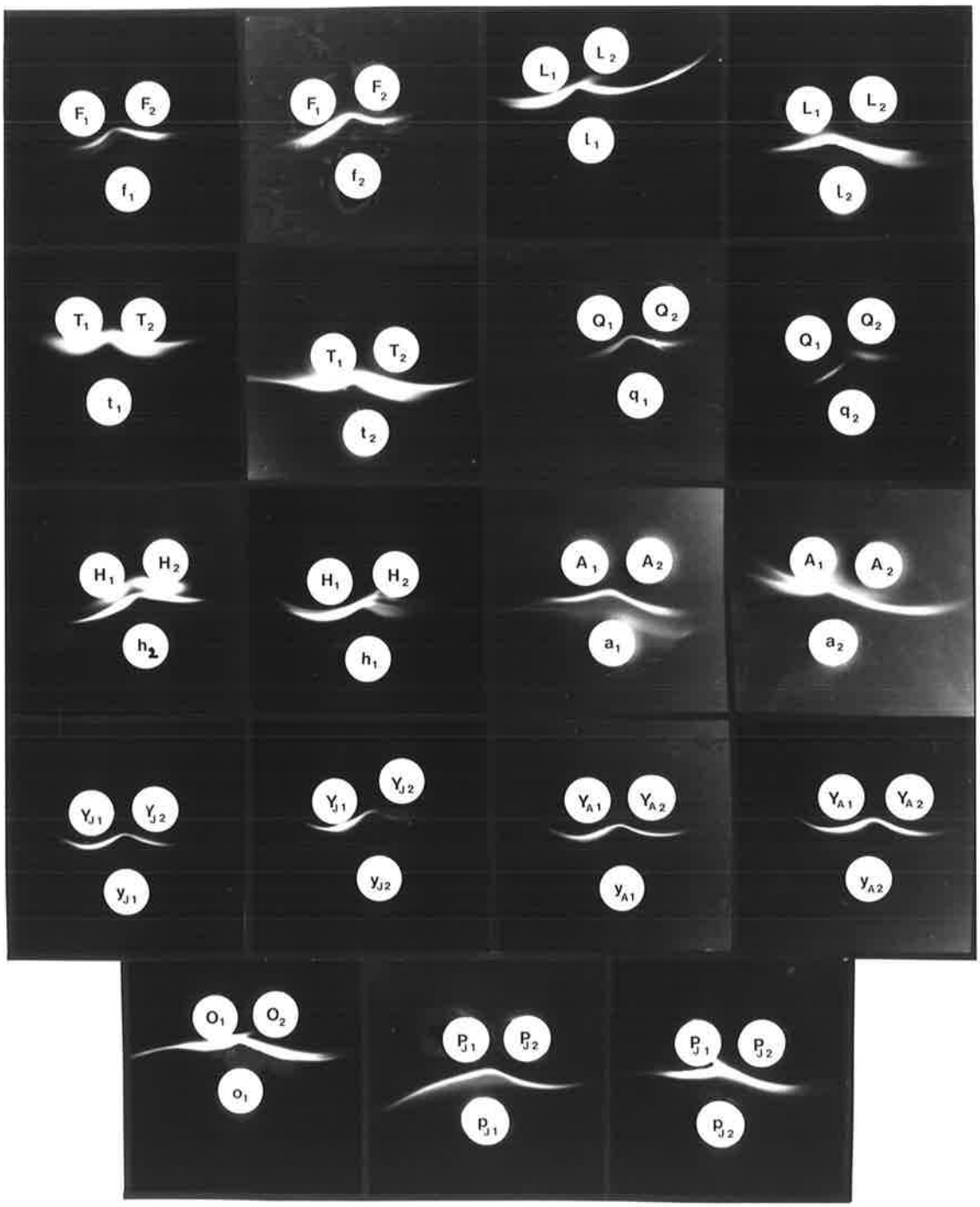

F1 F2 f1
F1 F2 f2
L1 L2 l1
L1 L2 L2
T1 T2 t1
T1 T2 t2
Q1 Q2 q1
Q1 Q2 q2
H1 H2 h2
H1 H2 h1
A1 A2 a1
A1 A2 a2
YJ1 YJ2 yJ1
YJ1 YJ2 yJ2
YA1 YA2 yA1
YA1 YA2 yA2
O1 O2 o1
PJ1 PJ2 pJ1
PJ1 PJ2 pJ2

**Table 6.2.** Reactivity of unfixed antigens with unfixed antiserum in gel immunodiffusion tests

| Antiserum | Standard antigen | Spur formed to the following antigens | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $Q_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
| $F_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | + | +++ | + | ±± | ± | +++ | +++ | +++ | ++ | +++ |
| $T_{WA}$ | $T_{WA}$ | - | - | - | - | - | - | - | - | +++ | +++ | +++ | +++ | - | +++ | +++ | +++ | +++ |
| $H_{NSW}$ | $H_{NSW}$ | - | - | - | - | - | - | - | + | +++ | +++ | + | + | - | +++ | +++ | +++ | +++ |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | - | - | - | - | - | - | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| $L_{NY}$ | $L_{NY}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $Q_{Qld}$ | $Q_{Qld}$ | + | + | + | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| $A_{NSW}$ | $A_{NSW}$ | ++ | +++ | +++ | +++ | + | +++ | +++ | - | - | - | - | - | - | - | - | - | - |
| $Y_{WA}$ | $Y_{WA}$ | +++ | +++ | +++ | +++ | ++ | +++ | +++ | - | + | + | + | + | ++ | + | + | + | + |
| $P_{Jpn}$ | $P_{Jpn}$ | + | ++ | ++ | +++ | ++ | +++ | +++ | - | - | - | - | - | - | - | - | - | - |

- single confluent precipitin line
+++ the spur formation was strong and + was very faint
± double precipitin lines, where spur was produced by the outer line and the inner line was confluent

In these tests, some strains formed very strong spurs to antigens in the adjacent wells (Table 6.3 shown by +++) and some were very faint (+). Some antisera produced double precipitin lines (±, =) and others only a single precipitin line (+, -) or crossing-over (Fig. 6.2, see footnote, Table 6.3 shown by *). For the double precipitin lines, the inner precipitin lines were generally confluent (-), and the outer lines were either confluent (=) or formed a spur (±) (Table 6.3, Fig. 6.2). For example, using antiserum to unfixed $F_{NY}$ and fixed $F_{NY}$ as the standard antigens, the outer precipitin lines formed spurs to fixed antigens of $Y_{WA}$, $L_{NY}$, $Q_{Qld}$, $A_{NSW}$, $S_{WA}$, $B_{SA}$, $W_{Tas}$. When fixed antisera to the subgroup II strains ($L_{NY}$, $Q_{Qld}$, $A_{NSW}$, $Y_{WA}$, $P_{Jpn}$) were used, the standard unfixed antigens did not form spurs to heterologous unfixed antigens of the subgroup I or II strains in adjacent wells (Table 6.3).

Fig. 6.2 shows double precipitin lines produced by the antiserum to fixed $F_{NY}$, in a reaction against standard fixed $F_{NY}$ antigen and heterologous unfixed antigens in adjacent peripheral wells. The outer precipitin lines formed spurs to unfixed $B_{SA}$, $S_{WA}$ and $E_{WA}$ antigens and it formed a confluent line with unfixed $C_{NSW}$ antigen. These kinds of spurs were also produced by the standard antigens of unfixed $P_{Jpn}$ to fixed $T_{WA}$ and $H_{NSW}$ antigens, using antiserum to unfixed $P_{Jpn}$ (Fig. 6.2).

These results show that this method could not be used for serotyping the strains of CMV (Table 6.3).

## 2. Antigenic differences between fixed and unfixed antigen in PTA indirect-ELISA

It has been demonstrated that indirect ELISA is reliable for detecting reactions between distantly related antigens (Devergne *et al.*, 1981). A test with distantly related strains of CMV was done with PTA indirect -ELISA (see Chapter 2.B.13.b).

### 2.a. Tests to determine dilution end points of antigen

Microtitre plates were pre-coated directly with either fixed or unfixed antigen of strains $T_{WA}$ (serogroup I), $L_{NY}$ (serogroup II) or $Y_{WA}$ (intermediate serogroup) in a

footnote

shown in Fig. 6.2 as a single precipitin line crossover between antigens Qf and Yf, and antiserum to Yu.

**Table 6.3.** Reactivity between combined placements of unfixed and fixed antigens with unfixed or fixed antisera in gel immunodiffusion tests

| Unfixed antiserum | Standard fixed antigen | Spurs formed against fixed antigen : | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | UQld | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
| $F_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | ± | ± | ± | ± | - | - | ± | ± | ± | - |
| $T_{WA}$ | $T_{WA}$ | - | - | - | - | - | - | - | - | - | +++ | +++ | ++ | +++ | ± | ++ | ++ | +++ | ++ |
| $H_{NSW}$ | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | +++ | +++ | +++ | - | + | +++ | +++ | +++ | +++ |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | - | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| $L_{NY}$ | $L_{NY}$ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | - | - | - | - | - | - | - | - | - |
| $Q_{Qld}$ | $Q_{Qld}$ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | - | +++ | - | + | +++ | - | ++ | ± | - | ± |
| $A_{NSW}$ | $A_{NSW}$ | - | - | - | - | - | - | - | - | +++ | +++ | ± | - | +++ | ± | ++ | ++ | ++ | ++ |
| $Y_{WA}$ | $Y_{WA}$ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | - | - | +++ | + | +++ | ++ | + | + | ++ | +++ |
| $P_{Jpn}$ | $P_{Jpn}$ | +++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | - | - | - | - | - | - | - | - | - |

| Unfixed antiserum | Standard unfixed antigen | Spurs formed against fixed antigen : | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
| $F_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | ++ | ++ | ± | - | ± | ++ | ++ | ++ | ++ |
| $T_{WA}$ | $T_{WA}$ | - | - | - | - | * | * | - | - | * | +++ | +++ | * | - | * | +++ | +++ | +++ | +++ |
| $H_{NSW}$ | $H_{NSW}$ | - | - | - | - | - | - | - | - | - | +++ | +++ | + | - | + | +++ | +++ | +++ | + |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | ± | - | - | - | - | - | - | +++ | +++ | +++ | +++ | - | ++ | +++ | +++ | +++ | +++ |
| $L_{NY}$ | $L_{NY}$ | +++ | +++ | ++ | + | * | +++ | +++ | +++ | ± | - | ± | - | - | - | ± | - | - | - |
| $Q_{Qld}$ | $Q_{Qld}$ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | - | + | - | + | - | + | +++ | ± | +++ | - |
| $A_{NSW}$ | $A_{NSW}$ | +++ | +++ | ± | ++ | ++ | +++ | +++ | ++ | - | - | - | - | * | - | - | - | - | - |
| $Y_{WA}$ | $Y_{WA}$ | ++ | ++ | + | +++ | +++ | +++ | +++ | + | - | + | + | + | - | +++ | ++ | ++ | ± | ++ |
| $P_{Jpn}$ | $P_{Jpn}$ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | - | - | - | - | - | - | - | - | - | - |

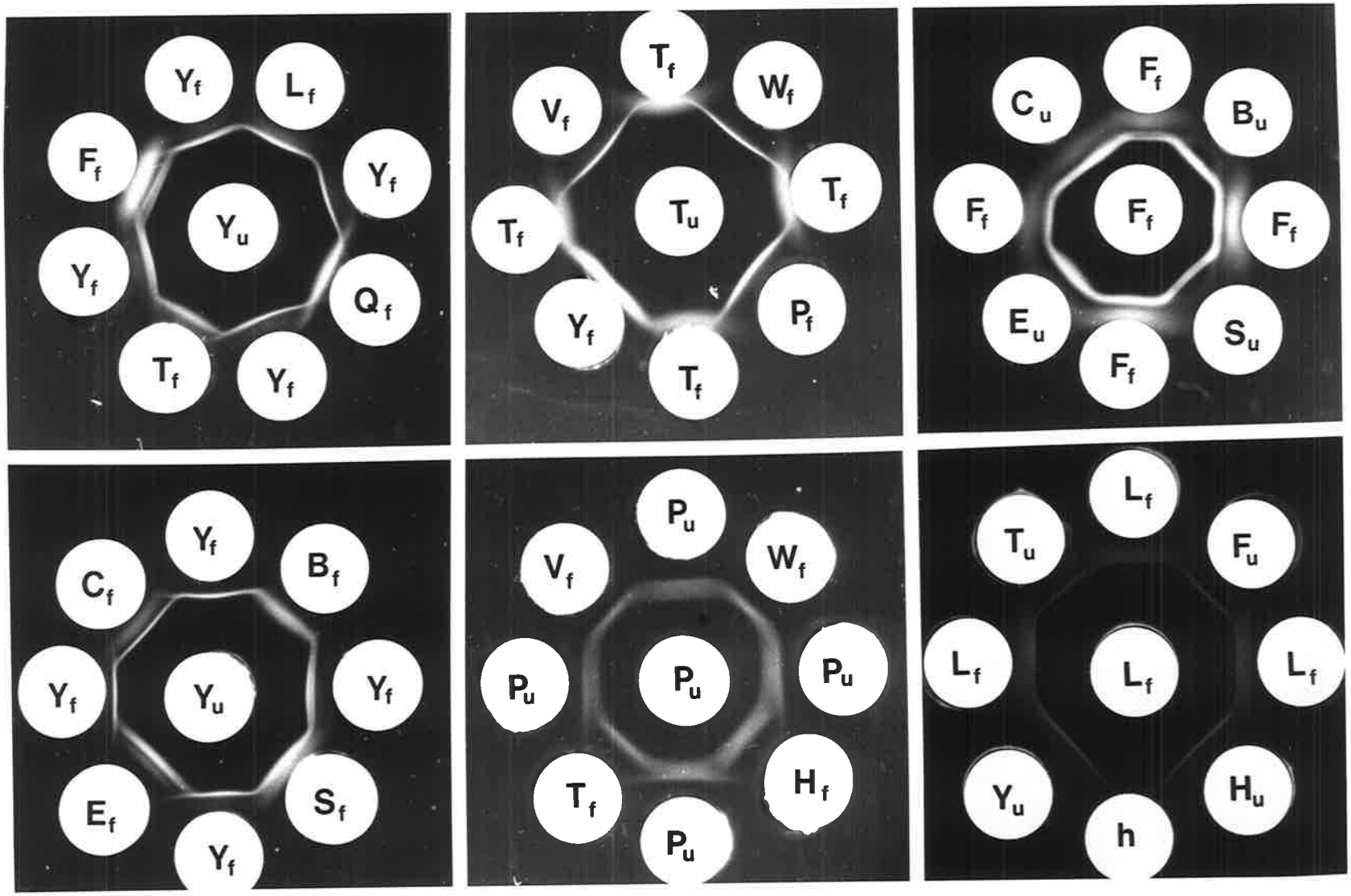
Table 6.3 (continued, p. 2)

| Fixed antiserum | Standard unfixed antigen | Spur formed against unfixed antigen : $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $F_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $T_{WA}$ | $T_{WA}$ | - | - | - | - | - | - | - | - | + | + | + | - | - | - | + | + | + | + |
| $H_{NSW}$ | $H_{NSW}$ | - | - | - | - | - | - | - | - | +++ | +++ | +++ | - | - | - | +++ | +++ | +++ | +++ |
| $L_{Vic}$ | $L_{Vic}$ | - | - | - | - | - | - | - | - | +++ | +++ | +++ | ± | - | ± | +++ | +++ | +++ | +++ |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | + | + | + | + | - | + | + | + | + | + |
| $L_{NY}$ | $L_{NY}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $Q_{Qld}$ | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $A_{NSW}$ | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $Y_{WA}$ | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $P_{Jpn}$ | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| Fixed antiserum | Standard fixed antigen | Spur formed against unfixed antigen : $F_{NY}$ | $T_{WA}$ | $H_{NSW}$ | $O_{Qld}$ | $L_{Vic}$ | $C_{NSW}$ | $E_{WA}$ | $Y_{Jpn}$ | $Y_{WA}$ | $L_{NY}$ | $Q_{Qld}$ | $A_{NSW}$ | $U_{Qld}$ | $V_{Qld}$ | $S_{WA}$ | $B_{SA}$ | $W_{Tas}$ | $P_{Jpn}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $F_{NY}$ | $F_{NY}$ | - | - | - | - | - | - | - | - | - | +++ | ++ | ++ | - | ++ | +++ | +++ | +++ | ++ |
| $T_{WA}$ | $T_{WA}$ | = | - | - | - | - | - | - | = | + | ++ | ++ | - | - | - | * | * | + | = |
| $H_{NSW}$ | $H_{NSW}$ | = | - | - | - | - | - | - | - | +++ | +++ | +++ | + | - | - | +++ | +++ | ++ | +++ |
| $L_{Vic}$ | $L_{Vic}$ | * | = | - | - | - | - | - | - | + | * | + | + | - | - | +++ | +++ | ++ | * |
| $Y_{Jpn}$ | $Y_{Jpn}$ | - | - | - | - | - | - | - | - | + | +++ | ++ | ±±± | + | ±±± | +++ | +++ | +++ | +++ |
| $L_{NY}$ | $L_{NY}$ | ± | ± | - | + | ± | ± | - | + | - | - | ++ | ++ | - | ++ | ±±± | ++ | ±±± | +++ |
| $Q_{Qld}$ | $Q_{Qld}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $A_{NSW}$ | $A_{NSW}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $Y_{WA}$ | $Y_{WA}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ± | - |
| $P_{Jpn}$ | $P_{Jpn}$ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ± | - |

± spur formation in the outer line whereas the inner line was confluent
= the inner and outer lines were confluent
* antigens produced cross-over lines
+ spur increased in intensity as shown by ++ and +++

**Figure 6.2**. Reactivity between combined placements of fixed and unfixed antigens with either fixed or unfixed antiserum in gel immunodiffusion tests. F = strain $F_{NY}$, T = strain $T_{WA}$, H = strain $H_{NSW}$, C = strain $C_{NSW}$, E = strain $E_{WA}$, L = strain $L_{NY}$, Q = strain $Q_{Qld}$, B = strain $B_{SA}$, S = strain $S_{WA}$, V = strain $V_{Qld}$, W = strain $W_{Tas}$, Y = strain $Y_{WA}$, P = strain $P_{Jpn}$, u = unfixed, f = fixed.


Yf
Lf
Ff
Yf
Yu
Yf
Qf
Tf
Yf
Tf
Wf
Vf
Tf
Tu
Tf
Yf
Pf
Tf
Ff
Cu
Bu
Ff
Ff
Ff
Eu
Su
Ff
Yf
Bf
Cf
Yf
Yu
Yf
Sf
Ef
Yf
Pu
Wf
Vf
Pu
Pu
Pu
Tf
Hf
Pu
Lf
Tu
Fu
Lf
Lf
Lf
Yu
Hu
h

twofold dilution series. The dilution series was started with a concentration of 0.5 µg/ml. Either fixed or unfixed antisera to $T_{WA}$ and $L_{NY}$ at 1/2000 dilution were used as the first antibody. Goat anti-rabbit AP-ase conjugate was used at 1/9000 dilution .

**Results :**

The lowest concentration of virus which reacted with both fixed and unfixed antiserum in these tests was 31.5 ng/ml. Fixed antiserum gave a slightly stronger reaction with homologous fixed antigens (Fig 6.3 A and C) than with homologous unfixed antigens. A slightly stronger reaction was also produced by unfixed antigens homologous to unfixed antiserum than by fixed antigens (Fig.6.3 B and D). With this test, strain $Y_{WA}$ was more closely related to strain $L_{NY}$ than to strain $T_{WA}$ (Fig. 6.3 B). Strain $L_{NY}$ was distinguished from strain $T_{WA}$ at higher antigen concentrations, although this difference was not as clear as achieved by direct DAS-ELISA (Chapter 4.B.1).

Therefore, another test with a dilution series of antiserum as described below was used to enhance differences between heterologous reagents.

**2.b. Tests to determine the titres of antisera**

Wells of microtitre plates were pre-coated directly with antigen (0.5 µg/ml) of either fixed $T_{WA}$, unfixed $T_{WA}$, fixed $L_{NY}$ or unfixed $L_{NY}$. Unfractionated fixed or unfixed antisera to $T_{WA}$ or $L_{NY}$ in a twofold dilution series were used as the first antibody, starting with a dilution of 1/1054. Goat anti-rabbit AP-ase conjugate was used at a 1/9000 dilution to detect bound antibody.

**Results :**

With this experiment, a very low dilution of antiserum (1/32000) could still detect the homologous antigen at a concentration of 0.5 µg/ml. Fixed antigens reacted a little more strongly with the homologous fixed antiserum than did unfixed antigens (Fig. 6.4). Unfixed antigens also reacted a little more strongly with homologous unfixed antiserum than did fixed antigens. However, direct DAS-ELISA distinguished the

**Figure 6.3.** Reactivity of unfixed and fixed virus in plate trapped antigen (PTA) indirect-ELISA. A twofold dilution series of either fixed or unfixed $T_{WA}$, $L_{NY}$ and $Y_{WA}$ antigen was used to coat the plate directly, then incubated with antiserum to fixed $T_{WA}$ (A), unfixed $T_{WA}$ (B), fixed $L_{NY}$ (C) or unfixed $L_{NY}$ (D) at a 1/2000 dilution. The hydrolysis time after the addition of substrate solution was 20 min. ---▲--- fixed $L_{NY}$ antigen, ---■--- fixed $T_{WA}$ antigen, ——▲—— unfixed $L_{NY}$ antigen, ——□—— unfixed $T_{WA}$ antigen, ---o--- unfixed $Y_{WA}$ antigen.

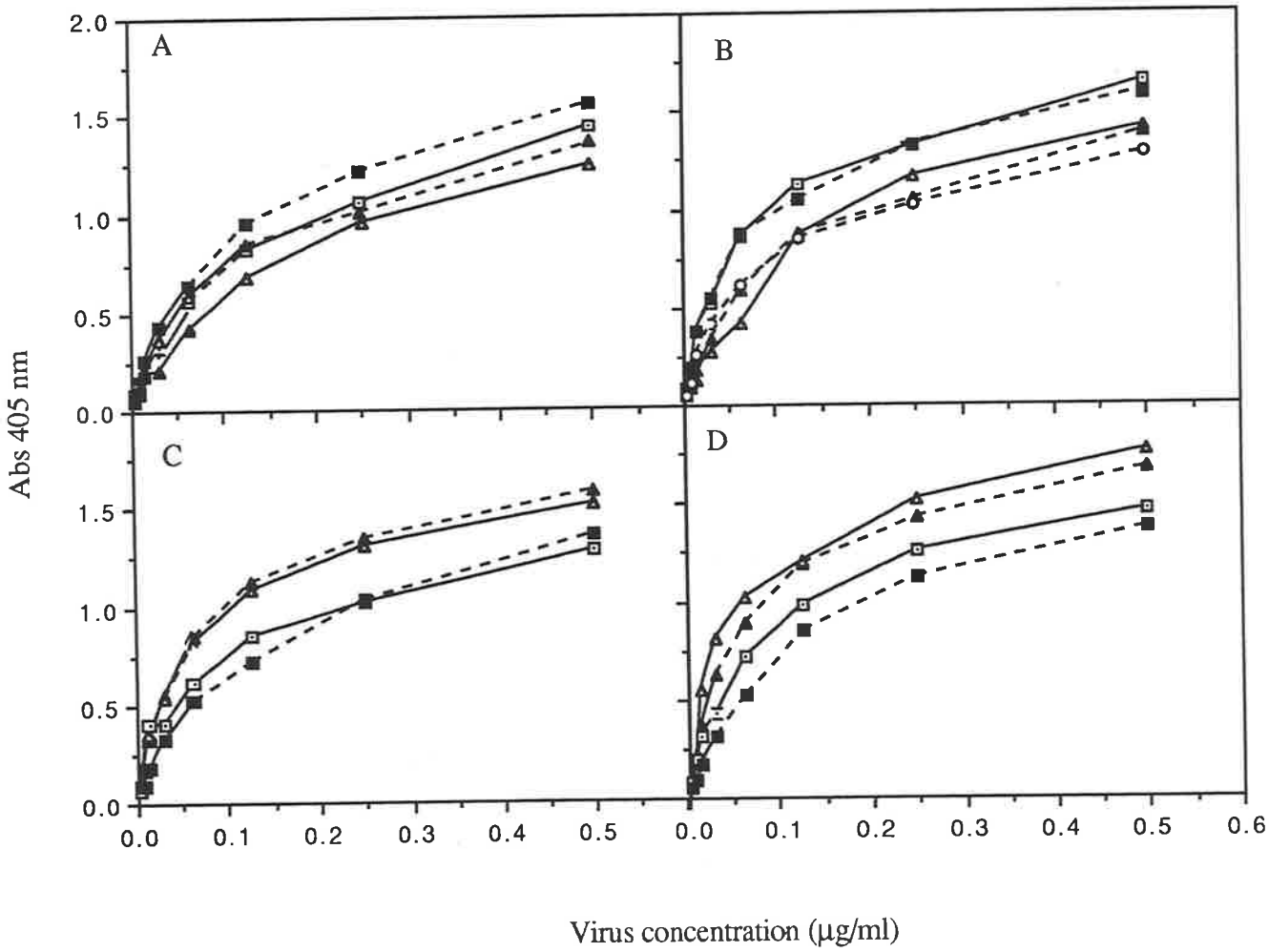
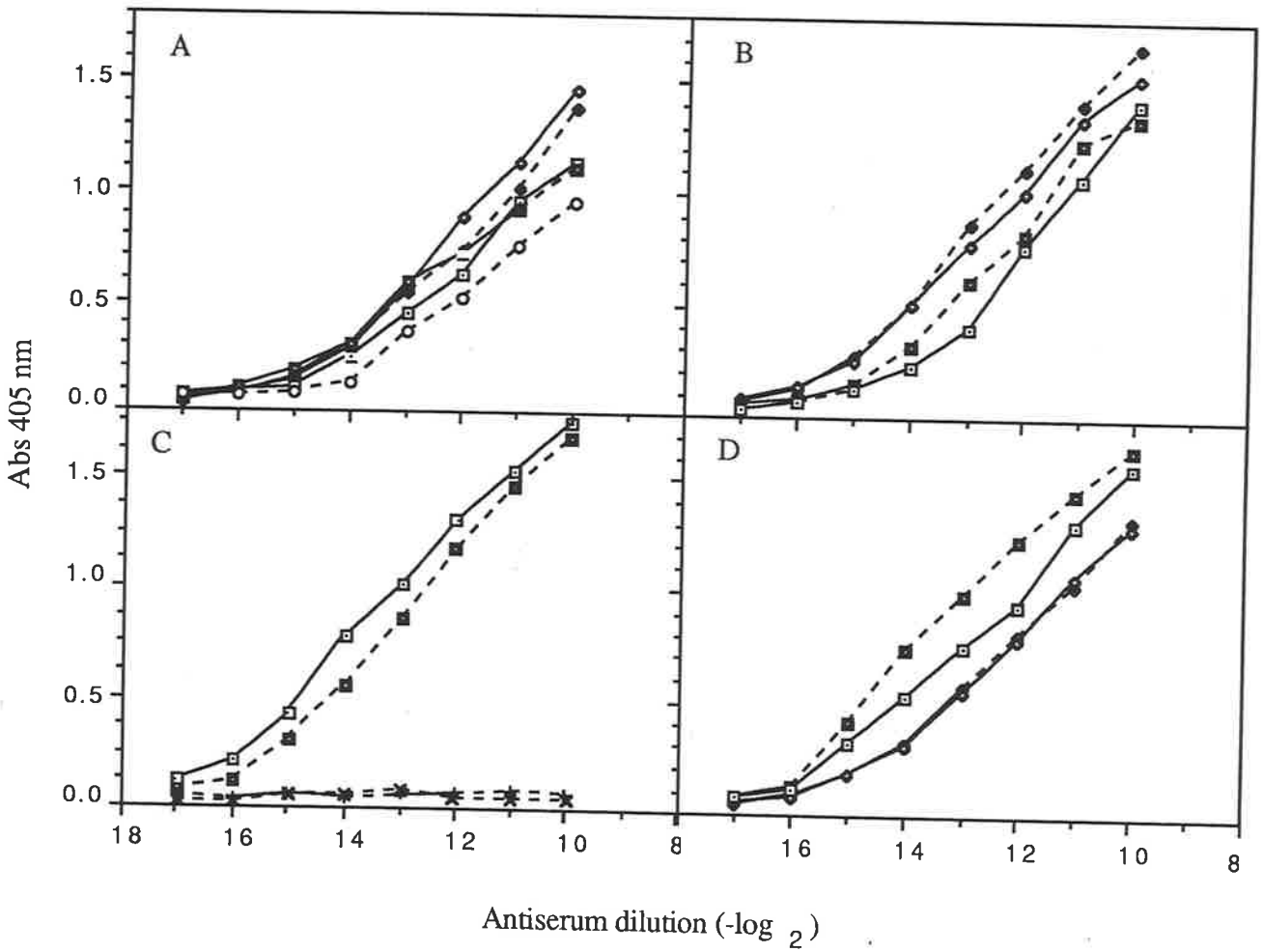

A
B
C
D
Abs 405 nm
Virus concentration (µg/ml)
2.0
1.5
1.0
0.5
0.0
0.1
0.2
0.3
0.4
0.5
0.6

**Figure 6.4.** Reactivity of fixed and unfixed antigen in PTA indirect ELISA . —■— fixed $L_{NY}$ antigen, —o— fixed $T_{WA}$ antigen, ---■--- unfixed $L_{NY}$ antigen, ---♦--- unfixed $T_{WA}$ antigen, ---o--- fixed $Y_{WA}$ antigen, ---+--- unfixed TMV-$U_1$ antigen, ---×--- unfixed TAV-V antigen. Unfractionated antisera to fixed $T_{WA}$ (A), to unfixed $T_{WA}$ (B), to fixed $L_{NY}$ (C), and to unfixed $L_{NY}$ (D) were used in a twofold dilution series.


A
B
C
D
1.5
1.0
0.5
0.0
18
16
14
12
10
8
Abs 405 nm
Antiserum dilution (-log 2)

serogroup I and II strains more clearly than PTA indirect-ELISA (see Chapter 4.B.1). TAV-V and TMV-$U_1$ were clearly not related to CMV strains (Fig. 6.4 C).

## B. Effect of Glutaraldehyde Fixation on the Stability of Virus During Long Term Storage

### 1. Analyses of physical changes in fixed and unfixed virus by sucrose density gradient centrifugation

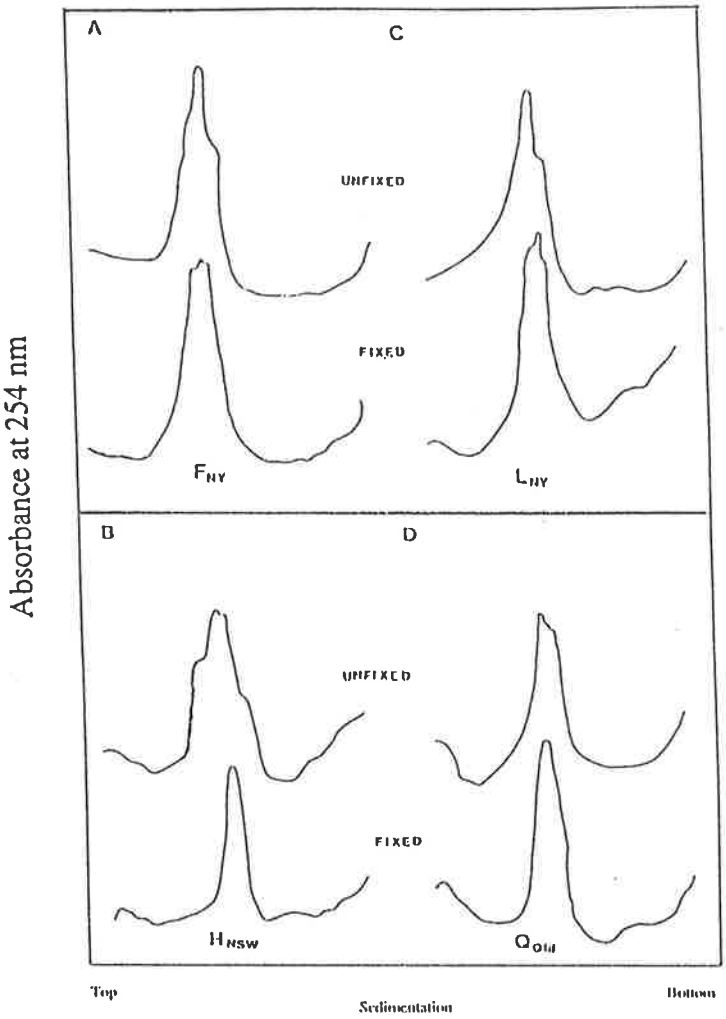
3 mg of purified fixed or unfixed virus were layered in 13 ml (SW 41) tubes containing 5 - 30% sucrose density gradients and run for 2 1/2 h at 25,000 rpm. The physical changes in virus particles were analysed with an ISCO density fractionator (see Chapter 2.B.2).

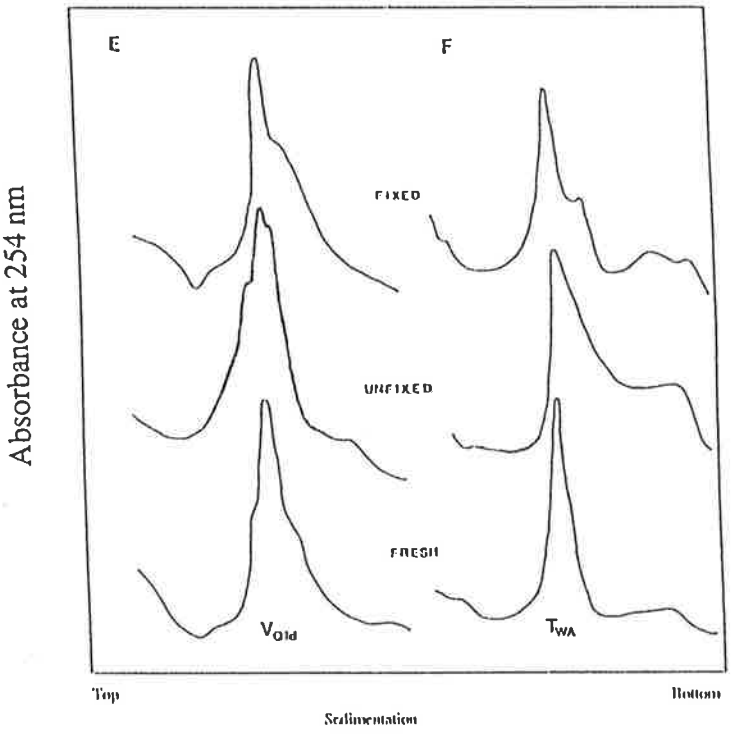
**Result :**

Fig.6.5 shows the sucrose density gradient analysis of fixed and unfixed virus preparations after long term storage (A,B,C,D) and a comparison with fresh virus preparation (E, F). The UV absorbance profiles for the fixed preparations of $F_{NY}$ and $L_{NY}$ virus which had been stored for 26 and 28 months, respectively, showed heterogeneity. Preparations of fixed $H_{NSW}$ and $Q_{Qld}$ which were kept for more than 30 months were more stable (shown by one single peak). However, preparations of unfixed $F_{NY}$ and $L_{NY}$ were more stable than unfixed $H_{NSW}$ and $Q_{Qld}$, after storage for 23, 27, 23 and 30 months, respectively. Fixed $T_{WA}$ (25 months) had two peaks, the peak preceding the virus peak possibly representing a dimer. The fresh virus preparations of $T_{WA}$ and $V_{Qld}$, and unfixed $T_{WA}$ showed only one peak (Fig. 6.5. E,F). The peak above the virus peak shown by unfixed $H_{NSW}$ and $V_{Qld}$, was possibly degraded particles.

It was concluded that strains of CMV have different stabilities after fixation with glutaraldehyde, as shown by the different shapes of the absorbance peak. Thus, fixed virus of some strains were stable and some degraded partially during storage.

**Figure 6.5**. Sucrose density gradient sedimentation analysis of fixed and unfixed virus after storage for more than one year, using an ISCO-density gradient fractionator. Preparations of fixed-$F_{NY}$ [stored for 26 months] and unfixed $F_{NY}$ [23 months] (A), fixed-$H_{NSW}$ [32 months] and unfixed $H_{NSW}$ [23 months] (B), fixed $L_{NY}$ [28 months] and unfixed $L_{NY}$ [27 months] (C), fixed $Q_{Qld}$ [34 months] and unfixed $Q_{Qld}$ [30 months] (D), were compared. Both fixed $V_{Qld}$ [21 months] and unfixed $V_{Qld}$ [21 months] (E) and fixed $T_{WA}$ [25 months] and unfixed $T_{WA}$ [25 months] (F) were compared with fresh virus preparations.


A
C
UNFIXED
FIXED
$F_{NY}$
$L_{NY}$
B
D
UNFIXED
FIXED
$H_{NSW}$
$Q_{Old}$
Absorbance at 254 nm
Top
Bottom
Sedimentation


E
F
FIXED
UNFIXED
FRESH
$V_{Old}$
$T_{WA}$
Absorbance at 254 nm
Top
Bottom
Sedimentation

## 2. Analysis of physicochemical changes by agarose gel electrophoresis

Virus preparations used in this experiment were the same as in the section 6.B.1, excluding the fresh virus preparations. Two μg of purified fixed or unfixed virus preparations was loaded on 1.5% agarose gels containing either TAE or TBE buffer, then subjected to electrophoresis at 100 V for 70 min. The gel was first stained with ethidium bromide to observe RNA patterns, then stained with coomassie blue to observe virus protein patterns (Hajimorad and Francki, 1991).

**Results :**

Both fixed and unfixed virus preparations of strains $F_{NY}$, $T_{WA}$, $H_{NSW}$, $C_{NSW}$, $L_{NY}$, $V_{Qld}$, $W_{Tas}$ and $Y_{WA}$ showed bands in gel electrophoresis. The strains of CMV produced different numbers of bands. For example, preparations of fixed $T_{WA}$, $V_{Qld}$ and $Y_{WA}$ virus produced 5, 4 and 3 bands, respectively.

Staining with ethidium bromide (Fig. 6.6 Top) showed that fixed virus contained a more rapidly migrating component than the unfixed virus. The mobility of bands from unfixed virus was slower than for fixed virus. Coomassie blue stained the bands of fixed and unfixed virus (Fig. 6.6 Bottom) after ethidium bromide (Fig. 6.6 Top).

The use of TBE as electrophoresis buffer separated virus components better than TAE buffer (data not shown).

## 3. Electron microscopy of virus particles

Particles of some CMV strains were observed under the electron microscope to determine whether particles were degraded during long term storage. Virus particles were stained with either 2% uranyl acetate or a mixture of 2% uranyl acetate with 3% K-phospho-tungstic acid pH 7.0 (addition of uranyl acetate prevents particle degradation due to PTA alone, Francki *et al.*, 1985).

The electron microscope observations showed that neither unfixed nor fixed $H_{NSW}$ virus particles were degraded (Fig. 6.7 A and B, respectively). The unfixed $V_{Qld}$ particles were apparently smaller than fixed particles, when stained with the mixture of

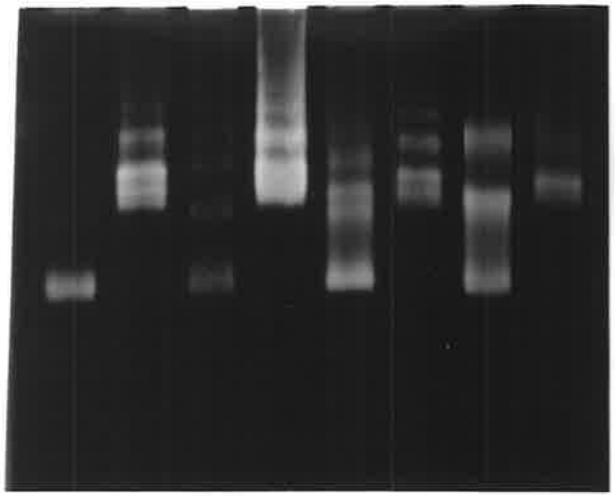
**Figure 6.6**. Analysis of the electrophoretic mobilities of fixed and unfixed virus in 1.8% agarose gels. Two μg of either fixed and unfixed virus from subgroup I : $F_{NY}$, $T_{WA}$, $H_{NSW}$, $C_{NSW}$ (A) and subgroup II : $L_{NY}$, $V_{Qld}$, $W_{Tas}$, $Y_{WA}$ (B) was loaded per well. Tracks no. 1, 3, 5 and 7 were fixed virus, and tracks no. 2, 4, 6 and 8 were unfixed virus. The gel was subsequently stained with ethidium bromide to visualize the viral RNA [shown by the bands] (Top), and coomassie blue to visualize the viral protein (Bottom).

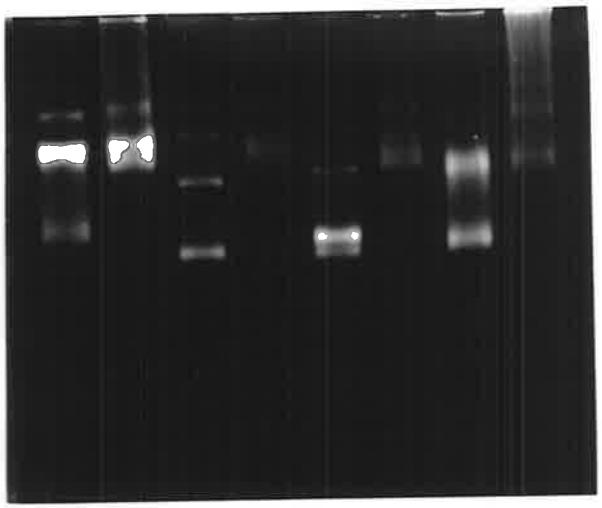
A. Subgroup I

1 2 3 4 5 6 7 8

B. Subgroup II

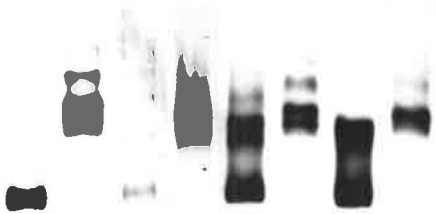
1 2 3 4 5 6 7 8





A

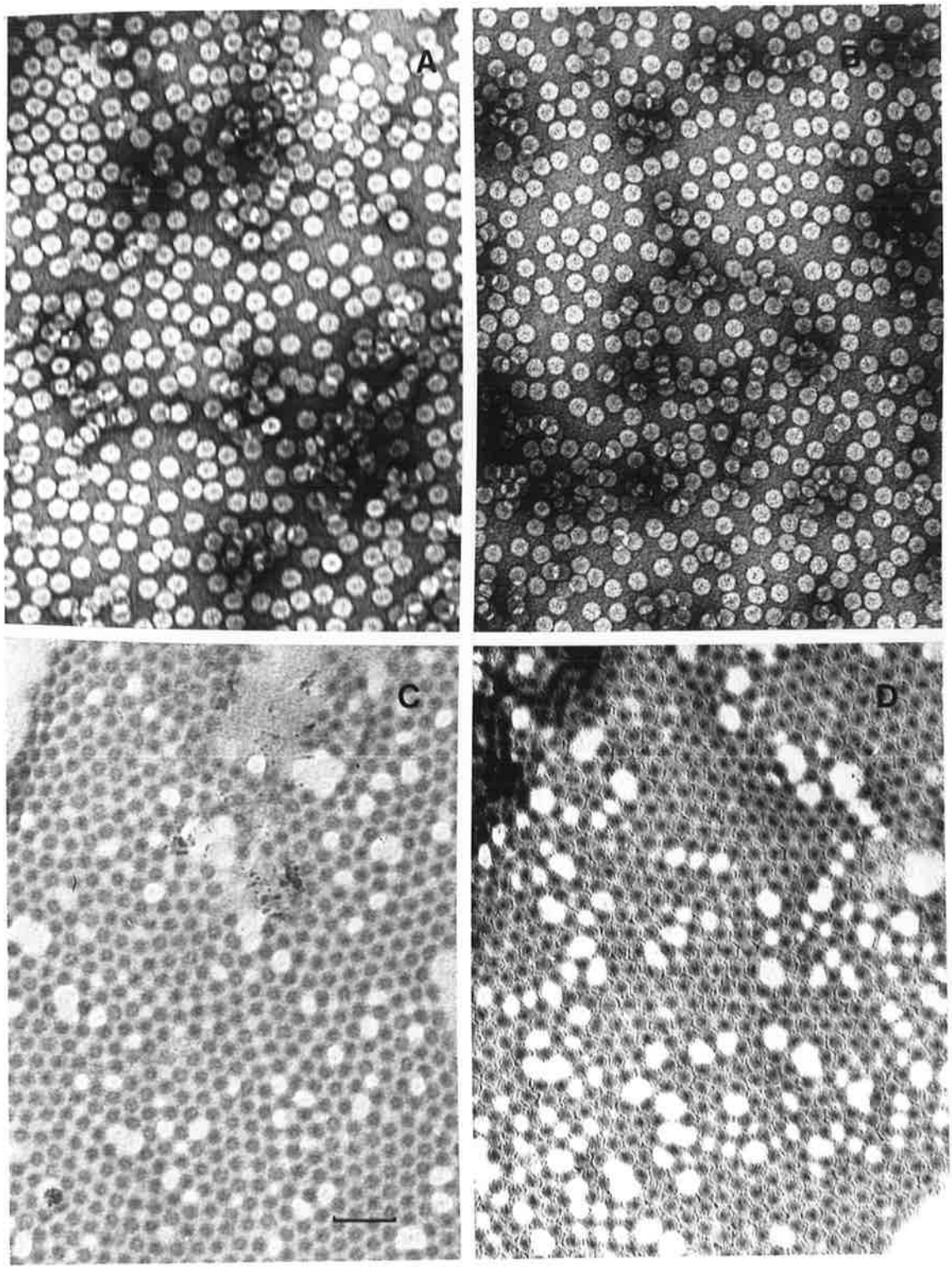
1 2 3 4 5 6 7 8



B

1 2 3 4 5 6 7 8

**Figure 6.7**. Electron micrograph of virus particles from unfixed and fixed purified virus preparations after long term storage. Preparations of unfixed (A), and fixed virus (B) of strain $H_{NSW}$ were stained with 2% uranyl acetate, unfixed (C) and fixed virus of strain $V_{QLD}$ (D) were stained with a mixture of 2% uranyl acetate and 3% phospho-tungstic acid (pH 7.0). Both fixed and unfixed particles had the same diameter. Bar scale = 100 nm.


A
B
C
D

uranyl acetate and phospho-tungstic acid. This was due to the core of the particles becoming partially positively stained, but the virus particles had the same diameter as those of fixed particles (Fig. 6.7.D). Preparations of unfixed and fixed antigens of strains $F_{NY}$, $H_{NSW}$, $C_{NSW}$, $T_{WA}$, $L_{NY}$, $Q_{Qld}$ $Y_{WA}$ were seen as intact particles after long term storage (data not shown).

## CONCLUSION :

1. With gel immunodiffusion tests using standard unfixed antigen homologous to unfixed antiserum, CMV strains could be serogrouped as in the same way as done for tests using fixed antigen and fixed antiserum. The exception was for strain $V_{Qld}$ with antisera to $T_{WA}$ and $H_{NSW}$.
2. With some strains of CMV, spur formation indicated that the antigen tested was distinct from the antigen homologous to antiserum. Spurs were also formed between homologous fixed and unfixed antigens with either fixed or unfixed antiserum.
3. The combined placement of homologous and heterologous fixed and unfixed antigen in gel immunodiffusion test showed that the reactions between subgroup I and II strains with either fixed or unfixed antiserum were variable.
4. DAS-ELISA distinguished the subgroup I and II strains more clearly than PTA indirect-ELISA using either dilution end points of fixed or unfixed antigen, or titres of fixed or unfixed antiserum.
5. During long term storage, some fixed and unfixed virus had degraded partially as shown by the appearance of double precipitin lines in gel immunodiffusion tests, and by the shape of the absorbance peak. However, the degradation of virus particles was not apparent by electron microscopy.
6. In agarose gel electrophoresis, the virus particles of CMV strains produced patterns of multiple bands. The mobility of fixed virus was faster than unfixed virus. The fixed virus showed a faster migrating component which was not present in preparations of unfixed stored virus.

# CHAPTER 7. RELATIONSHIPS BETWEEN CMV AND PSV

## INTRODUCTION

Peanut stunt virus [PSV] was first reported in the USA and Japan (Mink, 1972) then in Europe (Douine and Devergne, 1978, Xu *et al.*, 1986) and Korea (Kim *et al.*, 1988). PSV strains have been distinguished into Eastern [E] and Western [W] types on the basis of host species (Mink *et al.*, 1969) and serological tests (Mink *et al.*, 1967).

Thirteen PSV strains and isolates have been separated into four serogroups by gel immunodiffusion tests. These were, serogroups I, II, III and IV (Xu *et al.*, 1986). Using antiserum to either PSV-$B_2$, PSV-T, PSV-E, PSV-2 or PSV-W, the PSV-E antigen (serogroup I) could form either spurs or confluent lines with PSV-2 antigen (serogroup II). The relationships between serogroups I, II and III were close, and they were distantly related to serogroup IV [=PSV W]. Virus in the PSV serogroups I, II, and IV were able to infect peanut and pea cv. Perfected Wales (Xu *et al.*, 1986). Mink *et al.* (1969) observed that although the serological relationships between the Western type of PSV and some strains of CMV including Y (USA) were distant, the physicochemical properties of these viruses were very similar.

Natural recombination within members of the cucumoviruses should be able to occur, because most of these viruses have the same host species (Diaz-Ruiz and Kaper, 1983). Strain $Y_{WA}$ CMV is thought to belong to a separate serogroup of the cucumoviruses because it does not fall into either serogroup I strains or II strains (see Chapter 4.A.2). It seems possible that $Y_{WA}$ could be serologically related to PSV, firstly, because some CMV strains used in this thesis are serogically distantly related to TAV (Habili and Francki, 1975; Rao *et al.*, 1982), and secondly, $Y_{WA}$ reacted with some PSV antisera from Virginia and from Kentucky (see Chapter 2.A.2). PSV antigens were not available in our laboratory at this time (see Chapter 4, Table 4.4).

The SDI values between subgroups I and II of CMV is 1, between CMV and PSV is 3, between CMV and TAV is 6-7, and between PSV and TAV is 4-5 (Devergne and

Cardin, 1975). These values show that CMV and PSV are more closely than are CMV and TAV.

This chapter describes studies on the serological relationships between $Y_{WA}$ and PSV-E and PSV-2, and the RNA mobilities of these viruses.

## EXPERIMENTAL

### A. Comparison of serological properties of PSV, CMV and TAV

#### 1. Gel immunodiffusion tests

#### 1.a. Patterns of increase in titre of fixed and unfixed antisera to PSV-E during the immunization of rabbits

Antisera were prepared as described in Chaper 2.B.9. During the immunization of rabbits, the titre of antiserum to unfixed PSV-E in gel immunodiffusion, decreased rapidly after the highest titre was reached at 7-8 weeks (1/512). In contrast, the titre of antiserum to fixed PSV was increased to 1012 at 9 weeks and was then constant until 11 weeks (Fig. 7.1).

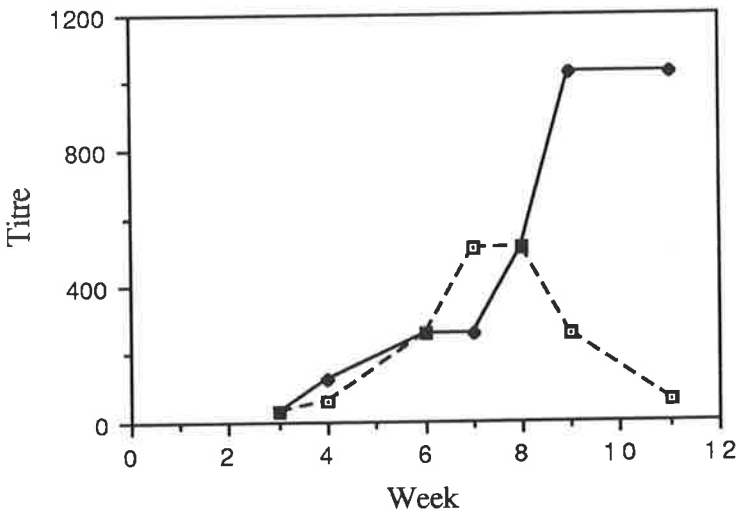



Figure 7.1. Titre of fixed and unfixed antisera to PSV-E in gel immunodiffusion, during the immunization of rabbits. —•— Fixed antiserum, ---□--- Unfixed antiserum. Rabbits received subcutaneous injections at weekly intervals.

### 1.b. Titres of antisera to fixed and unfixed PSV in tests against homologous and heterologous fixed and unfixed antigens

Antisera to PSV-E which had been prepared against fixed and unfixed antigens were titrated against both homologous fixed and unfixed antigens, and heterologous fixed and unfixed antigens of PSV-2, six CMV strains and one TAV strain.

Table 7.1 shows that the titre of fixed PSV-E antiserum in immunodiffusion tests against homologous fixed antigen was higher than to unfixed antigen, while the titre of unfixed antiserum to homologous unfixed and to fixed antigens were the same. PSV-2 was closely related to PSV-E. There was no reaction of these antisera to heterologous CMV and TAV antigens, except that unfixed PSV-E antiserum reacted with unfixed $Y_{WA}$, $A_{NSW}$ and $B_{SA}$ antigens by two, one and two twofold dilutions, respectively.

The SDI values between PSV-E and PSV-2, and strains of CMV, and TAV-V were 6-7, which indicates that their relationships are distant (Hamilton *et al.*, 1981).

Table 7.1. Titres of PSV-E antisera prepared against fixed and unfixed antigens to some strains of CMV and TAV in gel immunodiffusion tests.

| Antigen ** | Titre of antiserum prepared against : fixed PSV-E antigen (SDI) | | unfixed PSV-E antigen (SDI) | |
|---|---|---|---|---|
| fixed PSV-E | <u>1012</u> | | 256 | (0) |
| unfixed PSV-E | 512 | (1) | <u>256</u> | |
| fixed PSV-2 | 512 | (1) | 256 | (0) |
| unfixed PSV-2 | 512 | (1) | 256 | (0) |
| fixed CMV-$Y_{WA}$ | 0 | | 4 * | (6) |
| unfixed CMV-$Y_{WA}$ | 0 | | 0 | |
| fixed CMV-$T_{WA}$ | 0 | | 0 | |
| unfixed CMV-$T_{WA}$ | 0 | | 0 | |
| fixed CMV-$H_{NSW}$ | 0 | | 0 | |
| unfixed CMV-$H_{NSW}$ | 0 | | 0 | |
| fixed CMV-$A_{NSW}$ | 0 | | 2 * | (7) |
| unfixed CMV-$A_{NSW}$ | 0 | | 0 | |
| fixed CMV-$V_{Qld}$ | 0 | | 0 | |
| unfixed CMV-$V_{Qld}$ | 0 | | 0 | |
| fixed CMV-$B_{SA}$ | 0 | | 4 * | (6) |
| unfixed CMV-$B_{SA}$ | 0 | | 0 | |
| fixed TAV-V | 0 | | 0 | |
| unfixed TAV-V | 0 | | 0 | |

* No precipitin lines were observed after 24 h incubation, but faint bands appeared after 60 h incubation. The homologous titres are underlined.

** Antigens were adjusted to a concentration of 0.5 mg/ml and used at 10 µl per well

### c. Reactions between fixed and unfixed PSV, CMV and TAV antigens and antisera to fixed or unfixed PSV-E and CMV-$Y_{WA}$

Gel immunodiffusion tests with the combined placement of fixed and unfixed PSV, CMV and TAV antigens in adjacent wells were done as described in experiment 6.A.1.d.

The results of an experiment are shown in Fig. 7.2. Using antisera to fixed and unfixed CMV-$Y_{WA}$, no CMV strains reacted with fixed or unfixed PSV-E, PSV-2, or TAV-V antigens. Using fixed and unfixed antiserum to PSV-E, no precipitin lines were formed by standard PSV-E antigens to TAV-V, CMV-$Y_{WA}$ and CMV-$T_{WA}$ antigens.

These results lead to the conclusion that PSV-E and PSV-2 are serologically distinct from the strains of CMV and TAV-V tested.

## 2. Reaction in DAS-ELISA

The DAS-ELISA procedure was as described in Chapter 2.B.13.b. Antibodies (IgGs) from antisera prepared against fixed and unfixed PSV-E were used as the coating and antibody conjugate.

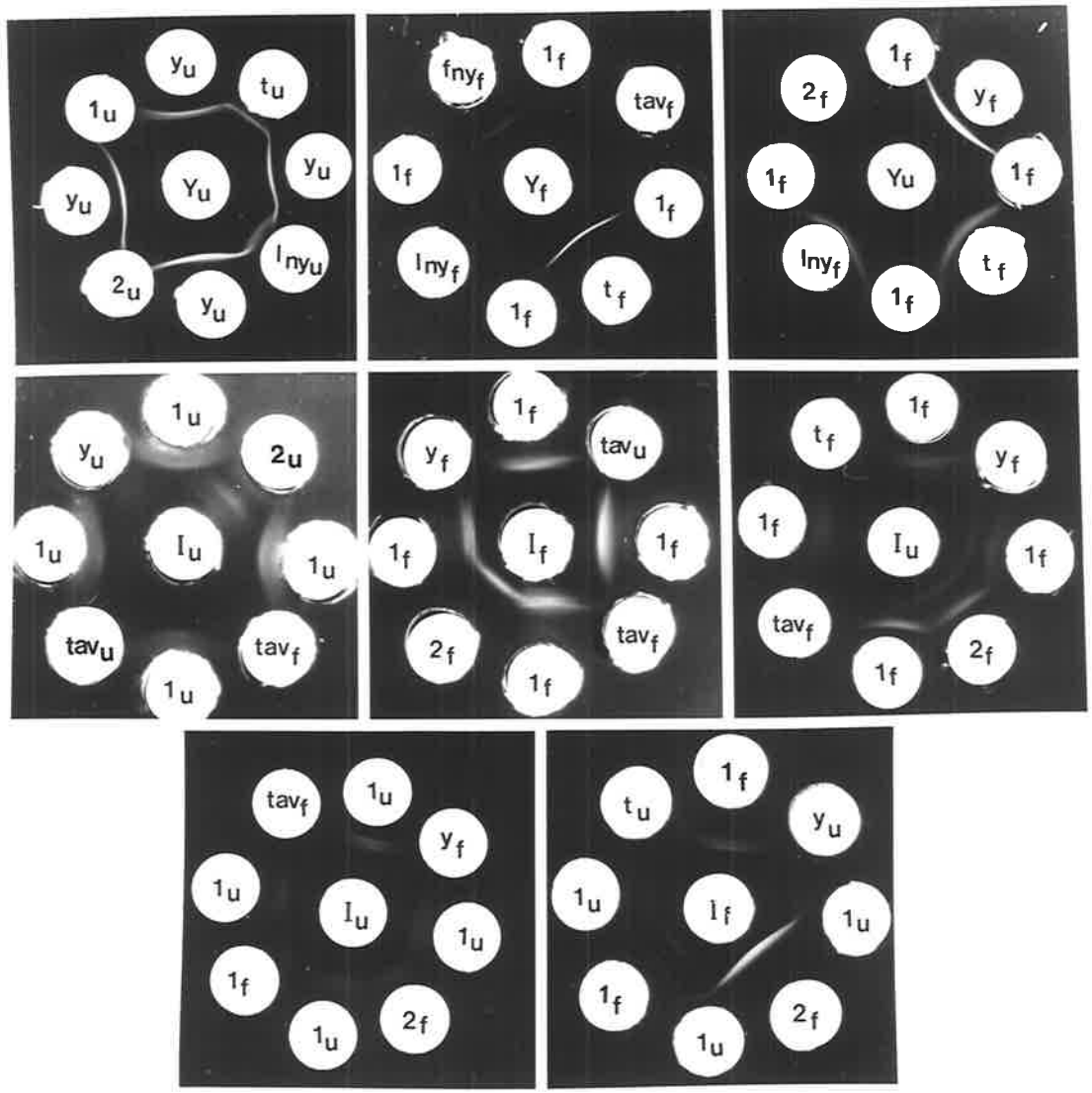
The results of DAS-ELISA showed that reactions between unfixed and fixed PSV-E antigens, and the IgG from antiserum to fixed PSV-E were the same (Fig. 7.3.A). However, when antibody from unfixed antiserum was used, the unfixed antigen reacted more strongly than fixed antigen (Fig.7.3 B).

In these tests, both CMV-$Y_{WA}$ and TAV-V were serologically distantly related to PSV-E.

## B. Comparison by northern blot hybridization

One μg of the RNAs of PSV-E and 2, and CMV-$L_{NY}$, $Y_{WA}$, $T_{WA}$ and $O_{Qld}$ were loaded on a 1.8% agarose gel and electrophoresed in TBE buffer for 60 min. The gels were stained with ethidium bromide to observe the RNA mobilities, then were transblotted to Hybond-N membranes. Preparations of cDNA to the total RNAs of

Figure 7.2. Reactions between fixed (f) and unfixed (u) antigens of some PSV, CMV and TAV strains, and antisera to either fixed (f) CMV-$Y_{WA}$, PSV-E or unfixed (u) PSV-E in gel immunodiffusion. Antigen concentrations were adjusted to 0.5 mg/ml and antisera were diluted to 1/16. Antigen 1 = PSV-E, 2 = PSV-2, t = $T_{WA}$, y = $Y_{WA}$, lny = $L_{NY}$, fny = $F_{NY}$, tav = TAV-V. Antiserum Y = CMV-$Y_{WA}$ and I = PSV-E.


yu
tu
1u
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Yu
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Yf
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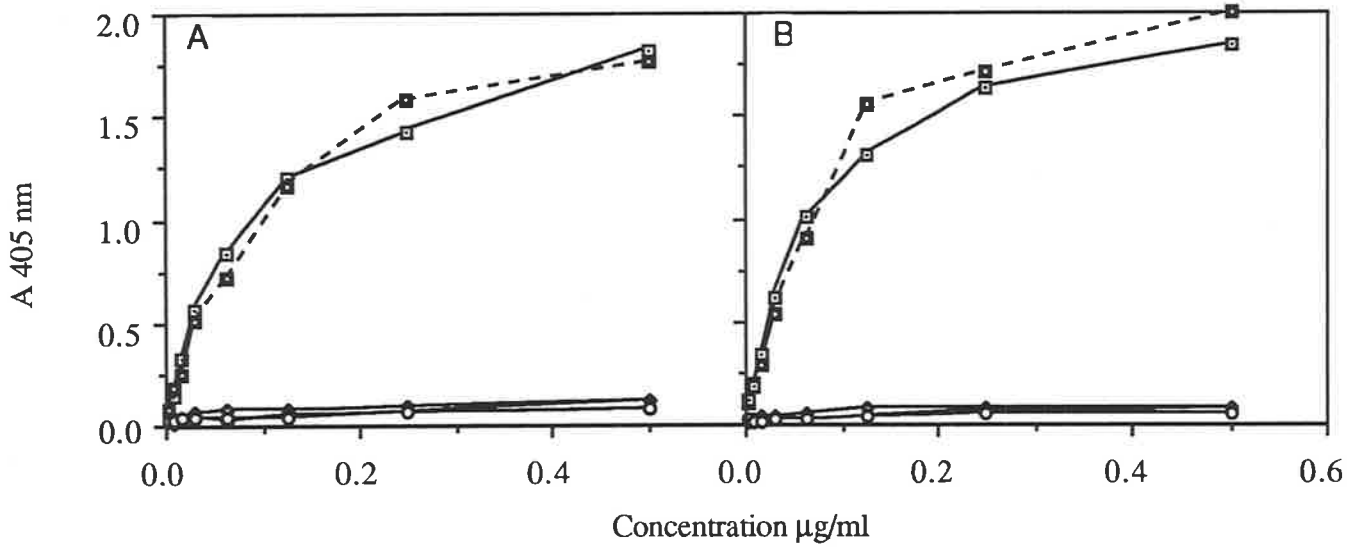



Figure 7.3. Serological reactions between PSV-E, CMV-YWA and TAV-V in DAS-ELISA. Enzyme-conjugates were against PSV-E IgG from antisera which were prepared to (A) glutaraldehyde fixed antigen (1/2000 dilution) and (B) native antigen (1/1000 dilution).

CMV-$Y_{WA}$ and PSV-E, and northern blot hybridization was as described in Chapter 2.B.6, 7 and 9.

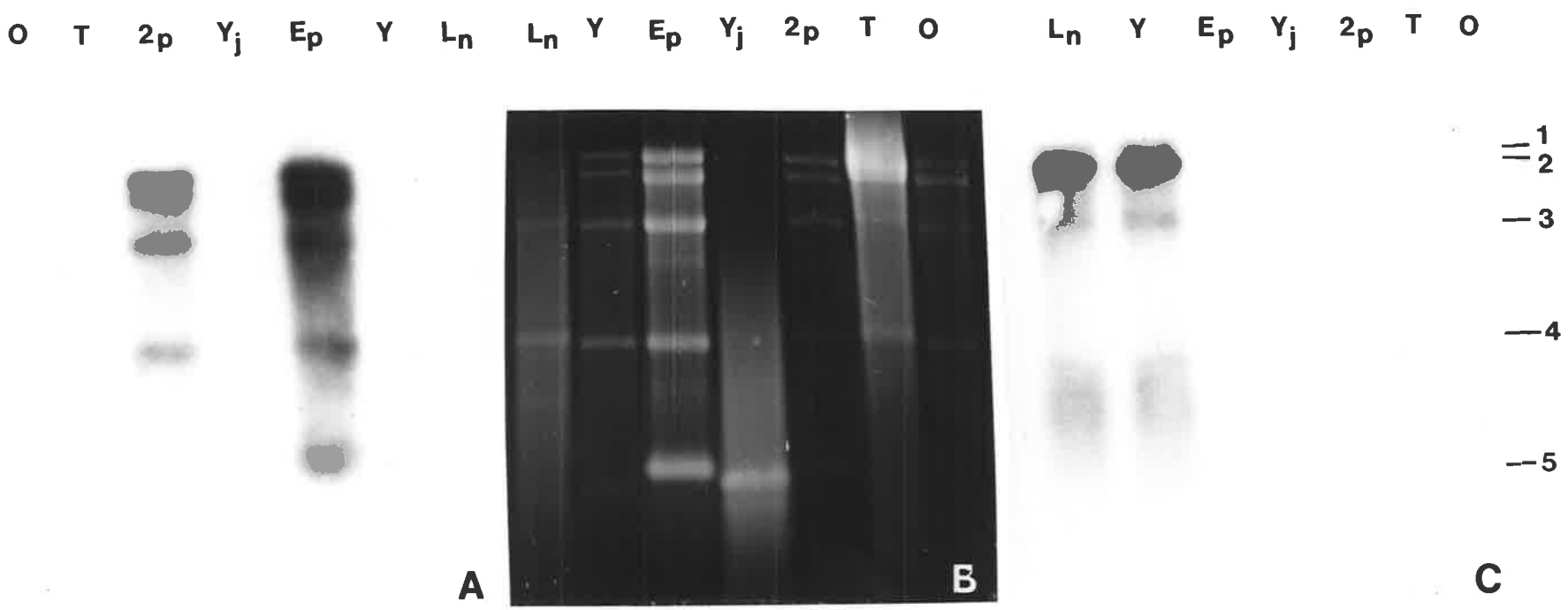
The mobilities of RNAs 1, 2, 3, and 4 of PSV and CMV were approximately the same. Both PSV-E and CMV-$Y_{Jpn}$ contained satellite RNAs. The satellite RNAs were also seen in PSV-2 and CMV-$Y_{WA}$ but were very faint. The mobilities of the satellites of PSV-E and 2 were approximately the same, but they were slightly smaller than $Y_{Jpn}$-sat (Fig. 7.4.B).

Northern blot hybridization showed that cDNA to PSV-2 produced strong signals to PSV-2 and PSV-E, but it did not cross hybridize with the RNA of any CMV strains (Fig. 7.4.A). Conversely, the cDNA to CMV-$Y_{WA}$ produced strong signals to CMV-$Y_{WA}$ and CMV-$L_{NY}$, but did not cross hybridize with the RNA of any PSV strains (Fig. 7.4 C).

## CONCLUSION

1. After 8 weeks immunization, the titre of antiserum to unfixed PSV-E decreased rapidly, while the titre of antiserum to fixed PSV-E increased.
2. In gel immunodiffusion tests and DAS-ELISA, PSV-E and 2 were distantly related to CMV and TAV-V. The SDI values in gel immunodiffusion tests were 6 to 7.
3. The fixed and unfixed PSV and CMV antigens reacted differently to fixed and unfixed antiserum preparations.
4. There was no cross hybridization between strains of PSV with strains of CMV, using cDNA to either CMV-$Y_{WA}$ or PSV-2 in northern blot hybridization tests.
5. Both PSV-E and 2 and CMV-$Y_{Jpn}$ contain satellites RNAs.

Figure 7.4. Comparison of RNA mobilities (a-f) in agarose gel electrophoresis (B). RNAs were then transblotted to a Hybond-N membrane and probed with cDNA transcribed from total RNAs of either PSV-E (A) or CMV-$Y_{WA}$ (C). The RNAs tested were CMV-$L_{NY}$ ($L_n$), CMV-$Y_{WA}$ (Y), PSV-E (Ep), CMV-$Y_{Jpn}$ ($Y_j$), PSV-2 (2p), CMV-$T_{WA}$ (T) and CMV-$O_{Qld}$ (O).


O T 2p Yj Ep Y Ln
Ln Y Ep Yj 2p T O
Ln Y Ep Yj 2p T O
1
2
3
4
5
A
B
C

# CHAPTER 8. EFFECT OF *Rhizobium* TREATMENT ON THE SUSCEPTIBILITY OF *Medicago truncatula* ssp. *truncatula* CV. JEMALONG AND *Lupinus angustifolius* CV. ILLYARRIE AND GUNGURRU TO CMV

## INTRODUCTION

In the symbiosis between legume plants and *Rhizobium*, the growth of nodules and the ability of *Rhizobium* to fix nitrogen are inhibited if nitrate is supplied in the growth medium (Streeter, 1985a; Kanayama *et al.*, 1990, Kanayama and Yamamoto, 1990). The efficiency of nitrogen fixation is influenced by the strain of *Rhizobium* (Orellana, *et al.*, 1980; Wongkaew, 1983; Streeter, 1985a), the concentration of nitrate supplement (Streeter, 1985a,b) and the species and cultivar of legume (Harper and Gibson, 1984; Orellana, *et al.*, 1987). The efficiency of nitrogen fixation can be measured by either the acetylene ($C_2H_2$) reduction activity (ARA) assay [ARA is calculated from the amount of ethylene ($C_2H_4$) produced from the $C_2H_2$] (Hardy *et al.*, 1968]), the nitrate reductase activity, the leghemoglobin content in nodules, the concentration of soluble nitrate in plant dry matter (Bergersen, 1980), or by $^{15}N$ incorporation (Sprent and Sprent, 1990).

Virus infection of nodulated legumes reduces plant growth (Tu, 1984). For example, the yield of soybeans infected with either soybean mosaic virus [SMV] or bean pod mottle virus [BPMV] (Ross, 1968) is reduced. PSV on *Vicia faba* (Ahmed, 1986), AMV on *M. truncatula* (Dall *et al.*, 1988), AMV on alfalfa (Tu and Holmes, 1980; Ohki *et al.*, 1986) and SMV on soybean (Tu *et al.*, 1970; 1970a) reduced the number, size and weight of nodules. Tobacco ringspot virus (TRSV) on soybean reduced leghemoglobin accumulation and $N_2$ fixation (Orellana *et al.*, 1977)

Virus content is higher in nodules than in roots of *P. vulgaris* infected with bean yellow mosaic virus [BYMV] (Orellana and Fan, 1978), peanut infected with peanut mottle virus (Wongkaew and Peterson, 1986) and red clover (*T. pratense*) infected with white clover mosaic virus [WCMV] (Khadhair and Sinha, 1983). The

presence of SMV in nodules of soybean was confirmed by observations under the electron microscope (Tu, 1973). However, nothing has been reported of the effect of nodulation on the virus-legume host interaction.

In this chapter, the effect of inoculation with *Rhizobium* on the susceptibility of *M. truncatula* spp. *truncatula* (barrel medic) cv. Jemalong and *L. angustifolius* (lupin) cv. Illyarrie and Gungurru to CMV was studied in plants grown in either soil or sand medium. The nitrogen fixation activity was measured by the ARA [see Chaper 2.B.19], and the number and size of nodules. Infected plants were identified by DAS-ELISA.

## EXPERIMENTAL

### A. EFFECT OF *RHIZOBIUM* ON CMV IN BARREL MEDIC

#### 1. Effect of a range of CMV strains on the susceptibility of barrel medic cv. Jemalong

The experiment described in this section was done to determine whether *Rhizobium* inoculation reduces the susceptibility of plants to CMV infection. It also compares some strains of CMV which were originally isolated from non legume spp. ($H_{NSW}$, $C_{NSW}$) with strains which were isolated from legume spp. ($E_{WA}$, $B_{SA}$, $S_{WA}$) [see Chapter 2.I.1] in their ability to infect barrel medic.

Barrel medic cv. Jemalong was grown in UC-soil medium (see appendix I-3). Sterilized seeds were either uninoculated [-] or inoculated [+] with the slurry of *Rhizobium* [R] (Nitrogerm, group A for lucerne and medic). Ten days after emergence (a.e.), plants were inoculated mechanically with CMV strains $H_{NSW}$, $C_{NSW}$, $E_{WA}$, $S_{WA}$, $B_{SA}$ or water (control). DAS-ELISA was done at 8 weeks a.i. The IgG used was from $O_{Qld}$ fixed antiserum.

**Result :**

Insect larvae were present in some pots and low survival of plants may have been due to larval feeding damage. Among the surviving plants, plants inoculated with

*Rhizobium* were less susceptible than uninoculated plants, and the number of plants infected with a range of CMV strains was variable (Table 8.1). CMV strains isolated from legume and non legume spp. appeared to have the same ability to infect barrel medic cv. Jemalong.

Table 8.1. Effect of *Rhizobium* treatment on the susceptibility of barrel medic to a range of CMV strains

| Treatment | Strain | No. of plants inoculated | No. of plants surviving | No. of survivors infected | % of survivors infected |
|---|---|---|---|---|---|
| -R | $H_{NSW}$ | 20 | 15 | 12 | 80 |
| | $C_{NSW}$ | 20 | 13 | 10 | 77 |
| | $E_{WA}$ | 20 | 14 | 11 | 79 |
| | $S_{WA}$ | 20 | 17 | 8 | 47 |
| | $B_{SA}$ | 20 | 11 | 5 | 45 |
| | Control | 20 | 12 | 0 | |
| +R | $H_{NSW}$ | 20 | 17 | 2 | 12 |
| | $C_{NSW}$ | 20 | 18 | 1 | 6 |
| | $E_{WA}$ | 20 | 14 | 0 | 0 |
| | $S_{WA}$ | 20 | 15 | 0 | 0 |
| | $B_{SA}$ | 20 | 12 | 1 | 8 |
| | Control | 20 | 14 | 0 | |

Virus was detected in leaf tips, shoots and root tips (Table 8.2) and nodules (data not shown). The virus content in leaf tips and root tips in the -R and+R treatment, and within plants infected with different strains also did not differ obviously. *Rhizobium* treatment increased the growth of healthy plants, but virus infection reduced plant growth (Table 8.2). The mean fresh weight of infected plants and roots in the -R and +R treatment, and within plants infected with different strains, did not differ markedly.

Table 8.2. Effect of *Rhizobium* inoculation and CMV infection on the growth of barrel medic cv. Jemalong. All values are means of infected plants. Rates of infection are shown in Table 8.1.

| Treatment | Mean fresh weight/plant (g) | (%) * | Mean root fresh weight (g) | (%) * | Abs 405 of root tips | Abs 405 of leaf tips |
|---|---|---|---|---|---|---|
| **- *Rhizobium*** | | | | | | |
| $H_{NSW}$ | 8.71 | 94 | 0.66 | 41 | 0.31 | 0.44 |
| $C_{NSW}$ | 7.99 | 86 | 0.88 | 55 | 0.26 | 0.59 |
| $E_{WA}$ | 5.64 | 61 | 0.62 | 39 | 0.27 | 0.15 |
| $S_{WA}$ | 8.96 | 97 | 0.82 | 51 | 0.15 | 0.45 |
| $B_{SA}$ | 8.60 | 93 | 1.04 | 65 | 0.12 | 0.51 |
| Control | 9.26 | | 1.60 | | - | - |
| **+ *Rhizobium*** | | | | | | |
| $H_{NSW}$ | 8.65 | 48 | 0.89 | 48 | 0.25 | 0.36 |
| $C_{NSW}$ | 5.62 | 65 | 1.22 | 65 | 0.34 | 0.22 |
| $E_{WA}$ | - | - | - | - | - | - |
| $S_{WA}$ | - | - | - | - | - | - |
| $B_{SA}$ | 9.60 | 78 | 1.42 | 76 | 0.27 | 0.29 |
| Control | 12.30 | | 1.87 | | - | - |

\- indicates data was not available

* As % of control

## 2. Effect of *Rhizobium* and nitrogen supplement on the susceptibility of barrel medic to CMV

To investigate whether nitrogen present in UC-soil medium used in the preliminary experiment above, may have affected the susceptibility of plants to CMV, barrel medic cv. Jemalong was grown in coarse sand medium (free of nitrogen).

To set up this experiment, sterilised seeds were inoculated with the slurry of *Rhizobium* and sown in sand medium in a square pot (6 x 6 inch; 16 seeds each). At 10 days after emergence (a.e.), plants were watered with nutrient solution containing either 0 mM ($NO_3$ free nutrient) or 7.5 mM $NO_3$ (see Chapter 2.B.18), twice weekly. The ARA is known to be completely inhibited at a $NO_3$ concentration of 7.5 mM (Silsbury *et al.*, 1986). Plants were inoculated with CMV-$C_{NSW}$ at 10 days a.e. Nitrogenase activity was measured by ARA, and the number of infected plants was determined by DAS-ELISA. Assays were done at 33 and 66 days a.i. Two days before the ARA assay, leaves were sampled for DAS-ELISA. The ARA assay was done as described in Chapter 2.B.19. All inoculated plants in each pot were assayed together because roots of individual plants were difficult to separate.

**Result :**

The assay at 33 days a.e. showed that the number of infected plants treated with -R+N and -R-N was the same, but at 66 days a.i. more plants were infected in the -R+N than in the -R-N treatment (Table 8.3). Following treatment with *Rhizobium*, infected plants were only detected in the +R+N+V treatment.The number of plants infected at 66 days a.i. was higher than at 32 days a.i. The ARA values in the +R+N+V and +R+N-V treatment were completely inhibited. The ARA values of healthy plants (+R-N) was higher at 32 days than at 66 days a.i. (data not shown).

Thus, this experiment showed that the presence of *Rhizobium* reduced plant susceptibility, but a high concentration of nitrogen (without *Rhizobium*) was associated with almost 100% infection.

Table 8.3. Effect of *Rhizobium* and nitrogen on the susceptibility of barrel medic cv. Jemalong to CMV-$C_{NSW}$

| Treatment * | No. of plants infected at 33 days a.i. | No. of plants infected at 66 days a.i. |
|---|---|---|
| -R, +N, +V | 8/32 | 30/32 |
| -R, +N, -V | - | - |
| -R, -N, +V | 8/32 | 15/32 |
| -R, -N, -V | - | - |
| +R, +N, +V | 1/32 | 9/32 |
| +R, +N, -V | - | - |
| +R, -N, +V | 0 | 0 |
| +R, -N, -V | - | - |

* +/- R indicates *Rhizobium* treatment, +/- N indicates nitrate concentrations of 7.5 mM or 0 mM, +/- V indicates inoculated with CMV-$C_{NSW}$ or uninoculated.
\- indicates data was not available.

**3. Effect of *Rhizobium meliloti* strain Rm 1021 and its mutant, Rm 1021 nif H⁻ : : Tn 5 on the susceptibility of barrel medic cv. Jemalong.**

*Rhizobium meliloti* strain Rm 1021 is able to fix nitrogen whereas its mutant, Rm 1021 nif H⁻ : : Tn 5 is unable to fix nitrogen. These *Rhizobium* cultures were kindly donated by Ms. M. Wexler and Dr. P.J. Murphy (Dept. of Crop Protection, The Waite Agric. Res. Inst.). The cultures of *Rhizobium* were maintained in T & Y medium.

Uninoculated or inoculated seeds with either of these two cultures of *Rhizobium* were grown in sand medium at 8 seeds/pot. At 10 days a.e., plants were inoculated mechanically with CMV-$T_{WA}$. At 12 days a.e., plants were watered with nutrient solution containing either 0 mM, 0.5 mM (a minimum concentration for plant growth), 5 mM or 7.5 mM $NO_3$ (a concentration which inhibits ARA). Assay for DAS-ELISA was done at 58 days a.i.

**Result :**

Plants were unhealthy when grown in the absence of $NO_3$, but they grew more vigorously following treatment with 0.5 mM N. Treatment with 5 mM and 7.5 mM N caused plants to grow even better, but with 7.5 mM N, nodulation was completely inhibited (data not shown). The number of active nodules (pink) on healthy plants inoculated with Rm 1021 and watered with either 0.5 or 5 mM N did not differ (Table 8.4). The number was fewer in infected plants (1 - 3) than in healthy plants, at these $NO_3$ concentrations.

The number of plants infected in the treatments 0.5 mM N+ Rm 1021 or 0.5 mM N+Rm 1021nif $H^-$ did not differ markedly. Treatment with a high concentration of $NO_3$ (5 mM and 7.5 mM) increased the number of plants infected, but this number did not differ for two *Rhizobium* inocula (Table 8.4).

Thus, both *Rhizobium* inocula, one of which was able to fix $N_2$ and the other not, had the same effect on the susceptibility of plants to CMV. Supply of a high concentration of $NO_3$ increased the number of plants infected to the same extent for both *Rhizobium* strains.

Table 8.4. Effect of treatment with *Rhizobium meliloti* strain Rm 1021 and its mutant, Rm 1021 nif H$^-$ : : Tn 5 on the susceptibility of barrel medic cv. Jemalong *

| Treatment | No. of plants infected | No. of nodules/ healthy plant | | Treatment | No. of plants infected | No. of nodules/ healthy plant | |
|---|---|---|---|---|---|---|---|
| | | pink | white | | | pink | white |
| 0 mM N+ Rm 1021 + V | 0/16 ** | 3 | 12 | 0.5 mM N+ Rm 1021 +V | 3/16 | 8 | 14 |
| 0 mM N+ Rm 1021 nif H$^-$ +V | 0/16 ** | - | 13 | 0.5 mM N+Rm 1021 nif H$^-$ +V | 4/16 | - | 22 |
| 7.5 mM N+ Rm 1021 +V | 5/16 | - | - | 5 mM N +Rm 1021 +V | 6/16 | 6 | 15 |
| 7.5 mM N+ Rm 1021 nif H$^-$ +V | 7/16 | - | - | 5 mM N +Rm 1021 nif H$^-$ +V | 8/16 | - | 19 |

* Assay was done at 58 days a.i. The mean number of nodules in infected plants was 1 - 3.

** Plants grew poorly (nitrogen deficiency)

N = nitrate concentration, Rm =*Rhizobium*, V= virus, CMV-$T_{WA}$

## B. EFFECT OF *RHIZOBIUM* ON THE SUSCEPTIBILITY OF lupin cv. Illyarrie and Gungurru to CMV

In the experiment with barrel medic, the distribution of virus and symptoms was uneven and a long time was required before symptoms appeared (see Chapter 5.3 and 4). Therefore, lupin was used instead of barrel medic in this set of experiments. With lupin, grafting or inoculation by aphid transmission was more easily done than with barrel medic. Lupin also produced clear symptoms and the time to induce symptoms was shorter than for barrel medic (see Chapter 5.1).

### 1. Effect of method of inoculation and different times of inoculation on the susceptibility of lupin cv. Illyarrie to strains $F_{NY}$ and $B_{SA}$

Seeds of lupin cv. Illyarrie were either inoculated or not inoculated with *Rhizobium* (Nitrogerm, Group G), sown in UC-soil and grown in the glasshouse. To determine when plants became immune to infection, they were inoculated at either 15, 25, 35, or 45 days a.e. The inoculation methods were either by aphids or patch grafting, with 8 replications. The aphid sp. used was *Myzus persicae*. Lupin infected with either strains $F_{NY}$ (subgroup I) or $B_{SA}$ (subgroup II) were used as inoculum. The number of plants infected was determined by DAS-ELISA as described in experiment 8.A.1. Samples were taken at 1 - 2 weeks a.i. for plants inoculated at 15 and 25 days a.e., and at 3 weeks a.i. for plants inoculated at 35 and 45 days a.e.

**Result :**

Success of inoculation at 15 days a.e. by aphid transmission in the -R treatment was nearly 100%. In the +R treatment, the number of plants infected with $F_{NY}$ was fewer than $B_{SA}$. Almost all plants were infected by patch grafting for both -R and +R treatments. Symptoms were first observed at 5-7 days a.e. (Table 8.5). For inoculation at 25 days a.e., the number of plants infected in the -R and +R treatment decreased for both aphid or patch grafting inoculation. The number infected was less in the +R than -R treatment. Symptoms first appeared at 1 - 2 weeks a.i. Inoculation with aphids at 35 and

Table 8.5. Number of lupin cv. Illyarrie infected by different methods at different times after emergence.

| Time of inoculation | CMV strain | Number of plants infected by : Aphid transmission -R [1] | Aphid transmission +R | Patch grafting -R | Patch grafting +R | Presence of nodules |
|---|---|---|---|---|---|---|
| 15 | $F_{NY}$ | 7/8 | 5/8 | 7/8 | 8/8 | - |
| | $B_{SA}$ | 8/8 | 7/8 | 8/8 | 8/8 | - |
| 25 | $F_{NY}$ | 4/8 | 2/8 | 6/8 | 3/8 | - |
| | $B_{SA}$ | 3/8 | 1/8 | 5/8 | 3/8 | - |
| 35 | $F_{NY}$ | 0 | 0 | 2/8 | 0 | + |
| | $B_{SA}$ | 0 | 0 | 1/8 | 0 | + |
| 45 | $F_{NY}$ | 0 | 0 | 0 | 0 | + |
| | $B_{SA}$ | 0 | 0 | 0 | 0 | + |

[1] with or without *Rhizobium* inoculation

45 days a.e. failed to infect any plants when assays were done at 3 weeks a.i. However, graft inoculation gave a low rate of infection in the -R treatment for the 35 day inoculation.

In general, plants were less susceptible when inoculated at 25 days a.e. and became immune after 35 days a.e. It was observed that nodules were formed at 30-35 days a.e. Plants were less susceptible just before and during nodulation. That is the number of plants infected were fewer in the +R than -R treatment, just before plants showed nodules. Symptoms induced by strain $F_{NY}$ appeared earlier and were more severe than for strain $B_{SA}$. Patch grafting was a more effective method than aphid transmission for inoculating plants at 35 days a.e.

**2. Effect of time of grafting, growth medium, day length and temperature of maintaining plants on the susceptibility of lupin cv. Gungurru to CMV**

Lupin cv. Gungurru was grown in either sand or soil medium. Plants grown in sand medium were maintained at 20° with a 13 h day length, and plants grown in soil medium were maintained in the glasshouse. Plants in sand medium were watered twice weekly with nutrient solution containing 1 mM $NO_3$, and plants in soil medium with tap water. Groups of plants were grafted at intervals of 5 days commencing after plants were 12 days a.e. Inoculum was from *N. glutinosa* infected with strains $F_{NY}$ or $B_{SA}$. Plants were assayed for DAS-ELISA and ARA at the times shown in Table 8.6.

**Results :**

When grafting was done at 12 days a.e., all plants in the +R and +R treatments (grown in either soil or sand medium) were infected with either $F_{NY}$ or $B_{SA}$ (Table 8.6). The number of plants infected decreased as time of grafting increased, yet the number infected remained higher in the -R than in +R treatment (Fig. 8.1 A and B). Plants became immune when grafting was done at and after 37 days a.e. The rate of infection of both -R and +R plants grown in either sand or soil medium did not differ (Fig. 8.1 A and B).

The efficiency of nitrogen fixation was lower in the infected plants than in the uninfected plants. This was shown by the ARA values and number of active nodules per plant (Table 8.6). The uninfected plants became active for fixing nitrogen at 30 days a.e., and the peak of ARA values was at 60 days a.e. (Fig. 8.1. C and D). The ARA values corresponded to the age of plant, and the size and number of active nodules, whereas the ARA values was less in infected plants (Table 8.6).

A difference in growth conditions affected the plant growth (Table 8.6). Lupins grown in sand at 20° grew very slowly from 3 weeks a.e., and -R plants still became infected when inoculations were done at 32 days a.e. (Fig. 8.1 A and B). At the same age, the number of nodules was less than those of lupin in the glasshouse (Fig. 8.1 C

Table 8.6. Effect of *Rhizobium*, growth-medium and time of inoculation on the susceptibility of lupin cv. Gungurru to CMV

| | | | UC-Soil medium | | | | | | | Sand medium + 1 mM N solution | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day of graft (after e-mergence) | Day of assay (after graf-ting) | Treatment | No. of plant infected with | | µmol $C_2H_4$ / plant /h | | No. of nodules per plant | | Diameter of nodules | No. of plant infected with | | µmol $C_2H_4$ / plant /h | | No. of nodules per plant | | Diameter of nodules |
| | | | $F_{NY}$ | $B_{SA}$ | $F_{NY}$ | $B_{SA}$ | Pink | White | (mm) | $F_{NY}$ | $B_{SA}$ | $F_{NY}$ | $B_{SA}$ | Pink | White | (mm) |
| 12 | 15 | +R+V | 5/5 | 5/5 | 0 [a] | 0 | 0 | 0 | - | 5/5 | 5/5 | 0 | 0 | 0 | 0 | - |
| | | -R+V | 5/5 | 5/5 | - | - | - | - | - | 5/5 | 5/5 | - | - | - | - | - |
| | | +R-V | - | - | 0 [b] | 0 | 0 | 0 | - | - | - | 0 | 0 | 0 | 0 | - |
| 17 | 17 | +R+V | 4/5 | 3/5 | 0 | 0 | 0 | 0 | - | 4/5 | 4/5 | 0 | 0 | 0 | 0 | - |
| | | -R+V | 5/5 | 5/5 | - | - | - | - | - | 5/5 | 5/5 | - | - | - | - | - |
| | | +R-V | - | - | 1.95 (0.07) | 1.81 (0.04) * | 8 (0.03) | 17 (0.05) | 1 - 4 | - | - | 1.01 (0.04) | 1.25 (0.07) | 5 (0.03) | 5 (0.05) | 1 - 3 |
| 22 | 18 | +R+V | 3/5 | 3/5 | 1.05 (0.09) | 1.27 (0.04) | 8 (0.03) | 9 (0.06) | 1 - 4 | 2/5 | 3/5 | 0.94 (0.09) | 0.87 (0.07) | 7 (0.04) | 18 (0.04) | 1 - 4 |
| | | -R+V | 5/5 | 5/5 | - | - | - | - | - | 5/5 | 4/5 | - | - | - | - | - |
| | | +R-V | - | - | 2.84 (0.25) | 3.03 (0.14) | 16 (0.06) | 22 (0.07) | 1 -5 | - | - | 2.72 (0.22) | 2.94 (0.16) | 12 (0.03) | 12 (0.04) | 1 - 4 |
| 27 | 21 | +R+V | 2/5 | 2/5 | 1.64 (0.08) | 1.47 (0.11) | 12 (0.07) | 18 (0.05) | 2 - 5 | 2/5 | 2/5 | 1.85 (0.12) | 1.11 (0.14) | 12 (0.08) | 28 (0.06) | 1 - 4 |
| | | -R+V | 5/5 | 5/5 | - | - | - | - | - | 5/5 | 4/5 | - | - | - | - | - |
| | | +R-V | - | - | 4.18 (0.12) | 4.87 (0.22) | 20 (0.06) | 22 (0.03) | 3 - 7 | - | - | 4.04 (0.23) | 3.63 (0.14) | 18 (0.07) | 29 (0.05) | 1 - 5 |

Continued (Table 8.6)

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 32 | 21 | +R+V | 0/5 | 0/5 | - | - | - | - | - | | 1/5 | 0/5 | 2.77 (0.21) | - | 12 (0.08) | - | 2 - 5 |
| | | -R+V | 2/5 | 3/5 | - | - | - | - | - | | 4/5 | 4/5 | - | - | - | - | - |
| | | +R-V | - | - | 5.25 (0.16) | 6.08 (0.21) | 24 (0.05) | 20 (0.03) | 2 - 8 | | - | - | 4.14 (0.17) | 4.43 (0.24) | 25 (0.04) | 22 (0.08) | 2 - 6 |
| 37 | 24 | +R+V | 0/5 | 0/5 | - | - | - | - | - | | 1/5 | 0/5 | 2.78 (0.19) | - | 14 (0.040) | - | 2 - 5 |
| | | -R+V | 0/5 | 1/5 | - | - | - | - | - | | 2/5 | 2/5 | - | - | - | - | - |
| | | +R-V | - | - | 6.95 (0.19) | 7.13 (0.22) | 32 (0.08) | 26 (0.05) | 2 - 8 | | - | - | 4.90 (0.07) | 4.65 (0.16) | 25 (0.10) | 28 (0.07) | 2 - 6 |
| 42 | 24 | +R+V | 0/5 | 0/5 | - | - | - | - | - | | 0/5 | 0/5 | - | - | - | - | - |
| | | -R+V | -0/5 | 0/5 | - | - | - | - | - | | 0/5 | 0/5 | - | - | - | - | - |
| | | +R-V | - | - | 5.12 (0.13) | 5.36 (0.20) | 26 (0.06) | 30 (0.07) | 2 - 7 | | - | - | 4.28 (0.12) | 3.89 (0.18) | 24 (0.06) | 29 (0.02) | 1 - 6 |
| 47 | 24 | +R+V | 0/5 | 0/5 | - | - | - | - | - | | 0/5 | 0/5 | - | - | - | - | - |
| | | -R+V | 0/5 | 0/5 | - | - | - | - | - | | 0/5 | 0/5 | - | - | - | - | - |
| | | +R-V | - | - | 4.95 (0.19) | 4.55 (0.13) | 24 (0.05) | 24 (0.12) | 1 - 7 | | - | - | 4.14 (0.23) | 3. 62 (0.18) | 22 (0.07) | 18 (0.02) | 1 - 6 |

* Standard error (±)
a ARA was measured only in infected plants
b ARA was measured in uninoculated plants as control

Figure 8.1. Effect of *Rhizobium* and time of grafting on the susceptibility and ARA of *L. angustifolius* cv. Gungurru grown in the UC-soil (A and C) and sand (B and D) media. Inoculum used for grafting was *N. glutinosa* infected with strain $F_{NY}$ or $B_{SA}$. Inoculation was done at 5 day intervals commencing after plants were 12 days a.e. Assay was by DAS-ELISA (A and B) and ARA (C and D). These graphs are another representation of the data in Table 8.6.

Symbols used in Fig. A and B

—▫— + strain $F_{NY}$ + *Rhizobium*

—◇— + strain $B_{SA}$ + *Rhizobium*

---▪--- + strain $F_{NY}$ - *Rhizobium*

---◆--- + strain $B_{SA}$ - *Rhizobium*

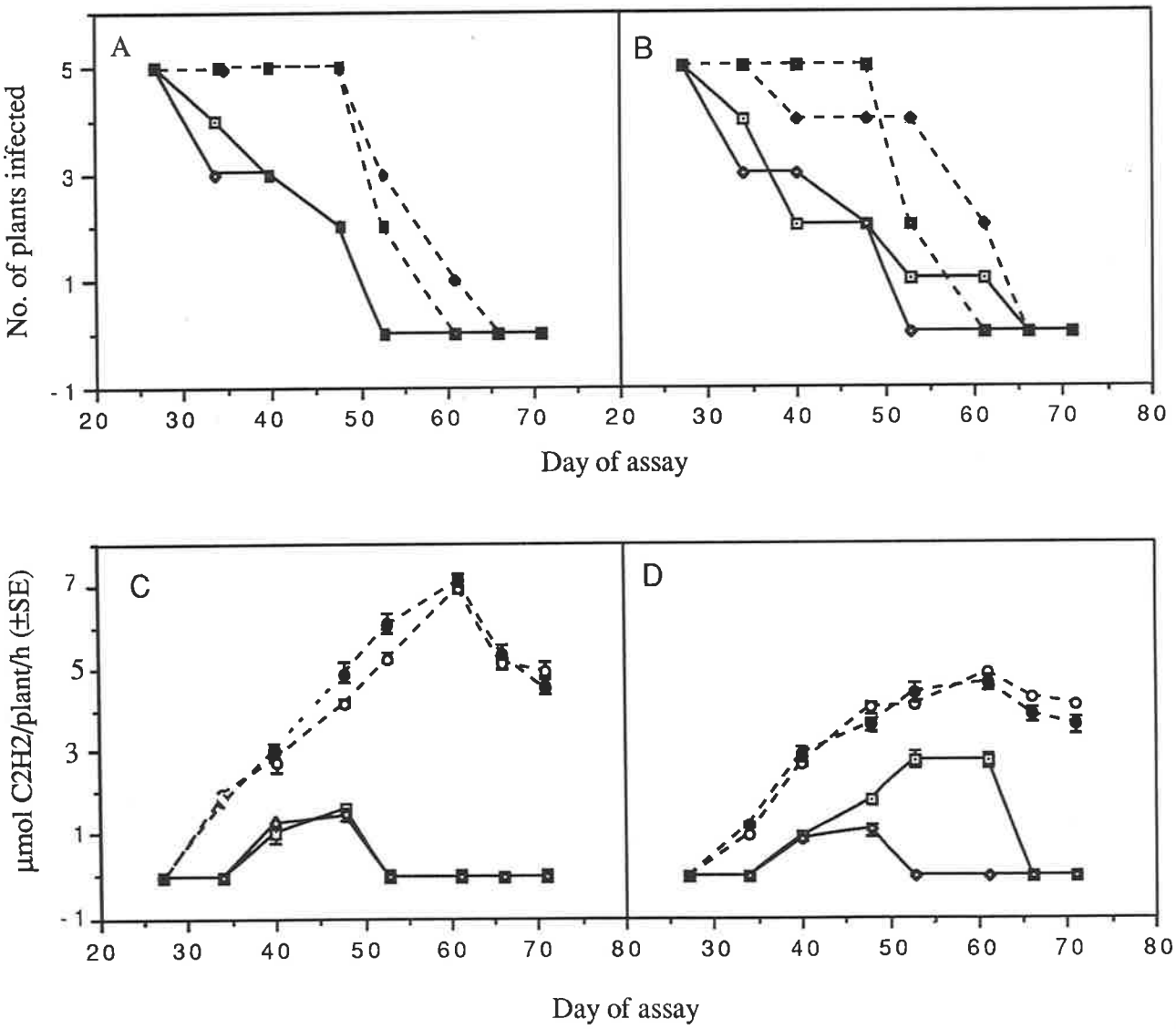
Symbols used in Fig. C and D

—▫— + *Rhizobium* + strain $F_{NY}$

—◇— + *Rhizobium* + strain $B_{SA}$

---○--- +*Rhizobium* - strain $F_{NY}$

---●--- +*Rhizobium* -strain $B_{SA}$


A
B
C
D
No. of plants infected
µmol C2H2/plant/h (±SE)
Day of assay
Day of assay

and D). Fig. 8.2 shows symptoms on lupin (+ and -R) grown in sand at 20°, when lupins were inoculated before or during nodulation. Success of grafting was achieved before nodulation, for example at 27 days a.e. for plants grown in the soil (glasshouse) and at 32 days a.e. for plants grown in the sand at 20°.

To test whether the source of inoculum affected these results, either *N. glutinosa* or lupin cv. Gungurru infected with $F_{NY}$ were used for grafting. Lupin (inoculated and uninoculated with R) were grown in UC-soil medium. Lupins were inoculated with either patch or tip grafting at 26 days a.e. (before the flowering stage) and 38 days a.e. (at the beginning of the flowering stage). The infected tip was grafted to healthy lupin at the third nodes from the tip.

This experiment showed that different sources of inoculum did not affect the number of plants infected (data not shown). +R plants were less susceptible than -R plants. Inoculum of infected lupin produced symptoms earlier than that of infected *N. glutinosa*. The tip grafting was unsuccessful for infecting plants. At the beginning of the flowering stage, plants seemed to become immune, both by symptom appearance and DAS-ELISA (data not shown).

### C. Electron microscope examination of nodules

The preparations of electron micrographs of roots and nodules from healthy and infected barrel medic cv. Jemalong with strains $C_{NSW}$ and $T_{WA}$ were done as described in Chapter 2.20. The aim of this study was to observe the nature of the association between virus and bacteroids of nodules.

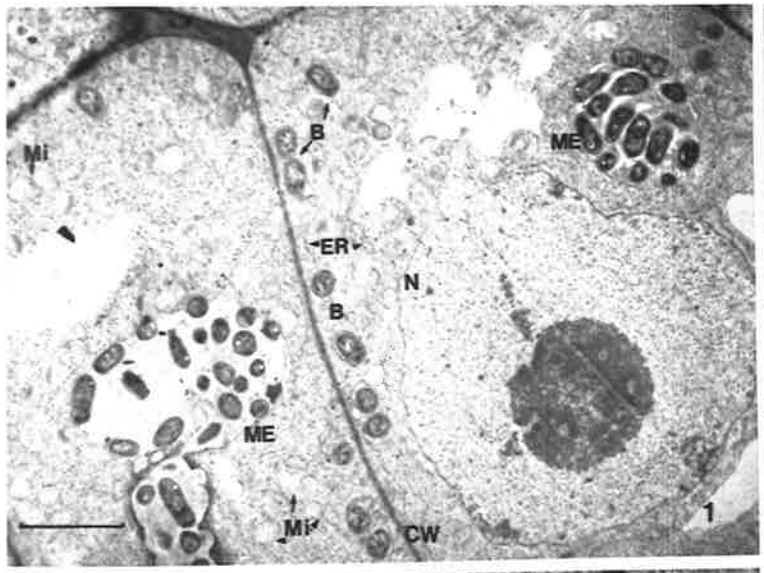
The development of bacteroids in nodules infected with commercial *Rhizobium* was studied in sections prepared without RNase treatment (Fig. 8.3). In two adjacent cells, the mature bacteroids in the membrane envelope (ME) were ready to release free bacteria into the cytoplasm, e.g. some bacteria can be seen along the cell wall (CW) [Fig. 8.3.1]. Other immature bacteroids (these were seen to be more compact in the membrane envelope than mature bacteroids) were observed in a thin membrane envelope located close to the nucleus, surrounded with abundant endoplasmic reticulum (ER) and

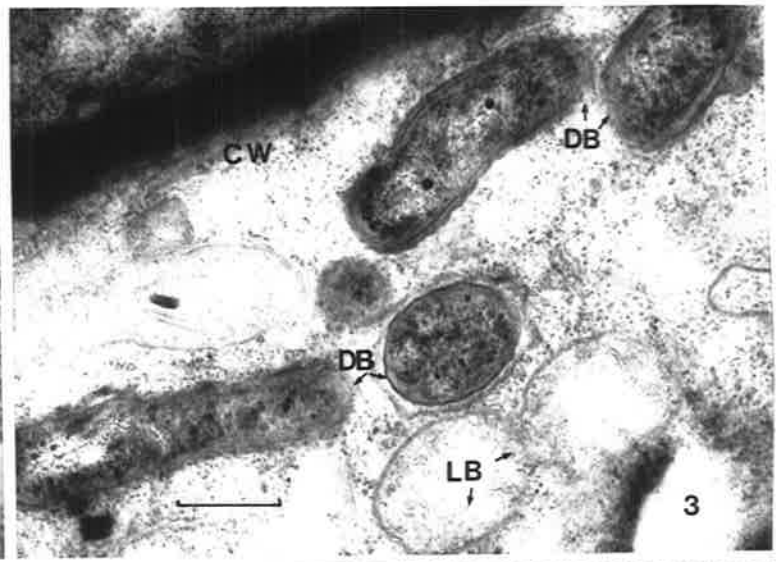
Figure 8.2. Symptoms on lupin cv. Gungurru grown in the sand medium (20° room). **1.** Plants treated without (-) *Rhizobium* (left) and with (+) *Rhizobium* (right) infected with strain $F_{NY}$ when inoculated before nodulation (17 days a.e.). **2.** Plants inoculated **(i)** with strain $B_{SA}$ at 27 days a.e. From left to the right, +r, uninoculated **(u)**; +r, infected; and -r, infected .

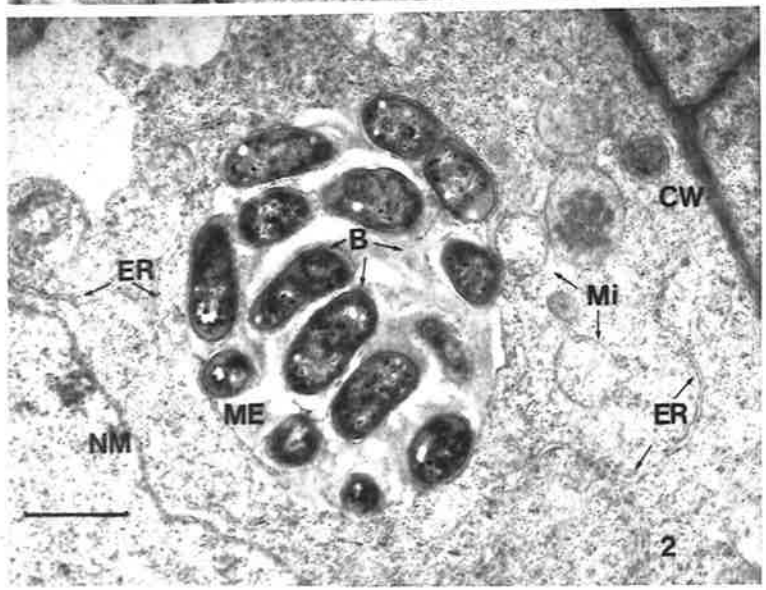

1
i - r
i+r

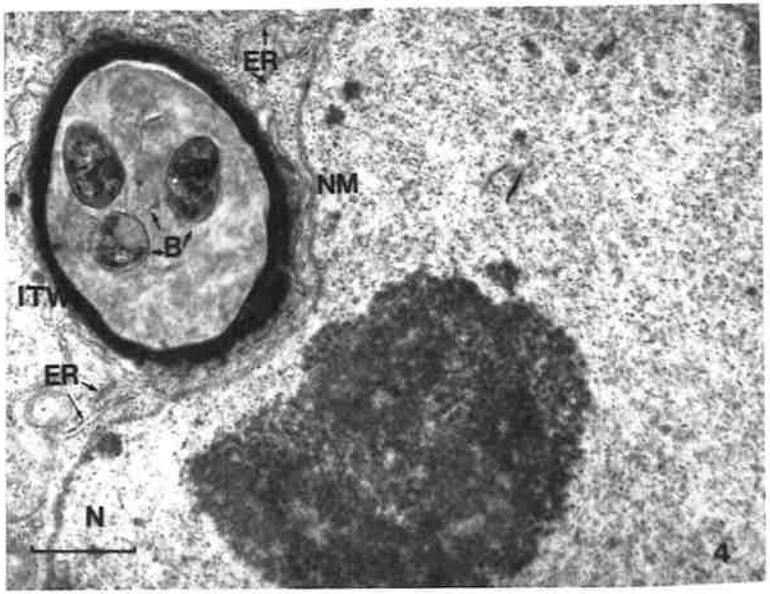

2
u+r
i+r
i-r

Figure 8.3. Electron micrographs of general development of bacteroid in the nodule of barrel medic cv. Jemalong infected with commercial *Rhizobium*. Plates 1 and 3 were from plants uninoculated with CMV, plates 2 and 4 were from plants infected with CMV-$C_{NSW}$. Plate 1, mature bacteroids with several free bacteria (B) which had been released from the membrane envelope (ME) are shown located along the cell wall (CW), bar = 3.75 μm. Plate 2, close up of section in plate 1, immature bacteroids (B) in ME, located close to the nucleus (N) and surrounded with abundant mitochondria (Mi) and endoplasmic reticulum (ER), bar = 1.5 μm. Plate 3, deteriorating bacteria (DB) with wrinkled peribacterial membrane, and lysing bacteria (LB) located close to the CW, bar =0.75 μm. Plate 4, the infection thread wall (ITW) located close to the nucleus membrane (NM), bar =1.5 μm.


Mi
B
ME
ER
N
B
ME
Mi
CW
1


CW
DB
DB
LB
3


CW
B
ER
Mi
ME
ER
NM
2

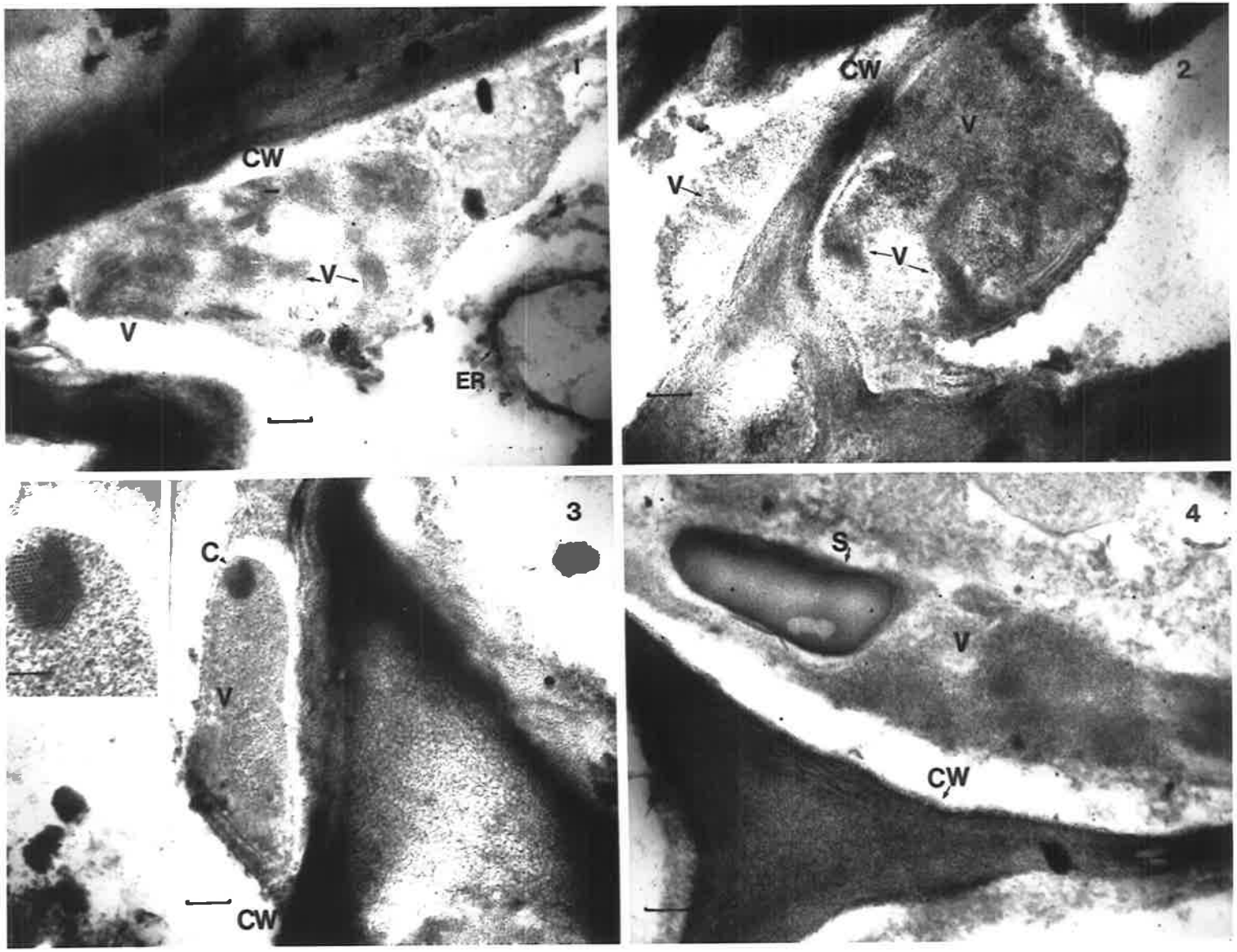

ER
NM
B
ITW
ER
N
4

mitochondria (M) [Fig. 8.3.2). In the late stage, bacteria seemed to deteriorate as shown by wrinkled peribacterial membranes which had ruptured in one side, then lysed (Fig. 8.3.3). The mature bacteroids in the infection thread which had a thick membrane (ITW) as shown in Fig.8.3.4, would infect the adjacent cell (Dart and Mercer, 1963; Dixon, 1963; Mosse, 1964).

Although Fig 8.3.1 and 2 were from healthy nodules, and Fig. 8.3.3 and 4 were from infected nodules, these figures only show the general development of bacteroids in the nodule cell. In these sections it was difficult to distinguish virus particles from ribosomes. Therefore, a batch of RNase treated root and nodule tissues was examined

In the cross-sections of root and nodule tissues treated with RNase, the individual virus and aggregation of virus particles can be distinguished from other cell organs. In the nodules infected with strain $T_{WA}$ which were taken from plants treated with *R. meliloti* strain Rm 1021 (this strain fixes nitrogen), individual virus particles and aggregates were found only in cells free of bacteroids. The cells containing virus could be either adjacent to or distant from the cells containing bacteroids. In many sections, aggregates of virus particles were located along the cell wall (Fig.8.4 ). In the nodules infected with strain Rm 1021 nif $H^-$ : : Tn 5 (this mutant does not fix nitrogen), the virus crystals were surrounded with some individual virus particles (Fig. 8.4.3). The virus crystals showed that the individual virus particles have a hexagonal structure (inset). Cross sections of infected roots of plants treated with 7.5 mM $NO_3$ and *Rhizobium* showed that virus aggregates and bacteroids were not present in the same cells (Fig.8.4.2 and 4).

Figure 8.4. Electron migrographs of aggregation of virus particles of CMV-$T_{WA}$ in the infected nodules [+ 0.5 mM $NO_3$] (plates 1 and 3) and roots [+ 7.5 mM $NO_3$] (plates 2 and 4). Nodule and root tissues were treated with RNase after glutaraldehyde fixation and post fixed with $OsO_4$. Plates 1 and 2 were from plants infected with *Rhizobium meliloti* strain Rm 1021, and plates 3 and 4 were from plants infected with a mutant of this strain (Rm 1021 nif $H^-$ : : Tn 5). CW cell wall, V aggregate of virus particles, ER endoplasmic reticulum, S starch, C crystal of virus. Bars = 1000 nm, inset figure (plate 3) = 400 nm.


1
CW
V
V
ER
2
CW
V
V
V
3
C
V
CW
4
S
V
CW

## CONCLUSION

1. Barrel medic and lupin plants were less susceptible to CMV infection in the presence of *Rhizobium*. The number of plants infected increased with the increase of concentration of $NO_3$. A $NO_3$ concentration of 7.5 mM completely inhibited nodulation, but it was associated with an increase in the number of plants infected.
2. The latency of virus on barrel medic was longer than on lupin.
3. The ARA values of infected plants were lower than uninoculated plants.
4. At 66 days a.i., barrel medic in the treatment +N -R was the most susceptible to CMV, while +N +R plants were the least susceptible.
5. The number of plants infected in the treatment with *Rhizobium meliloti* strain Rm 1021 and its mutant, Rm 1021 nif $H^-$ : : Tn 5, did not differ.
6. The ARA values of barrel medic or lupin nodules increased with the age of the plants and they declined with a high concentration of nitrate (5 and 7.5 mM $NO_3$) supplement.
7. Grafting was found to be a suitable method for inoculating lupin at the nodulation stage (e.g. after 30 days a.e.).
8. Virus was found in roots and nodules.
9. In the ultrathin sections of nodules and roots of barrel medic, aggregates of virus particles and bacteroids were present in different cells.

# CHAPTER 9. EFFECT OF *Rhizobium* OR A NITROGEN SUPPLEMENT ON VIRUS CONTENT IN LUPIN cv. Gungguru and *Nicotiana glutinosa*

## INTRODUCTION

In the absence of *Rhizobium* (R), virus content in infected plants supplied with nitrogen is greater than in plants without a nitrogen supplement (Spencer, 1941a; 1942a;b). In the presence of *Rhizobium*, mungbean cv. Pusa Baisakhi infected with CBMV had a higher virus content (based on a local lesion assay on *C. amaranticolor* ) when nitrate was added to the nutrient solution (Singh and Srivastava, 1983). The number of local lesions for the +R infected plants was slightly fewer than for the -R infected plants, but not significantly different. The increase in number of local lesions from different parts of plants (e.g. leaf, stem, roots and nodules) in the +R and -R treatment was positively correlated with the concentration of nitrate and time after inoculation.

L-glutamine and L-asparagine are intermediate nitrogen compounds in the nitrogen fixation pathways of nodulated legumes (Boland *cit.* Bergersen *et al.*, 1980). The effects of *Rhizobium* and nitrogen supplements including these amino acids on virus content was studied in this chapter. Virus content was assayed by ELISA.

## EXPERIMENTAL

### A. Effect of either L-glutamine or L-asparagine on virus content and ARA values in lupin

The purpose of this experiment was to investigate whether L-glutamine or L-asparagine have an effect on virus content in plants treated with *Rhizobium*. A further aim was to compare the effect of different concentrations of $NO_3$ on virus content.

Amino acid solutions were applied to the basal petioles through a wick (Oti-Boateng, 1989). The wick consisted of a hypodemic needle (25G, 0.5x16 mm) which

was threaded with a piece of cotton thread. An amino acid solution of 1.2 ml was placed into a 1.5 ml centrifuge tube, and the thread was dipped into this solution. The cap of the tube was partially closed to allow the solution to be taken up by the plant, by capillary action, and to minimize evaporation. Both wick and tube were covered with aluminium foil.

Lupin cv. Gungurru seeds which were either inoculated with *Rhizobium* (+R) or uninoculated (-R) were grown in sand medium. Two weeks a.e., plants were watered with either 1 mM or 5 mM nitrate (see Appendix 2). At 26 days a.e. (see Chapter 8.B.2) plants were inoculated by patch grafting with lupin infected with $F_{NY}$. At 35 days a.e, watering with nutrient solutions was stopped until plants were ready to assay. At 38 days a.e., wicks with either 10 mM L-glutamine, L-asparagine or water were applied to the basal petiole of infected plants. ELISA and ARA assays were done at 60 h and 106 h, respectively, after application of wicks.

Estimates of the virus content in tip leaves was done by PTA-indirect ELISA (see Chapter 2.14 c). Crude extracts of leaf sap were prepared by grinding 0.1 g of leaf tips with 400 µl ELISA sample buffer. Unfractionated antiserum to CMV-$F_{NY}$ (1/1000 dilution) was used as the specific antibody.

**Result :**

Table 9.1 showed that the number of lupins infected in the +R was fewer than in the -R treatments, whereas the number of infected plants following treatments with 1

Table 9.1. Effect of *Rhizobium* inoculation and nitrate concentration on the susceptibility of lupin cv. Gungurru to CMV

| Treatment | Number of plants infected : |
|---|---|
| + R, 1 mM $NO_3$ | 6/16 |
| + R, 5 mM $NO_3$ | 7/16 |
| - R, 1mM $NO_3$ | 11/16 |
| - R, 5 mM $NO_3$ | 13/16 |

and 5 mM $NO_3$ were similar. When a wick was applied to the stem at 12 days a.i., the infected lupins showed severe stunting, curling and distortion, and some of them showed necrosis of the basal stem.

Neither L-glutamine nor L-asparagine had a significant effect on virus content (Fig. 9.1). Moreover, virus content in the +R or -R lupins, and with different levels of $NO_3$, was the same.

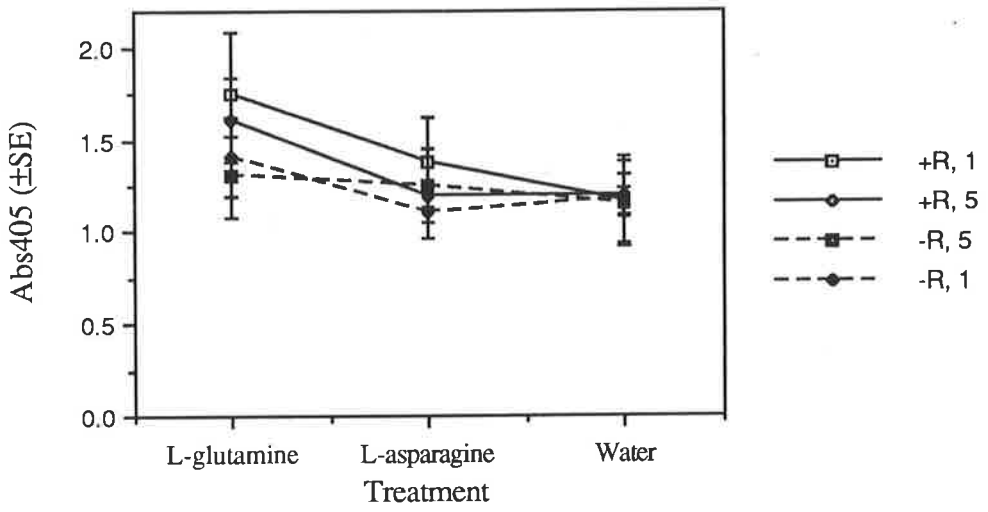



Figure 9.1. Estimate of virus content in infected lupin at 60 h after application of either L-glutamine, L-asparagine or water by wicks. +/- R indicates plants inoculated or not inoculated with *Rhizobium;* 1 and 5 indicate nitrate concentration (mM) supplied before insertion of the wick.

Neither amino acid affected the ARA values of infected lupins in the 1 and 5 mM N treatment (Fig. 9.2). The ARA values was higher in healthy plants than in infected plants. In the 5 mM $NO_3$ treatment, L-glutamine and L-asparagine increased the efficiency of nitrogen fixation more than with water. However, these amino acids did not significantly effect on the ARA values in the 1 mM $NO_3$ treatment. Treatment with 5 mM $NO_3$ reduced the ARA values.

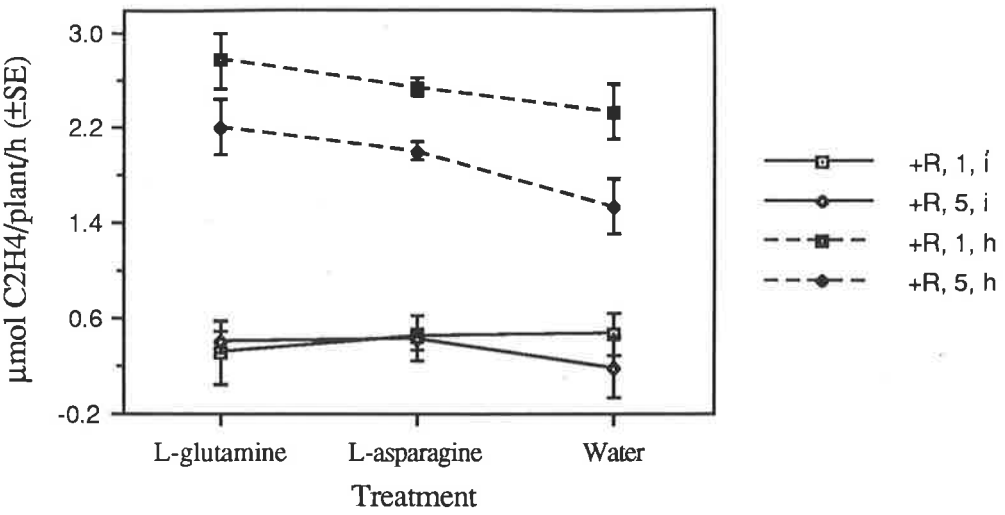



Figure 9.2. ARA values in lupin at 106 h after application of either L-glutamine, L-asparagine or water by wicks. R = *Rhizobium*; 1 or 5 indicate nitrate concentration (mM), i = infected plants, h =healthy.

**B. Effect of L-glutamine and L-asparagine on virus content in *N. glutinosa*.**

Experiment 9.A. showed that neither L-glutamine nor L-asparagine affected virus content in infected lupin, when they were applied after lupins were severely infected. In this experiment, *N. glutinosa* (which showed symptoms at less than 2 weeks a.i.) was used to study the effect of L-glutamine and L-asparagine on virus content.

*N. glutinosa* (4 weeks old) was mechanically inoculated with $C_{NSW}$, and the infected leaves were treated with amino acids either by slicing, application to the basal petiole of detached leaf (Matthews, 1991) or by the wick method (see section 9.A). The two former methods were done under sterile conditions to prevent contamination with other microorganisms which can grow in amino acid solutions. Leaf samples were surface sterilized by wiping with 70% alcohol then with SSDW.

**1. Slicing of leaf**

One half of each of two leaves of 48 plants were inoculated with CMV-$C_{NSW}$. Twenty four of the plants were maintained under continuous light (25°), and the other 24 were maintained in the glasshouse. Leaves were sampled at 3, 5, 7 and 9 days a.i., three plants for each sampling. Leaves were sliced at intervals of 3mm parallel to the veins on both sides of the midrib, then were floated on 2 ml of either 1 mM L-glutamine or water in plastic petri-dishes (10 cm in diameter). Plastic petri-dishes were then sealed with glad-wrap plastic, and incubated for 48 h at 25°. Assay of virus content was done by DAS-ELISA using IgG from polyclonal fixed-antiserum to $T_{WA}$. Each leaf was cut along the midrib to separate the inoculated from the uninoculated half leaf. Then each half was crushed and diluted with 1.2 ml of ELISA sample buffer.

**Result :**

In plants grown under continuous light (Fig. 9.3.A), virus content increased steeply in the inoculated half leaves floated on L-glutamine, but it remained constant in the inoculated half leaves floated on water. The virus was also detected at low levels in the uninoculated half leaves when assays were done at 7, 9 and 12 days a.i., due to the movement of virus between the adjacent halves of leaves. The virus content in uninoculated half leaves floated on either L-glutamine or water was not significantly different.

Similarly for plants grown in the glasshouse, virus content was higher in the inoculated half leaves floated on L-glutamine than in those on water (Fig. 9.3 B). Virus content was higher in plants grown under continuous light than in plants grown in the glasshouse.

**2. Application of amino acids to the basal petiole of detached leaf**

Eighteen plants were grown either in the glasshouse or under continuous light (25°), then were inoculated on one half leaf of 2 leaves for each plant. Samples were taken at 5, 6 and 7 days a.i. by cutting the petiole of the inoculated leaf. The basal

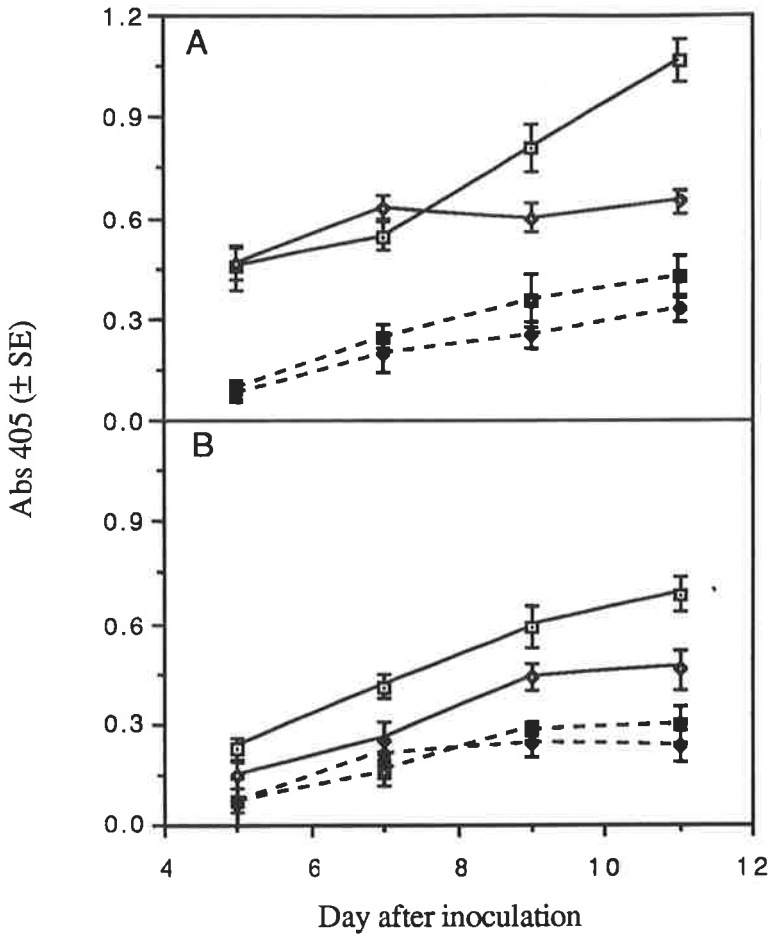



Figure 9.3. Analysis of the effect of L-glutamine on virus content in adjacent inoculated and uninoculated halves leaves. Leaf was sliced and floated on either L-glutamine or water for 48 h before assay. Leaves were removed from plants maintained under continuous light (A) or in the glasshouse (B) at 3, 5, 7 and 9 days after inoculation.

inoculated half-leaf floated on L-glutamine

uninoculated half-leaf floated on L-glutamine

inoculated half-leaf floated on water

uninoculated half-leaf floated on water

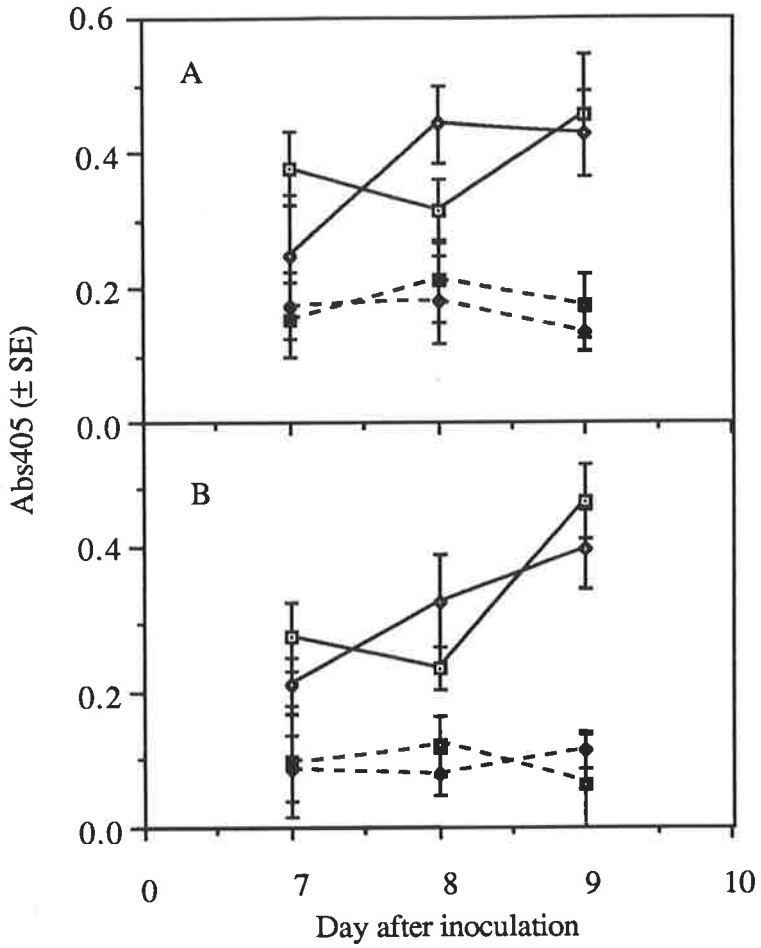



Figure 9.4. Analysis of the effect of L-glutamine on virus content in adjacent halves leaves which had been inoculated or uninoculated with $C_{NSW}$. L-glutamine was applied to the basal petiole of a detached leaves for 48 h before assay. Leaves were removed from plants which were maintained either under continuous light (B) or in the glasshouse (A) at 5, 6 and 7 days after inoculation.

inoculated leaf + L-glutamine
uninoculated leaf + L-glutamine
inoculated leaf + water
uninoculated leaf + water

petiole of the detached leaf was covered with cotton-wool saturated with either 10 mM L-glutamine or water and it was then placed in a plastic petri-dish for 48 h at 25°. Leaves were then cut along the midrib to separate the inoculated from the uninoculated half leaf. Each half of the leaf was assayed by DAS-ELISA as described in experiment 9.B.1.

**Result :**

There was no significant effect of treatment with L-glutamine on virus content compared with water, on inoculated half leaves. Plants maintained in the glasshouse and under continuous light showed the same results (Fig. 9.4.A and B). On plants maintained under continuous light, virus content in the inoculated half leaves treated with water increased steadily, and became relatively constant at 8 and 9 days a.i. On plants maintained in the glasshouse, virus content in the inoculated half leaves treated with water increased at 8 days a.i.

The virus content in inoculated and uninoculated half leaves was significantly different but again, there was no effect of L-glutamine on virus content in inoculated half leaves.

**3. Wick**

An experiment using a wick to supply amino acids was done in the glasshouse. A wick was injected on the basal petiole of inoculated leaf at 7 days a.i. The solution was allowed to be taken up by the plant for 48 h, then 5 leaf discs (diameter 0.5 cm) were taken by systematic sampling from each inoculated leaf (designated as leaf no. 1) and another two leaves immediately above the inoculated leaf (designated as leaf no. 2 and 3). Samples were crushed in 0.5 ml ELISA sample buffer and assayed by DAS-ELISA.

**Result :**

Plants were supplied with different concentrations of L-glutamine at 2.5, 5 and 10 mM. Fig.9.5 shows that the concentration of 10 mM had a significant effect on virus content in leaf no. 1, 2 and 3. Virus content increased steadily in leaf no. 2 and 3.

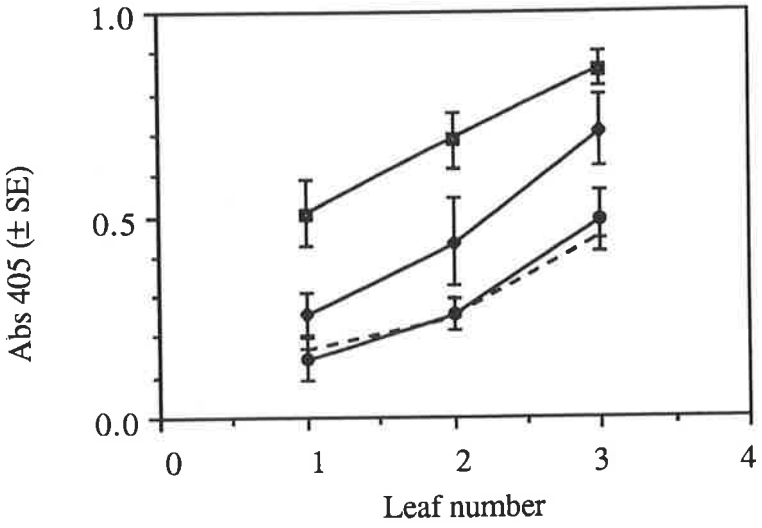



Figure 9.5. Effect of different concentrations of L-glutamine on virus content in inoculated leaf (leaf no.1) and the two leaves immediately above the inoculated leaf. L-glutamine was fed through the basal petiole of inoculated leaf by a wick method. Concentrations of L-glutamine were :

2.5 mM  5 mM

10 mM  H2O

Treatment with water was not significantly different from treatment with 2.5 mM L-glutamine.

Based on the result above, another experiment was done by using a wick with 1.2 ml of 10 mM of either L-glutamine or L-asparagine at 7, 10 and 13 days a.i. After 48 h incubation with these amino acids, 5 leaf discs were sampled from leaf no. 1 (inoculated leaf) to leaf no. 4 (immediately above the inoculated leaf), for DAS-ELISA.

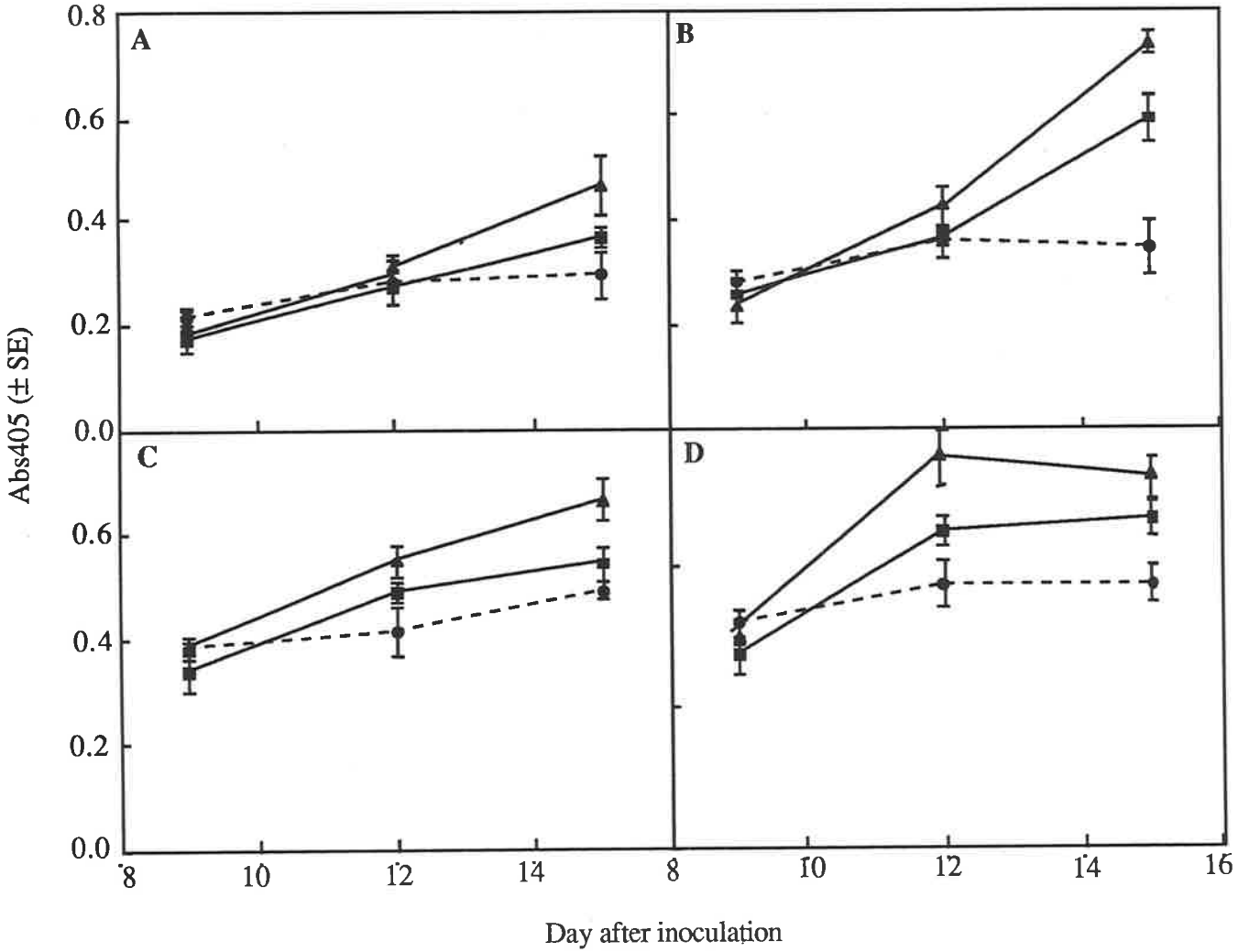
Treatments with either L-glutamine, L- asparagine or water had no significant effect on virus content at 9 days a.i. (Fig. 9.6 A,B,C,D). This treatment was also not significant when samples were taken from leaf no. 1 and 2 at 12 days a.i. (Fig. 9.6 A and B) but it was significant in leaf no. 3 and 4 (Fig. 9.6 C and D). At 15 days a.i., the effect of L-glutamine was significantly different than L-asparagine in leaf no. 1, 2, and 3 (Fig. 9.6. A,B,C), but it was not significant in leaf no. 4 (Fig. 9.6 D).

With these treatments, the virus content increased steeply in leaf no. 1 and 2, as shown by a sigmoid curve (Fig.9.6.A and B), and it increased exponentially in leaf no. 3 and 4 (Fig. 9.6. C and D). However, the virus content was higher in leaf no. 3 and 4 at the beginning of assay.

With this method, treatments with amino acids had a significant effect on virus content in plants. L-glutamine increased virus content more than for L-asparagine.

A preliminary trial using fractionation of the amino acids by electrophoresis (in the formic/acetic system), showed that a number of amino acids in lupin nodules (e.g. serine, valine and isoleucine) were lower in infected than in healthy lupins for both + and -R treatments. Some amino acids (asparagine, glutamine, aspartic acid and arginine) were slightly higher in nodules of infected lupins +R than in infected -R (data not shown).

Figure 9.6. Virus content in leaf infected with CMV-$H_{NSW}$ after application of either 10 mM L-glutamine —■—, 10 mM L-asparagine —▲— or water ---●--- through the wicks to the basal petioles of inoculated leaves for 48 h. Five discs/leaf (diameter = 0.5 cm) were sampled from leaf numbers 1 [inoculated leaf] (A), 2 (B), 3 (C) and 4 (D), at 9, 12 and 15 days after inoculation (including 2 days of amino acids application).


A
B
C
D
0.8
0.6
0.4
0.2
0.0
8
10
12
14
16
Abs405 (± SE)
Day after inoculation

CONCLUSION :

1. The number of lupins infected was fewer in the + R than in the -R treatment but the number was similar in the 1 and 5 mM $NO_3$ treatment for each the R treatment.
2. After application of L-glutamine or L-asparagine to lupins, virus content and ARA were not significantly different for the 1 and 5 mM $NO_3$ treatment, and also for the -R or +R treatment.
3. L-glutamine and L-asparagine increased virus content in *N. glutinosa,* when DAS-ELISA was done before 2 weeks a.i. A wick was a suitable method for this purpose.

# CHAPTER 10. GENERAL DISCUSSION

## I. PROPERTIES OF STRAINS IN SUBGROUPS I AND II

### 1. Nucleic acid properties

### 1.a. Subgrouping of CMV strains by hybridization to cDNA of total RNA

RNA-cDNA hybridization tests have established that the 14 Australian CMV strains studied can be divided into two distinct subgroups. Six of the virus strains belonged to subgroup I and the other eight to subgroup II, as defined by Owen and Palukaitis (1988), and were represented in this work by the type strains $F_{NY}$ and $L_{NY}$, respectively. The CMVstrains from Japan included in this study were also shown here to belong to these subgroups, strain $Y_{Jpn}$ to subgroup I and $P_{Jpn}$ to subgroup II. The unfractionated RNAs of the 18 strains examined in this thesis and another 12 strains studied by Owen and Palukaitis (1988), produced strong signals when probed with cDNA to either subgroup I or II, but never to both. This indicates that none of the strains arose by pseudorecombination between strains belonging to either of these subgroups. It has been shown that all the genomic RNAs of subgroup I,.e.g. strains K and M (P. Palukaitis, personal communication, 1989; Sleat and Palukaitis, 1990) are compatible with the subgroup II strains, e.g. strain $U_{Qld}$ (Rao and Francki, 1981; 1982). It has also been shown that pseudorecombinants constructed *in vitro* of cucumoviruses such as CMV and TAV produced strong signals in dot blot tests when probed with cDNA to either virus (Sackey and Francki, 1990).

The lack of cross hybridization between the RNAs of subgroup I and II strains when probed with cDNA to one strain of these subgroups was determined by dot-blotting, because the nucleotide sequence of RNA genomes of these two subgroups has been shown to be similar but distinct. There are only small differences in the numbers of nucleotides in the RNAs of different strains (see Chapter 1.II). In addition, a comparison of the complete nucleotide sequences of $F_{NY}$ (subgroup I) and $Q_{Qld}$ (subgroup II) showed 70 % similarity in their RNAs 1 (Rizzo and Palukaitis, 1989), 71

% similarity in RNAs 2 (Rizzo and Palukaitis, 1988), and 75% similarity in RNAs 3 (P. Palukaitis, personal communication, 1989; Owen *et al.*, 1990).

### 1.b. Subgrouping CMV strains by the electrophoretic mobilities of RNAs 1 and 2

When TAE buffer (pH 8.0) was used in agarose gel electrophoresis, the distance between RNA 1 and RNA 2 of subgroup II strains was greater than for the subgroup I strains (Fig. 3.2). However, when TBE buffer (pH 8.3) was used, the distance between RNA 1 and 2 of these two subgroups was the same, as shown in the comparison of RNAs of PSV and CMV (Fig.7.5). The reason for this is unknown. Electrophoresis in TAE buffer is therefore useful to distinguish between subgroup I and subgroup II strains, provided that markers are used.

## 2. Serological properties and separation into serogroups

### 2.a. Using fixed and unfixed antigens for separating CMV strains into serogroups

All the Australian CMV strains, except strain $Y_{WA}$, could be separated into two serogroups by the appropriate immunodiffusion tests or DAS-ELISA. These serogroups corresponded to the DTL and ToRS serogroups (Devergne and Cardin, 1973;1975) and the subgroup I and II of Owen and Palukaitis (1988) (see Chapter 1.II). Although strain $Y_{WA}$ was clearly a member of subgroup II by cDNA probing, it defied assignment to either subgroup by any of the serological methods used.

Serogrouping was also possible using unfixed antigens with antiserum to unfixed virus, with the exception of the reaction of $V_{Qld}$ with antisera to $T_{WA}$ and $H_{NSW}$.

### 2.b. Variability of reactions of CMV strains in gel immunodiffusion tests

Tests using the combined placement of heterologous and homologous fixed antigens with antiserum to fixed virus could not be used to serogroup CMV strains. It

appears that there are antigenic variations among the strains within each serogroup (Devergne and Cardin, 1975). With this test, some strains belonging to the same serogroup could be placed in the other serogroup on the basis of spur formation. Results similar to this were also demonstrated with two distinct isolates of wild cucumber mosaic virus (CMV-W, isolates 1 and 2) where a different placement of antigens in gel immunodiffusion altered their apparent relatedness (Grogan *et al.*, 1963).

Similar tests to that described above showed that the reactions between fixed and unfixed antigens with antiserum to fixed or unfixed antigen were also unpredictable (Table 6.2).

Thus, tests with the combined placement of heterologous and homologous antigens were not useful for distinguishing and serogrouping CMV strains.

### 2.c. Serological properties of CMV strains in ELISA with MAbs

According to Porta *et al.* (1989) MAbs 3.4 and 76.1 were specific for strain Co which is antigenically distinct from members of both the DTL and ToRS serogroups. However, tests with a larger panel of CMV strains showed that these MAbs reacted with some strains from both these serogroups. The reason for this was that MAbs specific for strain Co were obtained from mice immunized with CMV-D. MAb 76.1 and MAb 3.4 were obtained from mice immunized with unfixed CMV-D and fixed CMV-D coat protein, respectively. In my study, MAb 3.4 recognized all 16 strains whereas MAb 76.1 recognized none. It was possible that MAb 76.1 lost its reactivity in transit from France to Australia.

In another experiment with TAS and PTA-indirect ELISAs, MAbs 76.1, 42.3 and 3.4 ( IgMs) bound either the anti-mouse IgG or IgM enzyme conjugate, but MAbs 34.2 and 21.4 (IgG) were only recognized by anti-mouse IgG enzyme conjugate (Table 4.7 and 4.8). A possible explanation for this was that the anti-IgG enzyme conjugate was raised to the whole IgG, so it could be recognized by MAbs specific to subclasses IgG and IgM. The anti-IgM enzyme conjugate was raised specifically to the μ chain, therefore it was only recognized by MAbs to subclass IgM (Table 4.7).

Variability of antigen reactions in gel immunodiffusion tests and ELISA with either PAbs or MAbs showed that subgroup I and II strains were closely related (SDI= 1 to 2). Another important limitation of using serological tests for dividing CMV strains into 2 serogroups, is that serogrouping only reflects small differences in the sequence of nucleotides on the part of RNA 3 which carries the coat protein gene (see Chapter I).

### 3. Biological properties

Strains of CMV have been divided into certain groups on the basis of their reaction on selected host plants, particularly on *N. tabacum* cv Xanthi n.c. (Marrou *et al.*, 1975). The 16 strains of CMV compared in this thesis could not be assigned with confidence to either subgroup I or II (Table 51 and 5.2). On the basis of their biological properties, problems in identifying strains of CMV arise from variation in conditions at different time of the year. This variation could affect the susceptibility of plants and symptom production (Francki and Hatta, 1980).

None of the virus strains from either subgroup I or II consistently infected any one plant species at all the times of the year tested. It is uncertain whether my observation could be extended to the field situation where CMV subgroups have different thermosensitivities. For example, plants infected with subgroup I strains predominate during the summer in Europe (Quiot, 1980; Quiot *et al.*, 1983; Haack, 1986).

The study of host ranges and the data described in Table 5.1 and 5.2 will be useful for distinguishing CMV from other plant viruses (Edward and Gonsalves, 1983) rather than for distinguishing CMV strains into subgroups.

## II. PROPERTIES OF FIXED AND UNFIXED VIRUS AND ANTISERA PREPARED AGAINST FIXED AND UNFIXED VIRUS

### 1. Stability of fixed and unfixed antigens after long term storage

Glutaraldehyde fixation increased the stability of CMV particles *in vitro* (Rao and Francki, 1982). Analysis of fixed and unfixed virus preparations after long term storage

showed that the CMV strains varied in stability after fixation (see Chapter 6.B.1). Some CMV strains degraded partially in the storage container and this was detected by the appearance of a more slowly sedimenting component after analysis by sucrose density gradient centrifugation (Fig. 6.5).

Analysis of fixed and unfixed intact virus particles by electrophoresis in either TAE or TBE buffer showed that they separated into several bands (Fig. 6.6). Some of these could be separated protein subunits or nucleoprotein components. After staining with either ethidium bromide or coomassie blue, the mobility of the main component of unfixed virus appeared to be slightly slower than that of fixed virus. This result was in agreement with observations on the mobility of AMV particles (Hajimorad and Francki, 1991a). For example,the fixed AMV particles produced sharp bands while unfixed virus produced diffuse bands, indicating that the unfixed virus particles had lost their integrity as a result of an effect on their protein-RNA interaction. Furthermore, Hajimorad and Francki (1991a) could not detect any degradation of fixed and unfixed AMV particles after exposure to electrophoretic conditions. In my experiments, both fixed and unfixed CMV produced sharp bands. The fixed virus was distinguished by producing an additional more rapidly migrating band, but the composition of this band was not determined.

## 2. Does glutaraldehyde fixation enhance the immunogenicity and antigenicity of CMV?

In gel immunodiffusion tests, the titres of either fixed or unfixed antisera against their homologous fixed and unfixed antigens did not differ (Table 6.1). Only six of the 11 fixed antisera and one of the 9 unfixed antisera had titres which differed by one twofold dilution between fixed and unfixed antigens. Most unfixed CMV antisera reached a high titre of 1/512 to 1/1024, whereas 1/2048 was the highest titre reached by antiserum to $T_{WA}$. My results (Table 6.1) disagree with those obtained by the preparation of anti-AMV sera which demonstrated that antisera to glutaraldehyde fixed AMV have higher titres than antisera to unfixed virus (Hajimorad and Francki, 1991b).

Habili and Francki (1972; and the late R.I.B. Francki, personal communication, 1989) found difficulties in making antiserum to unfixed $Q_{Qld}$-CMV in mice. This suggests that the immunogenicity of unfixed virus in the rabbit differed from that in the mouse. A possible explanation is that the unfixed virus particles were degraded soon after injection, resulting in the inability of mice to produce antibodies specific to the injected strain (van Regenmortel, 1982). The degradation of unstable virus (CMV) into its constituent subunits may lead to the production of antibodies which do not react with intact virions. This has been observed for potato virus X which does not react with antiserum raised against its protein subunits (Shalla and Shephard, 1970). In addition, the anti-AMV serum raised against viral protein subunits failed to differentiate AMV isolates $H_4$, $N_{20}$, $S_{30}$, $S_{40}$ and $W_1$ when the coat proteins were used as test antigens (Hajimorad and Francki, 1991b). These AMV isolates were differentiated using fixed antigens rather than unfixed antigens.

In gel immunodiffusion tests, the same antigen preparations produced different precipitin lines when tested against either homologous fixed or unfixed antiserum (Fig. 6.1). Some antigens produced double precipitin lines which indicated that degradation of virus particles had occurred. It was possible that degradation occurred either when the virus was prepared, stored, or in the rabbit during immunization (Table 6.2 and 6.3). A systematic assessment of reasons for these problems was not achieved, because the combined placement of different antigen and antiserum preparations, led to the production of non-reproducible patterns of precipitin lines (Table 6.3). It was interesting to note that some strains produced double precipitin lines where the outer line formed a spur to the neighbouring distantly related antigen, whereas some produced a spur to the neighbouring closely related antigen of the same serogroup. The confluence of inner precipitin lines could be due to the degradation of unfixed or fixed antigens before or during the interaction with antibodies. Double precipitin lines were commonly produced in the interaction of fixed antigens with the antiserum to unfixed virus (Table 6.3), whereas these lines were not produced by fixed antigens and antiserum to fixed virus.

The explanation for this is that fixation of virus particles with glutaraldehyde may enhance their stability (Rao and Francki, 1982).

On the other hand, PTA indirect-ELISA with a serial dilution of either antigens or antiserum could not show the difference between fixed and unfixed antigens (Fig. 6.4 and 5). Hajimorad and Francki (1991b) also found no alteration in the antigenic conformation of AMV particles after glutaraldehyde fixation. Moreover, the fixation only influences the common epitopes which share the antigenic determinants for recognizing a strain (Rao and Francki, 1982). In PTA indirect-ELISA, the carbonate buffer (pH 9.6) causes complete dissociation of virus particles so that they could be adsorbed efficiently onto the plate (Hajimorad and Francki, 1991c), and this may explain the result shown in Fig. 6.4.

The results of PTA indirect-ELISA have not been compared with DAS-ELISA for these kinds of antigens and antisera, because the latter necessitates preparing antibody conjugates to each virus tested (Rochow and Carmichel, 1979). However, in another experiment with PSV, DAS-ELISA using IgG from either antiserum to fixed or unfixed PSV-E also failed to detect any differences between homologous fixed and unfixed antigens (Fig. 7.3).

These points all lead to the conclusion that the conventional ELISA methods and immunodiffusion tests used in this study were unable to detect any conformational changes in CMV or PSV particles as a result of fixation with glutaraldehyde.

## III. Relationships Between CMV-$Y_{WA}$ and PSV-E and PSV-2

CMV-$Y_{WA}$ was thought to be a separate member of the cucumoviruses, because it was serologically different from the other CMV strains tested (Wahyuni *et al.*, 1992; Fig. 4.1). Although CMV has been isolated from soybean (Hanada and Tochihara, 1982; Green and Lee, 1988), none of the CMV strains tested infected peanut or soybean by mechanical inoculation (Table 5.1). Therefore, CMV-$Y_{WA}$ did not belong to the PSV group by this test (Mink, 1972; Marchoux *et al.*, 1977; Xu *et al.*, 1986).

When PSV and CMV antisera to fixed and unfixed antigens were titrated against heterologous fixed and unfixed antigens of PSV, CMV and TAV, CMV-$Y_{WA}$ was distantly related to PSV-E or PSV-2 or to TAV-V with an SDI of 6 to 7. Therefore, CMV-$Y_{WA}$ was serologically different from the two CMV serogroups, and it was clearly not related to either of the strains of PSV tested.

In gel immunodiffusion tests, both PSV-E and 2 were more closely related to CMV than was TAV-V to CMV (Fig. 7.2). However, DAS-ELISA using immunoglobulin to PSV-E (fixed and unfixed) showed that both CMV-$Y_{WA}$ and TAV-V were distantly related to PSV-E (Fig. 7.4). This was also shown by Devergne *et al.* (1981) with indirect ELISA using IgGs from different species of animal. In their experiment, results of tests to distinguish the cucumoviruses and CMV serogroups were variable, depending on the method of ELISA, antiserum and virus strains used. PSV and TAV were distantly related to CMV in DAS-ELISA using IgG to either strain To or D, but in indirect ELISA, PSV was closely related to CMV (strain R) and TAV was closely related to CMV-Co. When IgG to PSV was used, TAV and CMV-D were distantly related to PSV in DAS- ELISA, but the position of TAV was between PSV and CMV in indirect ELISA.

Northern blot-hybridization showed that PSV-RNA did not hybridize with CMV-$Y_{WA}$ cDNA, and the RNA of strains of CMV did not hybridize with PSV-E cDNA, using high stringency washing conditions (Fig. 7.5). The relatedness of CMV-$Y_{WA}$ to the cucumoviruses was also shown by hybridization between $Y_{WA}$ RNA-cDNA and various CMV-RNA, so that now this strain could be placed into subgroup II of CMV (Wahyuni, *et al.*, 1992; Fig. 3.1).

## IV. SYMPTOM INDUCTION

### 1. Symptoms and virus distribution in *Medicago spp.*

Symptoms on some *Medicago spp.* (medic) were generally detected 6 weeks after inoculation. The positions of leaves with symptoms appeared not to be systematic, and not all shoots bore leaves with symptoms. The time for the induction of first

symptoms by different strains in different cultivars could be either short or long. For example, a long delay before production of symptoms in cv. Paragosa (Fig. 5.4D) indicated that CMV can have a long latency.

Virus concentration in leaves was variable (Fig. 5.4). The virus concentration detected could be higher in the middle shoots or in the symptomless tips. This was also observed in *P. vulgaris* infected with CMV (Davis and Hampton, 1986). The virus concentration in parts of plants with symptoms, however, declined with the time after showing the first symptom. This decline was shown by the lower concentration of virus in leaves with symptoms than in the leaf-tips. It was concluded that virus concentration in medic leaves is not correlated with symptom intensity.

*M. scutellata* cv. Sava did not show symptoms following mechanical or aphid inoculation (Table 5.1). The failure of *M. persicae* and *A. craccivora* to transmit CMV to this plant could have been due to the unsuitability of this host for these aphids, but the failure to transmit CMV by patch or approach grafting provides strong evidence that this cultivar is resistant to CMV.

### 2. Induction of necrosis on lupin

Induction of necrosis on lupin generally depended on the virus strain and cultivar of lupin (Table 1). Conduct of the lupin experiment in sterile culture provided results similar to those obtained in the glasshouse. All strains described in Table 5.5 caused necrosis but not all of them caused plants to die. The death of lupins grown in sterile culture supported the hypothesis that CMV itself is able to cause the death of lupin without involving other organisms.

## V. INTERACTION BETWEEN LEGUMES, *RHIZOBIUM* AND CMV

### 1. Susceptibility of barrel medic and lupin to CMV

A preliminary experiment with barrel medic grown in soil showed that *Rhizobium* treatment reduced the susceptibility of this species to infection with a range

of CMV strains (Table 8.1). Among the plants which were infected, the virulence of different strains was variable.

Generally, CMV reduced plant growth (Table 8.2). The size and number of nodules on infected plants was smaller and fewer than for the healthy plants. In lupin, the size and number of nodules of both infected and healthy plants increased with time after inoculation with *Rhizobium*, then decreased after 2 months. The efficiency of plants to fix nitrogen was correlated with the age of plant, and the size and number of active nodules. These results have also been observed for CBMV on pea, CMV on pea and SeMV on mungbean (Chowdhury *et al.*, 1987; Rao *et al.*, 1987; Rao and Shukla, 1988). In my study, the colour of active nodules of infected plants were pink, as observed also by Tu *et al.* (1970). However, this result disagrees with the observation of Joshi and Carr (1967) that infected nodules with CPM were mainly white (= inactive nodules), and they suggested that this was the cause of a reduction in the efficiency of nitrogen fixation.

The susceptibility of barrel medic to CMV after inoculation with either *Rhizobium* strain Rm 1021 or its mutant (Rm 1021 nif $H^-$ : : Tn 5) did not differ. This mutant (which does not fix nitrogen) was used to investigate whether nitrogen fixation activity was involved in this interaction. In healthy plants, strain Rm 1021 alone induced fewer active nodules (syn. pink nodules) than the commercial *Rhizobium* used in other experiments. The number of plants infected using strain Rm1021 in the 7.5 mM $NO_3$ treatment was fewer than using commercial strain. These experiments failed to determine the way in which *Rhizobium* itself (both commercial strain, and strain Rm 1021 and its mutant) affected the susceptibility of barrel medic.

In experiments with barrel medic, a long latent period was required for the development of symptoms. The ARA value of healthy plants was higher at 33 days a.i.than at 66 days a.i., whereas the number of infected plants increased with time after inoculation (Table 8.3). The time to nodulation in barrel medic (± 2 weeks after inoculation with *Rhizobium*) was shorter than in lupin (± 1 month). The time to nodulatation depends on the cultivar or species of plants, the strains of *Rhizobium* and

the conditions of maintaining plants (Tu *et al.*, 1970a; Orellana *et al.*, 1977; 1980; Sprent and Sprent, 1990).

In experiments with lupin, grafting was a more suitable method for inoculation before flowering than was aphid transmission. Varying the source of inoculum by using either infected lupin or *N. glutinosa* had no effect on the success of patch grafting (Table 8.4; 8.5 and data not shown).

The susceptibility of lupins declined when nodules were produced, but the ARA value increased with the age of plant (Fig. 8.6 and 8.7). The highest ratio of success of inoculation was achieved before plants were nodulated.

The *Rhizobium* treatment reduced the susceptibility of lupin to CMV. CMV infection also reduced the plant growth, nodulation and the efficiency of fixing nitrogen as shown for other viruses (Tu *et al.*, 1970; Orellana *et al.*, 1980; O'Hair and Miller, 1982; Singh *et al.*, 1984; Rao and Shukla, 1988).

**2. Is nitrogen supplementation involved in the increase of susceptibility of the plants to CMV?**

The presence of a high concentration of nitrogen (7.5 mM $NO_3$) in the growth medium completely inhibits nodulation (Silsbury *et al.*, 1986) and this was also observed in my study. Nitrate is reduced to nitrite, and as bacteroids do not have nitrite reductase, accumulation of nitrite can inhibit nitrogenase activity (Kamberger, 1977). It has been reported that an increase in nitrogen concentration decreases the size and the number of nodules on infected plants (Singh *et al.*, 1984). However, plant growth is inhibited without any addition of nitrogen to the sand medium, and nitrogen deficiency may also affect the susceptibility of plants to virus infection as reported by Bawden and Kassanis (1950).

In experiments with a minimum supply of nitrate for barrel medic (0.5 mM) and lupin (1mM), the susceptibility of plants without *Rhizobium* was still higher than with *Rhizobium* (Table 8.3 and 8.6). Nitrogen supplementation increases the susceptibility of barrel medic and lupin to CMV (Table 8.4 and 9.1). When inoculation with virus was

done just before nodulation, lupins with 1 mM $NO_3$ were slightly less susceptible than with 5 mM $NO_3$, although it was not significantly different (Table 9.1, Fig. 9.1). Plants uninoculated with *Rhizobium* following supply with a high concentration of $NO_3$ are the most susceptible to CMV. Thus, a high nitrogen supply indirectly caused plants to be more susceptible to virus infection (Spencer, 1935;1941a; 1942; Bawden and Kassanis, 1950).

These data led to the conclusion that both *Rhizobium* and nitrogen supplementation are involved in the susceptibility of barrel medic and lupin to CMV.

**3. Do temperature and day length conditions affect the susceptibility of plants?**

The different conditions (temperature, day length, medium) for growing lupins showed that lupins in sand (20° and 13 h day length) grew more slowly than in soil (glasshouse, 26° and 15 h day length). The size of nodules of both healthy and infected plants was smaller than in the glasshouse. This is in aggrement with Tu *et al.* (1970a) who observed that at 15.5° and 21°, the size of nodules and the fresh weight of infected soybean with SMV were less than at 26.5°. At 21° and 26.5°, the ARA were not different.

My results show that the ARA, the severity of symptoms and the susceptibility of plants did not differ in these growth conditions (Table 8.5 and 8.6)

**4. Are L-glutamine and L-asparagine involved in the increase of virus content or plant growth?**

The supply of 10 mM L-glutamine or L- asparagine (as nitrogen intermediates in the nitrogen fixation pathways) by a wick to both inoculated and uninoculated lupin with *Rhizobium* had no significant effect on virus content in young leaves. Maybe this was due to the severity of symptoms on lupins when assay was done at 17 days a.i. These symptoms were rosetting, stunting, production of smaller young leaves, and necrosis of the basal stem. However, these amino acids affected virus content on *N. glutinosa*

(Fig.8.4). Virus content increased after application through a wick of either 10 mM L-glutamine or L-asparagine at 9-12 days a.i. The effect of these amino acids was greater on leaves immediately above the treated leaf. L-glutamine had a greater effect than L-asparagine.

The effect of L-glutamine on virus content in *N. glutinosa* was more evident when applied through slicing of the leaf (Fig. 8.2) than by the wick. This amino acid could be absorbed directly by the sliced leaf by exposing it to the light for 16 h/day during the incubation period. A disadvantage of the slicing method, is that leaves are cut from the plant, and the amino acid is introduced by diffusion. The main advantage was that the effect of this amino acid on virus content in inoculated half leaf could be observed at the time when virus began to replicate. In this experiment, the highest virus content was at 9-11 days a.i.

Between 7 and 14 days a.i., virus content in inoculated half leaves of *N. glutinosa* which were maintained under continuous light, was higher than in inoculated half leaves of plants grown in the glasshouse [dark-light conditions] ( Fig. 8.2). Cheo (1971) suggested that under continuous light, the activity of carbohydrate, protein and RNA synthesis in infected plants is higher, and this would favour increased virus replication. However, the amount of virus accumulated depends on the plant species, virus and time after inoculation. For example, based on the local lesion assay, the amount of TMV was higher in *N. glutinosa* than in tobacco c.v. Xanthi, and virus content began to increase from 5 days a.i. (Cheo, 1971).

I conclude that L-glutamine and L-asparagine supplements increase virus content when plants begin to develop symptoms, and this effect was abolished when symptoms very were severe. Addition of these amino acids by the wick increased the efficiency of nitrogen fixation in healthy lupins (Fig. 8.9).

**5. How does CMV associate with bacteroids in barrel medic nodules?**

In the preliminary experiment with barrel medic (Table 8.2), virus was detected in root tips and nodules. RNase treatment allowed CMV particles to be distinguished

from ribosomes in nodule cells, so the location of virus and bacteroids in cells could be observed. In the study of ultrathin sections of nodules coinfected with *Rhizobium* and CMV-$T_{WA}$, individual virus particles and aggregrates of virus particles were located in cells different from those which contained bacteroids. Aggregates of virus particles were commonly located along the cell wall of cells free of bacteroids. This observation differed from that for SMV in soybean nodules (Tu, 1973) where the inclusion bodies and virus aggregates were found in the same cells as the bacteroids. Tu also found that virus affects nodule structure and ultrastructrure of bacteroid cells in soybean nodules (Tu, 1973, 1975, 1977). For example, reduction in the distance between bacteroids and membrane envelope, in number of vesicles, and in number of ribosomes, mitochondria and amount of endoplasmic reticulum in the infected SMV nodules. Futhermore, Tu (1973) found that SMV did not affect the growth of rhizobial infection threads and the process of releasing bacteroid into nodule cells. In my study, it was very difficult to compare the structure of healthy and infected nodules (Fig. 8.10).

## CONCLUSION

This thesis makes the following contributions to the knowledge of the subgrouping of CMV strains, and the interaction between legumes and CMV :

1. Fourteen strains of CMV from Australia could be separated into subgroups I and II by RNA-cDNA hybridization. Using the appropriate gel immunodiffusion tests and ELISA with PAbs or MAbs, CMV-$Y_{WA}$ fell in between these subgroups, although this strain is clearly a member of subgroup II by RNA-cDNA hybridization tests. Host range tests could not be used for placing the strains into subgroups I or II.
2. CMV-$Y_{WA}$ is serologically distantly related to PSV-E, PSV-2 and TAV-V. None of the CMV strains used in this study are related to PSV-E or PSV-2 by RNA-cDNA hybridization.
3. The reactions of important pasture and grain legumes in South Australia to strains of CMV are variable. The symptoms produced is affected by time of the year, and no strains of CMV belonging to either subgroup I or II preferentially infected plants at a

particular time of the year. The time to induce the first symptoms depends on the strains of CMV and the cultivar or species of plant. Some CMV strains are able to induce necrosis on lupin. Virus and symptom distribution in *Medicago* spp. is uneven and a long latent period is required for the development of symptoms. *M. scutellata* cv. Sava is resistant to all strains of CMV tested.

4. Gel immunodiffusion tests using the combined placement of fixed and unfixed virus with either antiserum to fixed or unfixed virus, are not useful for serogrouping strains. Purified virus of strains of CMV had different stabilities after fixation with 0.25% glutaraldehyde and storage for more than one year.
5. *Rhizobium* inoculation reduces the susceptibility of barrel medic and lupin to CMV. However, watering with a high concentration of $NO_3$ increases their susceptibility. The susceptibility of plants to CMV declined when inoculation was done during nodulation. The time to nodulation is longer in lupin than in barrel medic, and the efficiency of nitrogen fixation is higher in healthy than in infected plants.
6. Application of L-glutamine and L-asparagine by the wick to the plants at the beginning of symptom development, increases virus content.

Appendix I-1

**Composition of medium Murashige and Skoog for growing lupin in sterile condition**

| Chemicals | Concentration | Chemicals | Concentration |
|---|---|---|---|
| $NH_4NO_3$ | 1600 mg/l | KI | 0.83 mg/l |
| $KNO_3$ | 1900 mg/l | $Na_2MoO_4.2H_2O$ | 0.25 mg/l |
| $CaCl_2$ | 330 mg/l | $CuSO_4.5H_2O$ | 0.25 mg/l |
| $MgSO_4.7H_2O$ | 370 mg/l | Thiamine HCl | 1.00 mg/l |
| $KH_2PO_4.H_2O$ | 170 mg/l | Inositol | 10.00 mg/l |
| $NaH_2PO_4.H_2O$ | 170 mg/l | Pyridoxine HCl | 0.10 mg/l |
| Na-EDTA | 37.3 mg/l | Nicotinic acid | 0.10 mg/l |
| $FeSO_4.7H_2O$ | 27.8 mg/l | Sucrose | 30.00 g/l |
| $H_3BO_3$ | 6.2 mg/l | IAA | 200.00µg/l |
| $MnSO_4$ (.$4H_2O$) | 16.9 mg/l | Agar (Type 750) | 8.00 g/l |
| $ZnSO_4$ | 8.6 mg/l | | |

Adjust the pH to 5.8 with 1 M NaOH

Source : B.J. Ingham (1990), The Waite Agricultural Research Institute, The University of Adelaide (personal communication)

Appendix I-2

**Composition of nutrient solution containing nitrogen ($NO_3$) on the basis of 1/2x Hoagland solution**

| Chemicals (mg/l) | Concentration of nitrogen 0 mM | 1 mM | 5 mM | 7.5 mM |
|---|---|---|---|---|
| $MgSO_4.7H_2O$ | 246.38 | 246.38 | 246.38 | 246.38 |
| $KH_2PO_4.3H_2O$ | 34.0 | 34.0 | 34.00 | 34.0 |
| EDTA | 23.82 | 23.82 | 23.82 | 23.82 |
| $FeSO_4$ | 19.92 | 19.92 | 19.92 | 19.92 |
| $K_2SO_4$ | 217.75 | 189.44 | 72.51 | 0 |
| $CaS0_4.2H_2O$ | 430.00 | 372.50 | 0 | 0 |
| $KNO_3$ | 0 | 33.67 | 168.34 | 252.51 |
| $Ca(NO_3)_2.4H_2O$ | 0 | 78.64 | 393.18 | 589.77 |
| Trace element | | | | |
| $Na_2MoO_4.2H_2O$ | 0.12 | 0.12 | 0.12 | 0.12 |
| $CuSO_4.5H_20$ | 0.08 | 0.08 | 0.08 | 0.08 |
| $ZnSO_4.7H_2O$ | 0.22 | 0.22 | 0.22 | 0.22 |
| $MnCl_2.4H_2O$ | 11.81 | 1.81 | 1.81 | 1.81 |
| $H_3BO_3$ | 2.86 | 2.86 | 2.86 | 2.86 |

Source : Oti-Boateng, C. (1989). The inhibition by nitrogenase of dinitrogen fixation in *Vicia faba*. Thesis. Uni. of Adelaide. The Waite Agricultural Reseach Institute.

Appendix I-3

**Composition of University of California (UC) mix soil**

| | | | |
|---|---|---|---|
| Sand | | 2/3 $m^3$ | |
| Peatmoss | | 1/3 $m^3$ | |
| Fertilisers | : Calcium hydroxide | 700 g | |
| | Calsium carbonate | 480 g | |
| | Nitrophoska | 600 g, containing : | |
| | | 5% | $NH_4$ (ammonium form) |
| | | 4% | $NO_3$ |
| | | 1% | $NH_2$ (amide form) |
| | | 5% | IBDU |
| | | 3.9% | P-citrate soluble |
| | | 12.4% | $K_2SO_4$ |
| | | 1.25% | $MgCO_3$ |
| | | 3.4% | $Ca_2PO_4$ |
| | | 5.3% | $SO_4$ |
| | | 0.3% | $FeO_2$ |
| | | 0.2 ppm | $CuO_2$ |
| | | 7 ppm | ZnO |
| | | 10 ppm | $Ca_2BO_3$ |
| | | 0.3 ppm | MoO |

Source : E. Nagy (1989), The Waite Agricultural Research Institute, The University of Adelaide (personal communication).

Appendix II

## Publication

1. Wahyuni, W.S., Dietzgen, R.G., Hanada, K, and Francki, R.I.B. (1992). Serological and biological variation between and within subgroup I and II strains of cucumber mosaic virus (CMV). *Plant Pathology* **41**, 282-297.
2. Wahyuni, W.S. and Francki, R.I.B. (1992). Response of some grain and pasture legumes to 16 CMV strains. *Australian J. Agric. Res.* **43**, 465-477.

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