INTERACTIONS BETWEEN RESISTANT AND SUSCEPTIBLE RICE GENOTYPES AND ISOLATES OF RICE YELLOW MOTTLE VIRUS

by

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Dedicated to:

My Parents, Wife and Children

Akel

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ABSTRACT

Five rice cultivars were used to assess the reaction of rice to five rice yellow mottle virus (RYMV) isolates collected from West and East Africa. Rice seedlings at the 4-5 leaf stage were inoculated with RYMV and kept under glasshouse conditions. Based on symptom severity, disease incidence, incubation period, plant height and fresh and dry weight of shoots and roots, cultivars ITA 212 and BG 90-2 were considered as susceptible, and cultivars ITA 235 moderately and Lac 23 and Moroberekan highly resistant to the five isolates tested. Although all five isolates induced generally similar reactions in all cultivars, RYMV from Sierra Leone induced the mildest reaction, whereas a Niger isolate was aggressive, particularly in resistant cultivars. The virus-host relationship in resistant rice cultivars involved a delay in the appearance of symptoms, a lower disease incidence and the production of mild symptoms.

Studies on the nature of possible resistance mechanisms revealed that symptom severity was correlated with virus concentration in plants. The amount of RYMV in ITA 235 (moderately resistant) was greater than in Lac 23 (highly resistant) but much less than in ITA 212 (highly susceptible). Different components of resistance were found including resistance to virus infection and resistance to virus movement and multiplication. Resistance was probably associated with the presence of an inhibitor(s) in plant extracts. In addition, the number of epidermal trichomes was greater in susceptible ITA 212 than in resistant ITA 235 and Lac 23. There were no differences in the cuticle thickness between resistant and susceptible cultivars.

Serological studies indicated that all five isolates were serologically related. Gel-immunodiffusion, DAS-ELISA and ISEM tests showed that the Ivory Coast and Sierra Leone isolates were closely related, Niger and Kenyan isolates were undistinguishable, while the Nigerian isolate could be differentiated from the other four isolates.

The reaction of *Oryza glaberrima* accessions to infection showed that TOg accession 7235 showed RYMV symptoms when inoculated with the Nigerian isolate, whereas the other four isolates did not induce symptoms.

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S E C T I O N O N E

LITERATURE REVIEW AND PROJECT OBJECTIVES

1. LITERATURE REVIEW AND PROJECT OBJECTIVES

1.1 RICE

1.1.1 Origin and distribution of rice

Evidence for the origin of cultivated rice (*Oryza sativa* L.) based on known habitats of wild species of *O. sativa* and the physiological requirements for rice growth have suggested that rice originated in South-east Asia (Purseglove, 1985; Grist, 1986). Rice spread south and east from India and via the Malay archipelago was introduced to Indonesia by the Deutero-Malays when they migrated to the area about 1500 B.C. Arab traders probably brought rice from Persia to Egypt between the fourth and the first centuries B.C. (Grist, 1986). The Moors then introduced rice into Spain, from where the crop was taken to Central and South America. The Portugese introduced rice into Brazil (Purseglove, 1985; Grist, 1986). *O. sativa* was probably brought to West Africa by the desert caravan routes before the arrival of Portugese traders (Grist, 1986). Today, rice is cultivated in Asia, Africa, Europe, North, Central and South America and Oceana.

1.1.2 The rice crop

1.1.2.1 General

Rice is a member of the Graminea, tribe Oryzeae. The *c.* 25 valid species of the genus *Oryza* are largely distributed in the tropical and subtropical regions of Africa, South and South-east Asia, South China, South and Central America and Australia, with the largest number (9 species) indigenous to Africa (Purseglove, 1985).

The rice plant usually takes 3-6 months from germination to

maturity, depending on the variety and the environmental conditions. The crop is largely self-pollinated, but small and varying amounts of cross-pollination by wind may occur. The usual range for cross-pollination is 0.04-1.5 per cent, with an average of about 0.45 per cent (Yoshida, 1981; Purseglove, 1985).

Rice is the staple food for more than half of the world's population. It is the most important grain component of the diets of numerous people inhabiting tropical and subtropical regions of Asia, Africa and America. In 1986 (Table 1.1), rice ranked second to wheat in terms of the total area cultivated and slightly after maize in terms of production.

Table 1.1. World cereals: area and production in 1986.

Crop	Area (1000s ha)	Production (1000s tonnes)
Wheat	228,945	535,842
Rice	145,358	475,533
Barley	79,645	180,441
Maize	131,475	480,609
Rye	15,492	31,791
Oats	25,563	47,769
Millet	39,941	30,800
Sorghum	46,807	71,445

Source : FAO Production Yearbook, vol. 40, 1986

During the milling process much of the protein, fat, minerals and vitamins are removed. White rice may be enriched by treating a

portion of the grain with vitamins and minerals (Luh, 1980).

Rice plays an important role in traditional customs and religious rites in the East, where its religious significance is usually connected with fecundity and plenty. Rice cakes are eaten at certain festivals in Asia to symbolise long life, happiness and abundance. The custom of throwing rice at the bride and bridegroom in the West is borrowed from the East (Purseglove, 1985; Grist, 1986).

1.1.2.2 Rice growing regions

Rice is a highly adaptable crop and can now be grown in many different locations and under a surprising variety of climates. It is grown in North-eastern China at latitude 53°N; in Central Sumatra on the equator, and in Australia at 35°S (Moomaw and Vergara, 1965; Yoshida, 1981). However, at extreme latitudes an adequate water supply is the main factor limiting rice cultivation (Yoshida, 1981).

1.1.2.3 Rice production and the environment

Average rice yields in rice growing countries range from less than 1 to more than 6 tonnes/hectare. This variation in yield is largely a reflection of the suitability or otherwise, of the prevailing environmental conditions.

Temperature: The average temperature required throughout the life of the rice plant ranges from 20-38°C (Yoshida, 1981; Grist, 1986). The effect of low temperature, at specific growth stages, has been summarised as follows (Grist, 1986):

Growth stage	Low temperature effects
Germination → Seedling establishment	Poor germination, stunted seedlings
Seedling establishment → Panicle initiation	Slow seedling growth or reduced vigour and leaf discolouration
Panicle initiation → Ripening	Stunting, poor tillering partial panicle exertion, degeneration of panicle tips and sterility or delayed heading and irregular maturation

High temperature is a critical factor in rice grain production in some rice growing regions. Several reports (e.g. Datta, 1981; Yoshida, 1981) have indicated that a high percentage of sterility and empty spikelets may be associated with exceptionally high temperatures. Yoshida (1981) showed that air temperatures above 35°C adversely affected rice growth.

Photoperiod: Rice is predominantly a short day plant. However, varieties often differ in their response to photoperiod and are often divided into two groups according to their response to photoperiod:

Sensitive varieties: rice varieties which flower only when daylength is decreasing and when a critical daylength is reached which initiates flower induction.

Insensitive varieties: rice varieties which do not respond to differences in photoperiod and which grow at any season taking roughly the same time from sowing to harvesting regardless of the date of planting (Datta, 1981; Yoshida, 1981; Grist, 1986). Many photoperiod insensitive varieties are grown in temperate regions and are termed

japonica rices.

Light: Light has an important role in the growth of rice. The numbers of tillers and ears has been found to increase with the intensity and duration of light (Yoshida, 1981; Grist, 1986). Light is not necessarily a limiting factor in the early stages of growth but becomes progressively more critical as the plant ages. The total amount of sunlight required throughout the growing period of rice is very variable, ranging from 400 to 1200 hours (Grist, 1986). The intensity of light should be greatest at the later stages of growth, i.e. 200-240 hours of high intensity light during the final *c.* 4 weeks. It has been observed that in West Africa (Johnston, 1959) the rice crop may receive insufficient high intensity sunlight due to frequent cloud cover late in the growing season and at the same time response to fertilisers is less than normal.

Water supply: Rice is a semi-aquatic plant so, clearly, water supply is a major factor influencing the distribution of rice and also its growth and yield potential. The growth of all rice varieties is favoured by flooding; rice yields have been increased by 53% in flooded compared with adequately irrigated but unflooded culture (Senewiratne and Mikkelsen, 1961). The net water requirement for lowland rice grown in tropical Asia varies from 750-2500 mm with an average of about 1250 mm. For upland rice grown in regions where a reasonably predictable period of heavy rainfall occurs, water stress during the reproductive and ripening stages may be particularly damaging to grain production. Excessive rainfall during the flowering period, which is often accompanied by a lowering of temperature, may also adversely affect normal pollination and increase the percentage of sterile spikelets.

Soil factors: Rice can be grown on many types of soil from sandy loams to heavy clay provided there is an adequate water supply either from rainfall or irrigation. Heavy alluvial soils of river valleys and deltas are usually better suited to rice production than lighter soils. The optimum soil pH is 5.5-6.5 when the soil is dry, usually increasing to pH 7.0-7.2 on flooding (Purseglove, 1985; Grist, 1986).

1.1.2.4 Rice and rice cultivation in Africa

Six species of the genus *Oryza* are indigenous to Africa (Bezancon *et al*., 1978). They are: *O. breviligulata* Chev.and Roehr. (syn. *O. barthii* Che.); *O. brachyantha* Chev. and Roehr; *O. eichingeri*; *O. glaberrima* Steud., *O. longistaminata* Chev. and Roehr and *O. punctata* Steud.

O. glaberrima is a cultivated type which is self-fertile with some cross-pollination. This species was domesticated in parts of West Africa *c*. 3,000 years ago from the wild annual rice *O. breviligulata*. It was first grown in the central Niger delta (Carpenter, 1978). Subsequent selection by early African rice farmers has led to the development of a large number of cultivars. This species is restricted to West/Central Africa.

O. breviligulata is a wild type, mainly self-fertile but with some cross pollination. It is often found at the edges of rice fields frequently together with *O. longistaminata*. This species is restricted to West/Central Africa.

O. longistaminata is another old type. This species is very

widely distributed throughout sub-Saharan and tropical Africa. It perennates by means of rhizomes and is mainly cross-pollinated. *O. breviligulata* and *O. longistaminata* are closely related to the cultivated *O. glaberrima* and may be crossed with both *O. glaberrima* and *O. sativa*. It is a tall rice which tends to set few seeds (Carpenter, 1975).

The three remaining indigenous species (*O. brachyantha*, *O. eichingeri* and *O. punctata*) are shade-loving perennial plants of forest zones and are only distantly related to *O. breviligulata*, *O. longistaminata* and *O. glaberrima* (Sharma and Steele, 1978).

According to the system of cultivation, rice cultivars have been grouped into two main types, lowland and upland. Lowland rice cultivation refers to all types of rice cultivation where the soil is covered with water to a variable depth over a period which may encompass the entire growth cycle. Mangrove swamp or tidal swamp rice cultivation is a form of lowland cultivation where rice is grown in lowland coastal areas periodically subjected to inundation by salt water. A number of problems including salinity and acidification of the soil may be encountered. This type of rice cultivation accounts for about 10% of the area devoted to rice production in Africa and is a prominent method in coastal West Africa, e.g. Sierra Leone and Guinea (Jacquot and Courtois, 1987; Fomba, 1988). Fresh water lowland rice cultivation is practised with and without water control. Natural rainwater or the rise in water level of a river in a flood plain is commonly used for rice production. Water can be retained by installing bunds or levelling the ground. This practice is extremely common in Asia whereas in Africa it accounts for only 22.5% of the rice growing

area, mainly in parts of Nigeria and Sierra Leone. Lowland rice may also be grown with water requirements provided throughout the growing cycle either by means of gravity or by pumping. This method is highly developed and sophisticated in Asia but is a relative recent introduction to Africa, involving less than 5% of the rice-growing area (Jacquot and Courtois, 1987).

In contrast to lowland rice cultivation, upland rice is grown on land which is covered, only very exceptionally, by standing water. In the strictest sense the crop is grown on naturally well-drained soil where the water table is always below the rice roots. Adequate soil moisture supply is entirely dependent on rainfall. This system is the dominant and traditional means of cultivation in Africa where it accounts for about 60% of the rice-growing area. The major African countries which have adopted this system are Ivory Coast, Zaire, Sierra Leone, Liberia, Nigeria and Madagascar (Jacquot and Courtois, 1987). Upland rice may also be grown where the water supply is often partly provided from ground water close to the surface of the soil. This type of upland cultivation gives a higher, more regular, yield than strictly upland rice because the crop has access to two potential sources of water. However, this type of cultivation is uncommon in Africa with about 2.5% of the area using this method of production (Jacquot and Courtois, 1987).

1.1.3 The development of rice cultivation in Africa

As stated earlier, *O. glaberrima* or African rice, was probably cultivated in West and Central Africa (the centres of diversity of the species) before the very early introduction of *O. sativa* into these

regions and long before the arrival of the first European explorers. Cultivation was initially centered on the central Niger delta, Lake Chad and lower Niger delta areas of West Africa. Selection of suitable African rice genotypes over a period of 1-2000 years led to the development of numerous cultivars adapted for local conditions. Floating cultivars, weakly and strongly photoperiod sensitive types, swamp and upland types and short and long duration cultivars can still be collected today (Carpenter, 1978).

Eventually, cultivation of *O. glaberrima* began to be partly displaced by Asian rice, a process which began to accelerate from the fifteenth century onwards. European colonial powers systematically introduced rice from Asia and encouraged its production in tropical West Africa. The crop has become acclimatised in the region which might now be considered a new centre of diversity of *O. sativa* (Sharma and Steele, 1978). However, *O. glaberrima* is still found on upland rice farms in Guinea, Liberia and the Ivory Coast, and a few farmers still grow pure stands of African rice where yields of 3 tonnes/ha were obtained in the 1960s in parts of Nigeria where the species was grown as floating rice (Carpenter, 1978). In general, upland *O. glaberrima* lines cannot compete with the better *O. sativa* lines in terms of yield and, often, grain quality. However, there is still considerable overlap between the best *O. glaberrima* lines and the poorer *O. sativa* types under upland conditions.

During the period 1500 to 1900 AD, African farmers developed methods for extensive rice growing. For example, Carpenter (1978) describes how, in Sierra Leone, peoples who migrated into estuarine areas of that country at the end of the nineteenth century felled the

tidal mangrove forest and found that transplanted Asian rice could be grown when the early rains had washed away the sea salt left by the dry season tides. Rich silt deposits maintained soil fertility from year to year and the salt tides killed many weed species. *O. sativa* types with short duration and salt tolerance were selected. Intercropping with vegetables became common in Liberia and contributed to a preference for upland rice over flooded valley rice where the intercrops could not flourish. Farmers also selected cultivars of suitable duration and flood resistance for production in the rising and falling flood waters of many of the large West African rivers. At this time, features of intensive Asian rice cultivation such as small irrigation systems, levelled terraced fields and organic manuring were little used in Africa (Carpenter, 1978).

Since about 1920 three developments in rice culture were tried by African farmers. Firstly, large scale irrigation schemes appeared in countries such as Mali, Senegal and Guinea and, more recently, the Mwea scheme in Kenya. These schemes were also concerned with the resettlement of farmers in more productive circumstances and were inspired by the Gezira scheme in the Sudan.

Secondly, mechanisation was introduced, particularly for rainfed rice. Thus, contract ploughing services appeared in the 1950s and 1960s in many West African countries.

Thirdly, irrigated rice farming was intensified in the 1960s, largely by Chinese specialist advisors. Some of these schemes have been very successful, particularly in the Ivory Coast.

Although there have been successes, many innovations have not proved as beneficial as initially anticipated. Thus, often there was too much emphasis on engineering expertise and not enough on the basic agronomic requirements of high yielding rice. Most African farmers had no background in the traditions of intensive rice cultivation. Mechanised ploughing had led to erosion, increased leaching and rapid loss of productivity in some areas (Carpenter, 1978). The major costs of intensification of irrigated rice cultivation in West Africa are in land clearing and water management in contrast to the traditional Asian rice economics where intensified production is obtained by increased inputs of high yield cultivars, fertilizers and pesticides.

From the above, it is clear that over the long term the type of rice cultivated in Africa has changed from *O. glaberrima* to predominantly *O. sativa* and in more recent times methods of cultivation have been subjected to many changes in many areas. However, the bulk of rice production in Africa is still in the hands of smallholders using largely traditional methods and who grow the crop firstly for their own use and only secondly for the market.

1.1.4 Rice situation in Africa

Area and production: Demand for rice in sub-Saharan Africa is becoming more acute as a result of a general dietary shift from traditional foods and it is considered by many that this demand will likely continue to rise (Zan *et al.*, 1987). Rice consumption has increased steadily in West Africa also because of the development of urban communities and because of the higher value attributed to rice compared with traditional crops such as sorghum, millet and cassava

(Virmani *et al.*, 1978). Several rice-producing African countries are importing rice to satisfy demand (Table 1.2) and rice production must be increased to avert a serious economic drain on foreign exchange reserves. Rice is often the best cereal for large scale irrigation schemes because its yield potential is sufficiently high to compensate for costly irrigation schemes, in contrast to millet and sorghum which produce low yields and respond poorly to fertilizers (Djibril, 1978).

Table 1.2 Rice imports into Kenya and selected countries of West Africa

Country	Imports (tonnes)
Ivory Coast	375,000
Nigeria	275,000
Liberia	100,000
Guinea	80,000
Mauritania	80,000
Kenya	61,750
Ghana	54,000
Niger	35,000
Cameroon	31,000
Sierra Leone	9,000
Others	1,935,600
Africa	2,036,350

Source : FAO Trade Year Book Vol. 40 1986

Globally, rice production occupies about 14.5 million hectares

of which only 3.7% is located in Africa. Rice yield in Africa is low compared with other rice-growing areas of the world (Table 1.3) although total production is ranked third behind Asia and South America.

Table 1.3 Rice area, yield and production in the world

Region	Area (1000s ha)	Yield (tonnes/ha)	Production (1000s tonnes)
Africa	5,325	1.849	9,847
North & Central America	1,646	5.021	8,265
South America	6,905	2.219	15,323
Asia	130,337	3.349	436,652
Europe	407	5.455	2,216
Oceania	122	5.883	720
World	145,358	3.271	475,533

Source : FAO Production Yearbook Vol. 40 (1986)

Table 1.4 summarises the area and production of rice for the fourteen major rice growing countries in Africa for the periods 1979-1981 and 1986. Half of these countries showed a substantial increase in the area of rice cultivation. Mali showed the greatest increase in area (52%) and production (47%) of rice, whereas in Sierra Leone rice growing areas declined by about 13% but production increased slightly

Table 1.4 Rice area and production in Africa

Country[a]	Area (1000s ha)		Production (1000s tonnes)	
	1979-1981	1986	1979-1981	1986
Ivory Coast	383	380	438	460
Egypt	416	412	2,376	2,450
Ghana	107	90	89	95
Guinea	486	560	438	480
Liberia	203	210	251	295
Madagascar	1,182	1,168	2,055	2,138
Mali	164	250	169	249
Mozambique	73	70	67	60
Nigeria	517	700	1,127	1,416
Senegal	74	61	96	128
Sierra Leone	403	350	504	525
Tanzania	267	316	400	547
Zaire	293	335	236	300
Guinea - Bissan	70	105	105	125
Africa (Total)	4,958	5,325	8,685	9,847

Source: FAO Production Yearbook Vol. 40 (1986)

[a]Only countries with more than 40,000 ha listed

during the same period (1979-81 to 1986). Table 1.4 also shows that Madagascar, Egypt and Nigeria are the major rice producers in Africa.

The total rice area in Africa increased by 7.4% from 1979-1981 to 1986, whereas rice production increased by 13% in the same period.

The low yields obtained in general for Africa (Table 1.3) are probably due to various constraints including climatic factors (rainfall, temperature, solar radiation and photoperiod), soil resources, biological constraints (diseases, insect pests and weeds), management (growing season, land preparation, plant density and fertilizer management) and socio-economic factors (marketing, price policies, credit, improved seeds, research and extension, and the role of women). However, yields of over 5 tonnes/ha and 4 tonnes/ha have been consistently obtained in the irrigated paddy crops in Egypt and Kenya, respectively.

1.1.5 Diseases of rice

A major constraint in rice production, as with most other crops, is reduced yields and/or quality caused by various pathogens. At least 40 diseases have been identified which attack rice and are caused by fungi, bacteria and viruses.

1.1.5.1 Fungal diseases

The most important fungal diseases of rice are summarized in Table 1.5 (from Datta, 1981; Grist, 1986). The severity and importance of these diseases varies considerably according to region and often from year to year.

Table 1.5 Major fungal diseases of rice and the causal agent(s)

Disease	Pathogen
Blast	*Pyricularia oryzae*
Sheath blight	*Rhizoctonia oryzae*
Brown spot (sesame leaf spot)	*Cochliobolus miyabeanus*
Leaf scald	*Rhynchosporium oryzae*
Stem rot	*Leptosphaeria salvinii*
Bakanae disease	*Gibberella fujikurae*
Sheath rot	*Acrocylindrium oryzae*
Narrow brown leaf spot	*Cercospora oryzae*
Irregular stem rot	*Helminthosporium sigmoideum*
Seed rot and damping off	*Saprolegniaceous* and *Pythiaceous* fungi

In Africa, rice blast caused by *Pyricularia oryzae* is the most widespread and devastating disease especially in upland and drought-prone areas. Leaves, nodes and floral parts, particularly the neck of the panicle, are affected by the disease. The disease has been reported in Ghana, Mali, Nigeria, Upper Volta, Senegal, Sierra Leone, Malagasy and Cameroon (Zan *et al.*, 1985). African rice cultivars such as Lac 23 and Moroberekan and upland rice cultivars developed at the International Institute for Tropical Agriculture such as ITA 235 have some resistance to blast disease (Zan *et al.*, 1985). Brown spot disease caused by *Cochliobolus miyabeanus* (*Helminthosporium oryzae*) has been reported to cause severe infection in both upland and lowland rice ecologies in several African countries including Mali, Nigeria and Benin (Zan *et al.*, 1985). Leaf scald disease caused by *Monographella*

albescens (*Rhyncosporium oryzae*) has also been reported in both upland and lowland ecologies (Zan *et al.*, 1985). Sheath blight disease caused by *Thanetophorus cucumeris* (*Rhizoctonia oryzae*) is becoming a serious problem in some African rice growing countries and sheath rot disease (*Acrocylindrium oryzae*) is becoming important in many lowland areas, particularly those subject to low temperature (*c.* 20°C) during the flowering period (Zan *et al.*, 1985).

1.1.5.2 Bacterial diseases

Two bacterial diseases are particularly noteworthy with reference to rice (Datta, 1981; Grist, 1986) : firstly, bacterial leaf blight caused by *Xanthomonas oryzae* is prevalent throughout Asia, Africa (lowland) and Latin America and secondly, bacterial leaf streak caused by *Xanthomonas translucens*. The latter disease is limited to the tropics where the pathogen attacks parenchymatous tissue between the leaf veins giving rise to elongated, water-soaked greyish interveinal lesions which eventually turn necrotic.

1.1.5.3 Viral diseases

Rice viruses are transmitted mechanically, by aerial arthropod or soil-inhibiting fungal vectors but not, apparently, through seeds or pollen (Grist, 1986). Some important rice viruses are listed in Table 1.6. This table shows that three groups of insects are known to transmit rice virus diseases, the Delphacidae (plant hoppers), the Cicadellidae (leaf hoppers) and the Chrysomelidae (beetles).

The genomes of the viruses listed in Table 1.6 are all RNA; most in the single strand form, but others, e.g. ragged stunt and gall dwarf have a double stranded RNA genome. It appears that all rice viruses

Table 1.6 Some important rice viruses with details of symptoms, vectors, particle shape, genome composition and geographical distribution.

Virus group (virus group)	Major vector(s) (group)	Symptoms	Particle shape and genome composition	Geographical distribution	Reference (CMI/AAB description number)
Ragged stunt virus (Fiji virus group)	Nilaparvata lugens (Plant hoppers)	Stunting, twisted and ragged leave, vein swelling and delay in flowering	Polyhedral (ds RNA)	Asia	Milne, R.G., Boccarda,G and Ling, K.C. 1982 (248)
Tungro virus (Maize chlorotic dwarf virus group)	Nephotettix virescens N. nigropictus (Leaf hoppers)	Plant stunting, leaf yellowing and rusty blotches	Isometric and bacilliform forms (ss RNA)	Asia	Galvez, G.E., 1971 (67)
Hoja blanca virus (Rice stripe virus group)	Sogatodes orizicola S.cubanus (Plant hoppers)	Chlorotic stripes on the leaf blade, stunting, partial filling of spikelets	Filaments (ss RNA)	America	Morales, F.J. and Niessen, A.I., 1985 (299)
Gall dwarf virus (Phytoreovirus group)	Nephotettix nigrapictus N. cinctinceps (Leaf hoppers)	Stunting, small whitish galls along the underside of the leaf blade and outside of leaf sheath	Polyhedral (ds RNA)	Asia	Omura, T. and Inoue, H. 1985 (296)
Stripe virus (Rice stripe virus group)	Laodelphax striatella Unkanodes sapporona (Leaf hopper)	Twisting of young leaves yellowish-white stripes parallel to midrib	Filaments (ss RNA)	Asia	Toriyama, S., 1983 (269)
Dwarf virus (Phytoreovirus group)	Nephotettix apicalis N.cinctinceps (Leaf hoppers)	Pale green or yellow young leaves, stunting, early infected plants die	Isometric (ds RNA)	Asia	Iida, T.T., Shinkai, A. and Kimura, I., 1972 (102)
Transitory yellowing virus (Rhabdo virus group)	Nephotettix apicalis N. cincticeps (Leaf hoppers)	Leaf yellowing, starting from the tips of a leaf blade, mottling and rusty flecks	Bullet-shaped (ss RNA)	Taiwan	Shikata, E., 1972 (100)
Black-streaked dwarf virus (Fiji virus group)	Laodelphax striatella Unkanodes sapporona (Plant hoppers)	Stunting, deformation of leaves and white waxy or black-streaked swelling along the vein	Isometric (ds RNA)	Japan	Shikata, E., 1974 (135)
Yellow mottle virus (Sobemovirus group)	Sesselia pusilla Chaetocnema pulla (Beetles)	Yellow streak on the youngest leaves, twisting and stunting. Early infection kills susceptible lowland genotypes	Isometric (ss RNA)	Africa	Bakker, W., 1975 (149)

are geographically restricted in that they are each found on only one continent. Thus, RYMV is restricted to Africa and hoja blanca to America, and the remaining viruses listed in Table 1.6 are found either generally in South-East Asia or restricted to Taiwan (transitory yellowing virus) or Japan (black streaked dwarf).

Only two viruses have been recorded as pathogens of rice in Africa, rice yellow mottle, which is discussed later in this section, and rice stripe necrosis virus (RSNV).

RSNV has recently been associated with a condition known for many years on upland rice in West Africa as rice crinkling disease (Louvel and Bidaux, 1977); the probable viral etiology was established in the Ivory Coast by Fauquet and Thouvenel (1983) and recently reviewed by Fauquet *et al.* (1988). Symptoms are extremely variable but may include yellow leaf striping, stunting, reduction in tillering and leaf and panicle distortion. Infected plants tend to show a patchy distribution which recurs erratically in the same areas from season to season. The disease is associated with the presence of the soil fungus *Polymyxa graminis* in the roots of infected plants and this fungus is considered to be the likely vector (Fauquet *et al.*, 1988). The virus is not mechanically transmissible to rice but can be mechanically transmitted from rice to *Chenopodium amaranticolor* and *Nicotiana benthamiana* in which it induces local lesions. Virions are rod-shaped with three different particle lengths of 110-160, 270 and 380 nm. Present evidence suggests that RSNV may be a member of the furovirus group.

The disease is not a severe problem in West Africa but has been

reported in the Ivory Coast, Nigeria, Liberia and Sierra Leone (Fauquet *et al*., 1988). Although limited evidence suggests the disease appears not to be spreading, the presence of resistance genes in some varieties indicates that appropriate selection and breeding programmes offer a suitable strategy for control. The virus is restricted to West Africa.

1.2 RICE YELLOW MOTTLE VIRUS

1.2.1 Geographical distribution

Rice yellow mottle virus (RYMV) was first reported from Kenya in 1966 (Bakker, 1974). Subsequently, the virus has been found in Sierra Leone (Rayamundo and Buddenhagen, 1976); Ivory Coast (Fauquet and Thouvenel, 1977) and Nigeria, Liberia and Tanzania (including Zanzibar) in 1988 (Rossel *et al*., 1982). Recently, RYMV has been found to be particularly severe in Niger, Burkina Faso and Mali (John *et al*., 1984) and it has also been found in Guinea (Fomba, 1988). The disease seems to be restricted to Africa (Fig. 1.1); it has not been reported from South-east Asia (Reckhaus and Adamou, 1986) or other rice-growing areas of the world.

1.2.2 Importance

Yellow mottle disease is considered a major problem affecting rice production in areas of Kenya and Niger (IITA, 1982; Reckhaus and Adamou, 1986) and of potential economic significance wherever the pathogen has been found.

Significant reductions in grain weight and the number of fertile

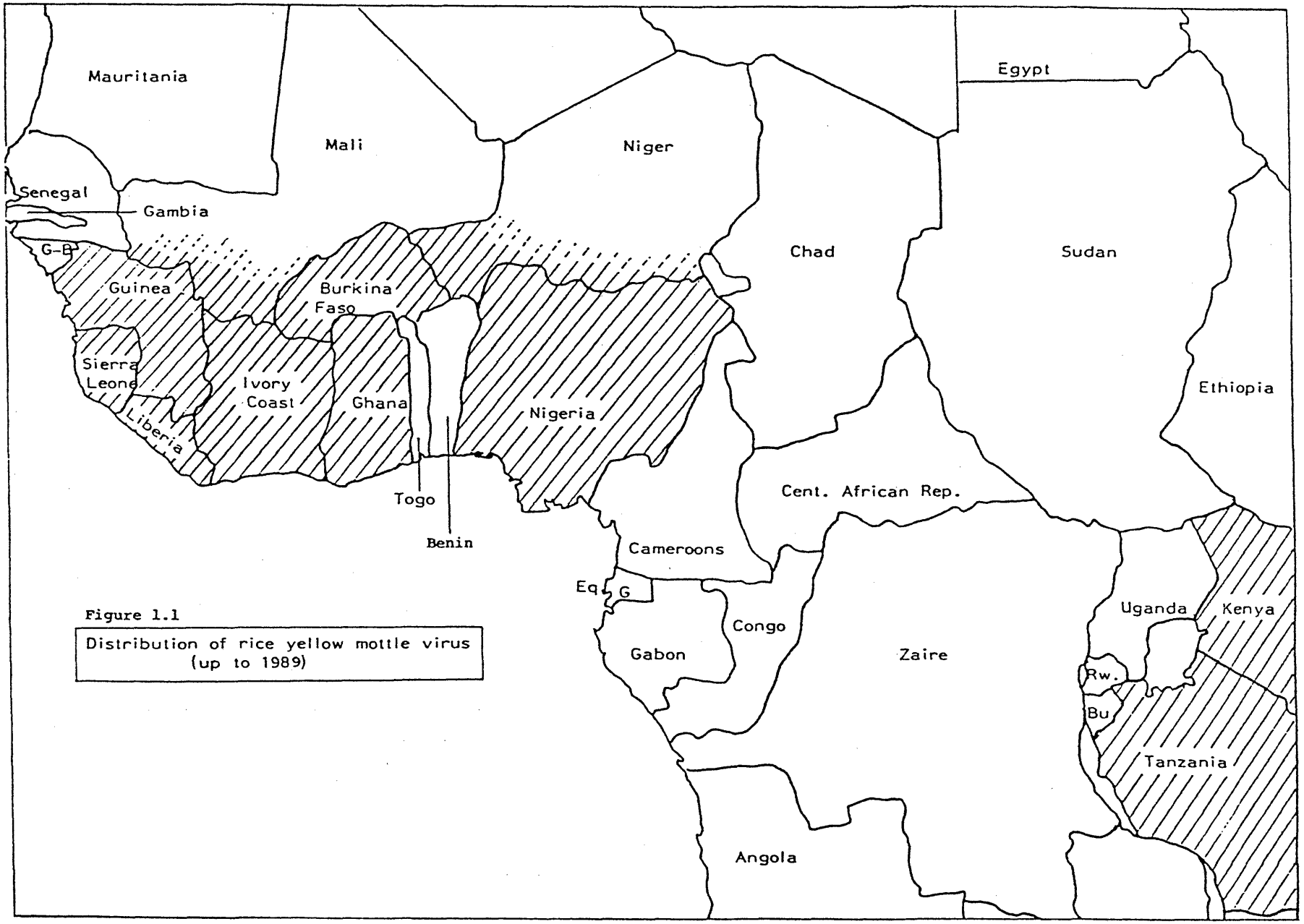


Figure 1.1

Distribution of rice yellow mottle virus (up to 1989)

flowers per panicle were caused by a Niger isolate of RYMV (Reckhaus and Adamou, 1986). In Nigeria, inoculation of cv. OS6 (resistant cultivar) with RYMV 20 days after germination reduced yield by 18.6% and increased sterility to 84%, whereas in the susceptible cultivar, BG 90-2, yield reduction was 93.5% and sterility 61.7% (IITA, 1982). Yield was depressed by 10-40%, depending on the cultivars tested, but increased sterility was not observed when 15 IRAT cultivars were inoculated with an Ivory Coast isolate of RYMV (Fauquet and Thouvenel, 1977).

RYMV appears to be most common in irrigated lowland areas where the exotic lowland Asian varieties are planted (John *et al.*, 1985). Recently, the disease was identified in plants growing at the African Upland Rice Advanced Variety Trial (AURAT) at Tombokro, Ivory Coast, under a typical upland rice ecology (Awoderu *et al.*, 1987).

1.2.3 Host range and symptoms

The host range of RYMV is narrow and limited to species within the Gramineae, mainly in the tribes Oryzeae and Eragrostidae (Bakker, 1974). Hosts infected and in which symptoms were induced included *O. sativa* (79 varieties tested), *O. barthii*, *O. glaberrima* and *O. punctata* within the Oryzeae; *Dinebra retroflexa*, *Eragrostis aethiopica* and *E. ciliasis* within the Eragrostidae and *Phleum arenarium* (Phalarideae); hosts from which virus was recovered from inoculated leaves only included *O. alta* and *O. latifolia* (Oryzeae); *Eragrostis macilenta* and *E. tef* (Eragrostidae), *Bromus hordeaceus* (Bromeae) and *Setaria viridis* (Paniceae) (Bakker, 1974). Bakker (1974) further found that the Kenyan RYMV isolate he used failed to infect 24 dicotyledenous

hosts tested.

Characteristic symptoms of infection in susceptible rice cultivars included foliar mottle and yellow streaks, malformation, partial emergence and sterility of panicles and overall stunting. Sometimes young leaves were spirally twisted. In severe cases, particularly when plants were infected at early growth stages, the virus was lethal (Bakker, 1974; Fauquet and Thouvenel, 1977). Old leaves usually developed chlorosis followed by the appearance of orange pigments and then necrosis, a sequence which started at the leaf tip then progressed basipetally.

In resistant cultivars, disease severity depends upon the variety and the age of the plant when infected (Rossel and Thottappilly, 1985). Infected leaves of resistant cultivars usually develop a general mottling and chlorotic streaks but do not turn orange or necrotic (Bakker, 1974).

Susceptible rice cultivars usually showed symptoms 5 to 7 days after inoculation whereas it was difficult to discern the first symptoms in a resistant cultivar (Basmati 21). With *O. barthii* and *O. punctata*, initial symptoms appeared 14 days after inoculation (Bakker, 1974). Hosts outside the Oryzeae, e.g. *Dinebra retroflexa*, showed a systemic yellowish green discolouration and mild mottling of the newly-formed leaves.

1.2.4 Transmission

a) Sap transmission

The virus is readily transmitted by mechanical inoculation using sap from leaves showing clear symptoms. The virus has been recovered from sap obtained from the roots of infected plants and from the guttation fluid of diseased rice plants (Bakker, 1974).

b) Natural vectors

Work in Kenya by Bakker (1971, 1974) showed that the Kenyan isolate was transmitted by various Chrysomelid beetles belonging to the subfamilies Criocerinae (e.g. *Oulema dunbrodiensis*), Galerucina (e.g. *Sesselia pusilla*), Halticinae (e.g. *Chaetocnema* spp.) and Hispinae (e.g. *Trichispa sericae*). These chrysomelid beetles were usually encountered in and around rice fields in Kenya (Bakker, 1974).

Conocephalus merumontanus (long-horned grasshopper) was also shown to transmit RYMV (Bakker, 1974). The beetle, *Chaetocnema pulla*, was found to be a virus carrier in the field, and Bakker (1974) considered that this insect might be the main natural vector of the virus in Kenyan rice fields.

Acquisition and inoculation feeding periods for *S. pusilla*, *C. pulla* and *T. sericea* were in the order of 15 minutes but the efficiency of transmission increased with increasing length of acquisition and inoculation feeding periods up to 2-3 days. *S. pusilla* and *C. pulla* retained the virus for 2-3 days and sometimes 8 and 5 days respectively, whereas *T. serica* retained the virus for one day only (Bakker, 1974). It is possible that other beetle vectors may exist.

Extensive searches and tests for natural vectors in West Africa have yet to be made - a pressing need if the detailed ecology and spread of the virus is to be fully understood.

The virus was not transmitted from rice to rice by a range of other invertebrates including nematodes, aphids and mites (Bakker, 1974).

c) Seed transmission

No seed transmission was found in tests with a total of 974 seeds collected from nine cultivars of infected rice plants (Bakker, 1970, 1974). Fauquet and Thouvenel (1977) tested a total of 6,000 seeds from 15 cultivars and failed to obtain evidence of seed transmission.

The above experiments with Kenyan and Ivory Coast isolates of RYMV involved 'growing on' tests only, absence of symptoms was the evidence for lack of seed transmission. Experiments have yet to be made to determine whether or not virus is present in the embryo, testa or endosperm of seeds produced on infected plants and whether or not absence of symptoms is consistently associated with freedom from virus as determined by, for example, serological or dot-blot hybridization-type tests.

1.2.5 Sap properties

Experiments with the original Kenyan isolate of RYMV by Bakker (1974) showed that with sap from rice (cv. Sindano) tested on seedlings of the same variety, the dilution end point (DEP) was 10^{-6} and the

thermal inactivation point (TIP) 65°C. Infectivity in sap was retained for 99 days at 20°C and for 260 days at 4°C. Virus in infected leaves stored over $CaCl_2$ at 4°C survived for at least one year (Bakker, 1975). Fauquet and Thouvenel (1977), working with an Ivory Coast isolate, reported that the DEP fluctuated between 10^{-6}-10^{-9} and the TIP was 55°-70°C. The virus remained viable in sap for at least 34 days at 27°C but not for 41 days, and for 84 days at 4°C. RYMV remained infectious in dry leaves stored for more than 56 days at laboratory temperature and for several months at -20°C. The DEP and longevity *in vitro* data suggested that RYMV reaches high concentrations in rice leaves and that particles are stable when infected leaves are stored at 4°C or below.

1.2.6 Virus particles

The virus consists of small isometric particles 28±3 nm in diameter (Bakker, 1974; Fauquet and Thouvenel, 1977), sedimenting as a single component. Each virion contains a single RNA species with a M.W. of 1.4 x 10^{-6} comprising about 23% of the particle by weight (Hull, 1977).

Samples of purified RYMV-K (Kenyan isolate) stained with PTA were examined by Bakker (1974). The virus particles usually had an angular outline, this was hexagonal particularly when particles were packed into semi-crystalline arrays. When frozen purified suspensions were examined, virus particles appeared less angular, many were broken and no crystalline arrays were seen (Bakker, 1974).

In an electron microscopy study at different stages of leaf growth and symptom development, Bakker (1974) found that in young,

infected 'Sindano' leaves (initial symptom development) the virus particles were seen in thin sections of tissue between veins. The mesophyll cells showed the highest virus concentration. In the second stage of leaf and symptom development (early enlargement of the mesophyll cells, mottle symptoms), aggregations of particles were more common in the cytoplasm. In the later stages (mature leaf with mottling and chlorotic areas) the virus particles were distributed in the cytoplasm of mesophyll cells and crystalline structures composed of virus aggregated particles appeared which were sometimes surrounded by a membrane. At this stage also, fibres were often completely filled with crystals of virus particles particularly in the chlorotic regions of the leaf. Two other types of inclusion were also seen, long flexuous tubules and an inclusion made up of an electron-dense centre surrounded by a less electron dense area enveloped by a membrane (Bakker, 1974).

1.2.7 Relationships

RYMV is presently classified as a membrane of the sobemovirus group on the basis of morphology, molecular weight of RNA and capsid protein and beetle transmission in which it resembles the type member of the group, Southern bean mosaic virus (SBMV) (Hull, 1977). However, RYMV differs from SBMV and some other groups members in the absence of seed transmission (Bakker, 1974). No serological relationships have yet been demonstrated between RYMV and other sobemoviruses (Bakker, 1974; Hull, 1977).

Hull (1988) considered ten viruses as members of the sobemovirus group. These viruses are: Southern bean mosaic virus (SBMV), cocksfoot

mottle virus (CFMV), rice yellow mottle virus (RYMV), sowbane mosaic virus (SMV), blueberry shoestring virus (BBSSV), lucerne transient streak virus (LTSV), solanum nodiflorum mottle (SNMV), turnip rosette virus (TRosV), velvet tobacco mottle (VTMoV) and subterranean clover mottle (SCMV).

Some characteristics of sobemovirus are: mechanically transmitted, a relatively narrow host range, isometric particles that sediment between 110 and 120 S and having a single viral coat protein species of molecular weight about 30,000. The genome is a single species of single-stranded RNA of molecular weight about 1.4×10^{-6}.

1.2.8 Epidemiology

In Kenya, diseased plants were easily identified in rice fields about 2-3 weeks after transplanting. In a small trial at the Mwea irrigation research station, the disease spread randomly through the trial plots, whereas in large fields diseased plants were more common only at the edges of the fields 2-3 weeks after transplanting (Bakker, 1974).

In the Niger, the first indication of the disease was seen in a few diseased plants scattered in a rice field which was part of a rice scheme at Say (near Niami). During succeeding growing seasons, RYMV spread into other state rice schemes and finally the disease became established in all major rice-growing areas (Reckhaus and Adamou, 1986). At the present time, there have been few reports detailing the frequency and incidence of RYMV in growing crops.

Virus source: RYMV is readily sap-transmissible and appears to be

quite stable, both inside and outside the host. As stated earlier, several insects with biting mouthparts, especially within Chrysomelidae were identified as vectors of RYMV by Bakker (1974). Moreover, Bakker (1974) also identified several members of the Gramineae which developed systemic symptoms after mechanical inoculation and others, which though infected, failed to show symptoms. Many such plant species within the tribes Oryzeae and Eragrostidae, were found around Lake Victoria in and around lowland rice crops growing as part of Kenyan Mwea irrigation scheme. Bakker considered that the virus was likely to be present in wild indigenous grasses (Oryzeae and Eragrostidae) around Lake Victoria before irrigated lowland rice cultivation was introduced to the area. The Kenyan irrigation scheme and regime for lowland rice cultivation was found to enhance the growth of grasses such as *Dinebra retroflexa*, *Oryza barthii*, *Eragrostis aethiopica* and *E. ciliaris* which Bakker found to be systemic hosts for RYMV and hosts (food and shelter) for the beetle vectors.

From the considerations above, primary inoculum of RYMV could be found in firstly, wild hosts belonging to Oryzeae such as *O. barthii* reported to be susceptible to the Kenyan isolate (Bakker, 1974). Later, John *et al.* (1984) identified RYMV in the wild host, *O. longistaminata*, commonly seen growing in marshy areas as well as in rice fields in the Niger. They suggested that this plant may be a natural reservoir of the virus. A second virus source is probably the rice fields themselves. The existence of rice throughout the year (as a result of irrigation practices) increases the inoculum potential especially in the presence of susceptible cultivars and with an abundance of vectors. Thirdly, infected nursery seedlings are a potential source of infection. Although only a low percentage of

symptomless infected seedlings was observed by Bakker (1974), it seems very likely that these nurseries could contribute to RYMV spread. Lastly, infected volunteer rice plants on bunds or ratoon plants are of great potential importance as sources of primary inoculum to initiate new infections. It is unfortunate that little epidemiological work has been carried out in West Africa along the lines of that done by Bakker in Kenya.

Virus spread within the rice crop: Very little is known about the dynamics of within-crop spread of RYMV in rice crops. The presence of beetle vectors, especially *Chaetocnema* spp. and *Sesselia pusilla* throughout the year in and around Kenyan rice fields, suggested that beetles were the main agency for secondary spread of RYMV (Bakker, 1974).

Agricultural practices such as transplanting and weeding may increase the possibility of mechanical transmission.

Spread of RYMV to other rice growing areas: With increases in the area planted with lowland and upland rices, given a natural source of RYMV, the chance of spread of the disease may increase. The introduction of new highly-susceptible lowland cultivars could exacerbate the problem.

1.2.9 Species and cultivar reactions

According to Bakker (1974), the following species within the Oryzeae were systemic hosts for RYMV isolated from Kenya when test hosts were grown under glasshouse conditions: *Oryza sativa*, *O. barthii*, *O. glaberrima* and *O. punctata*. Rossel *et al.* (1982) failed to recover

RYMV from *O. longistaminata*, *O. barthii* or *O. glaberrima* when samples of populations of these species in a large swamp area in central Nigeria were tested serologically. Later, however, John *et al.* (1984) found that samples of *O. longistaminata* collected from the Niger gave a positive reaction with RYMV antisera and exhibited clear symptoms. Most lines of *O. glaberrima* were found to be susceptible to a Nigerian isolate of RYMV by Attere and Fatokun (1983). They reported that 13 accessions out of 455 accessions tested of African rice were resistant because, although infected, they showed no symptoms.

In a later study, John *et al.* (1985) showed that all 19 accessions tested of *O. glaberrima* exhibited a marked resistant reaction. In the Ivory Coast, artificial inoculation with an Ivory Coast isolate of RYMV to the following rice cultivars and species *O. sativa* 'IR 8', 'IR 5', 'Zenith', 'Moroberekan' and 'OS6'; *O. barthii*; *O. australiensis* and *O. glaberrima* resulted in infection and symptom appearance (Fauquet and Thouvenel, 1977). In a varietal reaction trial with RYMV undertaken at IITA (Nigeria), John *et al.* (1985) found that the progression of RYMV symptoms in tested rice varieties differed considerably only two weeks after inoculation. In cultivars ITA 212, BG 90-2 and IR 5, bred specially for lowland situations, virus symptoms progressively increased in severity with time. Upland cultivars 'TOs 415' and Moroberekan and several *O. glaberrima* lines remained symptomless, whereas IR 9224-162-3-2-3 and some other TOs lines expressed mild symptoms throughout their growth. Some varieties, typified by cultivar Kulu, initially showed disease symptoms but later produced leaves which appeared healthy, whereas other cultivars such as IR 42, which were initially symptomless, later produced conspicuous yellow mottle symptoms.

Using a combination of visual assessment of symptoms and relative virus antigen concentration in tissues determined by ELISA, workers at IITA (Nigeria), (IITA, 1985) found that cultivars BG 90-2, IR 5 and IR 20 were highly susceptible to RYMV whereas Lac 23, Moroberekan and OS 6 were highly resistant but contained the virus. Lac 23 had the lowest content of RYMV antigen. However, no details were given concerning the method used to determine virus concentration and no specific values for virus content were provided.

1.2.10 Control

Practical direct measures for the therapeutic control of virus diseases do not exist, therefore, indirect prophylactic approaches are commonly adopted attempting, for example, to eliminate or reduce sources of infection within and around susceptible crops. Although RYMV is present in wild and weed grasses belonging to the Eragrostideae and Oryzeae (Bakker, 1974), and the elimination of these plants is recommended, it is clearly a difficult objective to achieve practically. Infected ratoon rice and volunteer rice plants should also be eradicated. Other cultural practices such as drainage of rice fields between two consecutive growing seasons and the planting of rice within a short period to prevent successive infections at different growth stages are also recommended (Bakker, 1974; Reckhaus and Adamou, 1986). The use of chemical control against the vectors seems to be an impractical approach to RYMV control in West and East Africa and the high cost of insecticides precludes such measures because of the weak economies of most rice growing states in Africa. Ecological and toxicological problems further argue against such an approach (Bakker,

1974; Reckhaus and Adamou, 1986). The most cost-effective and practical method is undoubtedly the use of resistant varieties. The indigenous wild rice *O. barthii* has been found to be immune to a Nigerian isolate of RYMV (IITA, 1984). In addition, resistance screening of *O. sativa* germplasm has shown that traditional African upland varieties are significantly more tolerant to the virus than exotic rice lines and cultivars from South-east Asia (Raymundo and Konteh, 1980). Some accessions of *O. glaberrima* e.g. TOg 5764 and TOg 5681, have been identified as having high levels of resistance to the virus (IITA, 1986). Therefore, resistance in traditional African upland cultivars such as Lac 23, Moroberekan and OS 6 is being used in breeding programmes at the IITA as a basis for the development of high-yielding cultivars with adequate resistance to RYMV.

1.3 PROJECT OBJECTIVES

Rice yellow mottle virus, first reported in the early 1970s from Kenya, was subsequently shown to be present and sometimes widespread in rice growing regions of other East and West African countries in the late 1970s and early 1980s.

Half of the land devoted to rice in Africa is now producing lowland-type rice (Zan *et al.*, 1985). The introduction of lowland Asian varieties, most of which are susceptible to RYMV, is likely to increase and become a potential constraint to rice production in Africa.

Since the virus had been isolated from several countries in East and West Africa, as might be expected, some biological and serological

differences have been reported (Fauquet and Thouvenel, 1977; IITA, 1987). Nevertheless, very little attention has been given to the relationships between RYMV isolates from different geographical regions of Africa and their comparative effects on disease symptoms and crop growth when tested under the same or similar environmental conditions.

Also at IITA researchers have identified several rice genotypes resistant to Nigerian isolates of RYMV. The resistance was first found in traditional African upland cultivars such as Lac 23 and Moroberekan and these and other resistant cultivars are being used in breeding programmes at IITA. However, no work has been done to attempt to elucidate the nature of resistance or tolerance or the possible resistance mechanism(s) involved.

Therefore, the present project was undertaken firstly to assess the reaction of several RYMV-N resistant and susceptible rice cultivars under glasshouse conditions to five isolates of RYMV collected from different countries in Africa. Further objectives were to determine the relationships between the age of the plant at inoculation and the resulting disease incidence, symptom type and severity. Experiments were planned to look at the nature of resistance in resistant cultivars inoculated with RYMV-N and to pinpoint some of the possible resistance mechanisms involved. Lastly, it was planned to commence studies on the relationships between the five isolates and to determine if any biological and/or serological differences existed between them.

S E C T I O N T W O

GENERAL MATERIALS AND METHODS

2.0 GENERAL MATERIALS AND METHODS

2.1 Plant materials

The five rice cultivars used were ITA 212, BG 90-2, Lac 23, Moroberekan and ITA 235 and seed of all cultivars was kindly supplied by Dr. T.M. Masajo, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria (Table 2.1).

Table 2.1 Rice cultivars used in the present study

Cultivar	Origin	Reaction to RYMV-N	Ecology	Stature
ITA 212	Asia	Susceptible	Lowland	Short
BG 90-2	Asia	Susceptible	Lowland	Short
Lac 23	Africa	Resistant	Upland	Tall
Moroberekan	Africa	Resistant	Upland	Tall
ITA 235	Asia x Africa	Resistant	Upland	Tall

2.2 Virus isolates

Five rice yellow mottle virus (RYMV) isolates were obtained from East and West Africa for use in the present study (Table 2.2). The isolates were coded according to their country of origin. All isolates were received in 1986 and recovered from rice leaves by mechanical inoculation to cv. ITA 212. Virus isolates were subsequently maintained in rice (cv. ITA 212) plants or stored as

leaf material at -20°C or over $CaCl_2$ at 4°C. The Kenyan isolate obtained from Dr. Bos was a culture of the original RYMV isolate discovered and described by Bakker (1974) and maintained in leaf material dried over $CaCl_2$ at Wageningen (The Netherlands). The Niger isolate, obtained from Dr. Leseman, was originally collected in Niger by Dr. P.M. Reckhaus (Reckhaus and Adamou, 1986).

Table 2.2. Rice yellow mottle virus isolates used in the present study

Country of origin	Code	Obtained from	Address
Ivory Coast	RYMV-1C	Dr. C. Fauquet	Laboratoire de Virologie, Office de Recherche Scientific et Technique, Outre-Mer, Abidjan, Ivory Coast
Kenya	RYMV-K	Dr. L. Bos	Institute of Phytopathological Research, Wageningen, The Netherlands
Niger	RYMV-Nr	Dr. D. Leseman	Biologische Bundesanstalt for Land und Forstwirtschaft, Braunschweig, Federal Republic of Germany
Nigeria	RYMV-N	Dr.G.Thottappilly	IITA, Ibadan, Nigeria
Sierra Leone	RYMV-SL	Dr. S.N. Fomba	West African Rice Development Association, Regional Mangrove Swamp Rice Research Station, Freetown, Sierra Leone

2.3 Seed germination and plant growth

Seeds of each cultivar were soaked with tap water in a beaker and incubated at 30°C for 24 hours. Soaked seeds were sown (1 cm deep) in plastic trays containing vermiculite and watered with half-strength nutrient solution (see below). Trays were covered with polyethylene sheets and kept in the glasshouse. Immediately after seedling emergence, the polyethylene covers were removed. When seedlings had reached the 1-2 leaf stage, they were transplanted into plastic pots. To minimize the risk of possible virus transmission to uninoculated control plants by root to root contact, to ensure a good water supply and to prevent possible virus spread via water, all pots were isolated from others by placing them on individual plastic saucers.

The growing medium was prepared by collecting heavy clay soil from Sturdes-Pound field on the Wye College farm. This field was situated over Gault clay. After drying, the soil was crushed and sieved, and sieved soil was mixed with peat (1:1 v/v) and the pH adjusted, if necessary, to 6.5 by adding lime.

Transplanted seedlings were immediately fed with rice nutrient solution and kept in the glasshouse. Subsequently, pots were watered with nutrient solution each week. Plants were examined daily to ensure water was never limiting and watered with distilled water when required. Pot size, light duration and quality and temperatures varied between experiments, and these variables are detailed later in the materials and methods for specific experiments.

The nutrient solution used in all experiments was prepared as described by Yoshida *et al*. (1976) (Tables 2.3 and 2.4).

2.4 Mechanical inoculation

RYMV inoculum was prepared by grinding frozen or fresh systemically infected rice leaves 3 weeks after inoculation (unless stated otherwise) in a mortar containing cold 0.067 M Sorensen's buffer, pH 7.0. The tissue:buffer ratio was 1:10 (w/v) unless stated otherwise. Celite (1 mg/ml) was incorporated as an abrasive. Inoculations were made by stroking both sides of the receptor rice leaves three times using forefinger and thumb or a cotton wool swab. Test plants were inoculated at the 4-5 leaf stage unless stated otherwise. For experimental purposes the day of inoculation was designated day 0.

Considerable care was taken to prevent possible contact transmission of virus from infected to healthy plants or to and from plants infected with different RYMV isolates. Thus, for example, plants were well separated on the glasshouse bench, isolated from the bench by standing pots on plastic saucers and after handling plants infected with a given isolate, hands were washed thoroughly before moving on to the next isolate. The capillary matting on the glasshouse bench was changed between experiments.

Table 2.3 Preparation of stock nutrient solutions

Element	Reagent	Amount (g/litre)
N	NH_4NO_3	91.4
P	$NaH_2PO_4.2H_2O$	40.3
K	K_2SO_4	71.3
Ca	$CaCl_2$	88.6
Mg	$MgSO_4.7H_2O$	324.0
Mn	$MnCl_2.4H_2O$	1.5
Mo	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.074
B	H_3PO_4	0.939
Zn	$ZnSO_4.7H_2O$	0.035
Cu	$CuSO_4.5H_2O$	0.031
Fe	$FeCl_3.6H_2O$	7.7
	Citric acid (monohydrate)	11.9

Table 2.4 Composition of nutrient solution

Element	Volume (ml) of stock solution per 4 litres of distilled water[a]	Final concentration of elements in nutrient solution (ppm)
N	5	40
P	5	10
K	5	40
Ca	5	40
Mg	5	40
Mn		0.5
Mo		0.05
B		0.2
Zn	5	0.01
Cu		0.01
Fe		2

[a] The solution was stirred after adding each reagent to avoid salt precipitation, then the pH was adjusted to 5.0 by the addition of 1 N NaOH.

2.5 Assessment of disease development

2.5.1 Production of foliar symptoms

Leaf position on the main shoot was indicated by numbering each expanded leaf using a waterproof marker to permit measurement of individual leaf lengths and disease assessment of all emerged leaves on each plant. Disease development was recorded after the first appearance of symptoms on susceptible and resistant cultivars. The

disease score for each leaf was assessed visually according to a disease rating scale of 0.0-7.0. Disease ratings and symptoms were:-

0.0 = symptomless

1.0 = chlorotic stripes or mottle covering 1-20% of the total leaf area

2.0 = chlorotic stripes or mottle covering 21-40% of the total leaf area

3.0 = chlorotic stripes or mottle covering 41-60% of the total leaf area

4.0 = chlorotic stripes or mottle covering 61-80% of the total leaf area

5.0 = chlorotic stripes or mottle covering 81-100% of the total leaf area

6.0 = as 5.0 but leaves also turning yellow into orange

7.0 = as 6.0 but leaves turning orange and necrotic

The above categories are illustrated in Fig. 3.1A

As a guideline to the overall reaction of cultivars to infection by the five RYMV isolates, a disease severity value for the whole plant was calculated by dividing the total score for all unfolded leaves showing symptoms by the number of such leaves present on the main shoot. For more accurate comparisons, disease severity scores for specific leaf positions were used. The same 0-7.0 disease rating scale was used for susceptible and resistant cultivars.

2.5.2 Disease incidence

Disease incidence was the proportion of inoculated plants showing symptoms expressed as a percentage of the total number of plants.

2.6 Enzyme-linked immunosorbent assay (ELISA)

The direct, double antibody sandwich (DAS) form of ELISA was used in all tests.

Antisera to RYMV isolates N, K and IC were kindly supplied by Dr. G. Thottappilly (IITA, Nigeria), Dr. D.A. Peters (Agricultural University, Wageningen), and Dr. C. Fauquet (ORSTROM, Ivory Coast) respectively.

Preparation of gamma (γ)-globulin fraction

Gamma-globulins were fractionated from crude antiserum as described by Clark and Adams (1977) and Hill (1984). Gamma globulins were separated from the serum by ammonium sulphate precipitation at room temperature. The antiserum was diluted 1:10 (v/v) with distilled water and globulins precipitated by adding an equal volume of saturated ammonium sulphate. The mixture was allowed to stand for 30-60 minutes at room temperature during which time a white flocculent precipitate developed. The preparation was centrifuged at 2,600 g for 15 minutes. The supernatant was discarded and the pellet resuspended in a 10 ml volume of distilled water. Saturated ammonium sulphate (8 ml) was added, then the preparation was allowed to stand and centrifuged as described above. The pellet was suspended in 2 ml of half-strength PBS at 4°C to remove the ammonium sulphate. The final dialysed globulin fraction was adjusted by sequential dilution with half-strength PBS to give a reading of 1.4 at A_{280} (approximately 1.0 mg protein/ml). The gamma-globulin was stored at -20°C in silicon-coated (dimethyldichlorosilane) vials.

Preparation of enzyme conjugate

The objective was to prepare an alkaline phosphatase (ALP) conjugate such that most of the enzymological and immunological activities of the complex were retained. ALP was coupled with antibody protein using a single-step procedure with the dialdehyde glutaraldehyde (GA). GA forms a bridge between the two proteins by linking amino groups on the two different proteins.

The enzyme ALP, suspended in sodium chloride (Sigma Type VII-T), was mixed well with RYMV gamma-globulin using 2.5 mg of enzyme (usually about 0.25 ml) and 1 ml of thawed gamma globulin (about 1 mg). The weight ratio of enzyme to globulin was about 2.5:1. Glutaraldehyde (B.D.H. 25%) was diluted to 2% with distilled water and added to give a final concentration of 0.05% (w.v.). Following gentle mixing the mixture was left to stand for 4 hours at room temperature. A faint yellow brown colour developed with some, but not all, preparations. The final mixture was dialysed against four changes of PBS at 4°C for 24 hours to remove any uncombined glutaraldehyde. Bovine serum albumin (Sigma, Fraction V) was added to give a final concentration of 5 mg/ml and the preparation stored in silicone-coated tubes at 4°C.

ELISA tests:

Several buffers were used in the various steps involved in the ELISA tests. They were:-

Phosphate buffered saline (PBS) containing 8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4.12H_2O$ and 0.2 g KCl dissolved in a litre of distilled

water giving a pH of 7.4

Phosphate buffered saline - Tween (PBS-Tween) PBS containing 0.05% (v/v) Tween 20 (polyoxyethylene (20)-sorbitan monolaurate).

Test sample buffer PBS-Tween buffer containing 2% (w/v) polyvinyl pyrrolidone (PVP) m.w. approx. 44.000.

Conjugate buffer PBS-Tween containing 2% (w/v) PVP and 0.2% (w/v) ovalbumin.

Coating buffer containing 1.59 g $NaCO_3$, 2.93 g $NaHCO_3$, pH 9.6

Substrate buffer made from 97 ml diethanolamine and 800 ml distilled water. The pH was adjusted to 9.8 with 1 N HCl.

Sodium azide (NaN_3) was added to all the buffers as a preservative (final concentration 0.02%).

The test procedure adopted followed those described by Clark and Adams (1977) and Hill (1984). Preliminary tests showed that a γ-globulin concentration of 1 μg/ml and a conjugate dilution of 1:1000 (v/v) were optimal for low background values and high positive values using healthy leaf or RYMV-infected leaf material, respectively. The same globulin concentrations and conjugate dilutions were found to be optimal and were used for all of the several conjugates prepared during the work. A single conjugate was used for a given experiment or series of related experiments.

Tests were made in polystyrene flat-bottom microtitre plates, type M29AR (Dynatech Ltd., Billingshurst, Sussex) with an array of 8 x 12 wells. However, in early work, high variation was encountered between and within plates which caused problems, particularly with quantitative tests. After tests with several batches of Dynatech plates, a change was made to Nunc-immuno plates type Maxisorb F96 (Gibco Ltd., Paisley, Scotland). Variation was much reduced using these plates and was no longer a major problem.

Plates were coated with 200 µl aliquots of diluted gamma-globulin per well, covered with clingfilm and incubated for 3 hours at 30°C. Well contents were discharged and the wells washed by flooding with PBS-Tween. This process was repeated three times. The test samples prepared at an appropriate tissue:test sample buffer ratio, were added as 200 µl aliquots to each coated well. Plates were then covered with clingfilm and stored overnight at 4°C. The wells were washed as before, and 200 µl of diluted enzyme-labelled gamma globulin was added to each well. The plates were then covered with clingfilm, incubated for 3 hours at 30°C and then 250 µl aliquots of freshly prepared substrate were added. The substrate was p-nitrophenyl phosphate (Sigma) at a concentration of 0.6 mg/ml in substrate buffer. Plates were incubated at laboratory temperature until the hydrolysis reaction had progressed sufficiently to induce the production of a yellowish coloration (p-nitrophenol).

Results were recorded by measuring the absorbance at 410 nm (A410) with a Microelisa Mini Reader (Model MR590, Dynatech Ltd., Billingshurst, Sussex). Readings were usually taken at 15 minute intervals from 15 to 120 minutes after substrate addition.

Incubation time was kept constant within an experiment. Extracts of leaves from RYMV-free or RYMV-infected plants were incorporated into all tests as negative and positive control, respectively. Test values were considered positive when they exceeded healthy control values by a factor of two.

S E C T I O N T H R E E

INTERACTIONS BETWEEN RICE CULTIVARS AND ISOLATES OF RYMV

3. INTERACTIONS BETWEEN RICE CULTIVARS AND ISOLATES OF RYMV

3.1 INTRODUCTION

Yellow mottle disease is the only rice virus disease of economic importance in Africa. This African-specific virus disease is now a major problem in some irrigated rice areas in West and East Africa, especially where dwarf lowland Asian high-yielding varieties have been introduced. Identification of rice yellow mottle virus (RYMV) in West and East African countries has been based mainly on field disease symptoms but the mode of transmission, particle size and shape and serological tests have sometimes been used. Some studies have suggested that RYMV isolates may be very similar (Raymundo and Buddenhagen, 1976). However, Fauquet and Thouvenel (1977) showed that an isolate from Ivory Coast and Bakker's original Kenyan isolate were serologically distinguishable. Biological differences such as between Kenyan and Nigerian isolates have also been reported (IITA, 1986). Nevertheless, it is not known if isolates of RYMV differ in their effect on rice growth and productivity. Knowledge of the interactions between RYMV isolates and rice genotypes should be of value in devising appropriate resistance breeding programmes. For example, would rice genotypes bred at IITA and selected using a Nigerian RYMV isolate as being virus resistant, prove to be resistant to RYMV isolates in, for example Liberia (assuming the rice genotype is suitable for release in appropriate regions of Liberia). There is clearly some potential danger in testing IITA rice genotypes to RYMV isolates imported from other African countries for assessment at IITA; such exotic isolates may be more aggressive than indigenous isolates and accidental escape into the countryside could be devastating. Such considerations have doubtless

inhibited studies using a range of geographically well separated isolates.

The best way to compare the biological properties of virus isolates is to test them under the same conditions (Walkey, 1985). Therefore, a series of experiments were conducted under glasshouse conditions to compare the reaction of five rice genotypes (two susceptible and three resistant to RYMV-N) to five RYMV isolates obtained from different geographical areas of Africa.

The three general reaction experiments were followed by an experiment to examine the effect of the age of rice plants at inoculation on symptom type and development in susceptible (ITA 212) and resistant (Lac 23) rice genotypes as there is often a direct relationship between plant age at inoculation and susceptibility and symptom severity. For example, Bakker (1974) reported that the reaction of three susceptible rice varieties to RYMV-K was markedly influenced by host age at inoculation. Some susceptible varieties have been found to be killed when inoculated early (3-4 leaf stage) with RYMV (Bakker, 1974; Fauquet and Thouvenel, 1977). In a screenhouse experiment conducted in Nigeria, inoculation of four resistant and 10 susceptible cultivars with RYMV-N at 27, 32, 37 and 47 days after sowing showed that two tolerant varieties (CT 19 and ITA 235) had reduced tolerance when infected early, whilst the other two tolerant genotypes were similarly affected, as were the susceptible cultivars. All cultivars developed maximum leaf symptoms between 22 and 46 days after inoculation (Kumwenda, 1988).

3.2 MATERIALS AND METHODS

Spatial constraints precluded testing all five isolates in each of the three experiments made. Thus three isolates were used in experiments 1 and 2 with RYMV-N common to both. In the third experiment, all isolates were tested.

3.2.1 Plant materials

Seeds were sown in vermiculite on 28th March, 1987 (experiment 1); 5th June, 1987 (experiment 2) and on 22nd September, 1987 for experiment 3. Seedlings were transplanted into plastic pots 7-10 days after sowing with two plants per pot. Glasshouse temperature was computer regulated and set to produce a mean temperature of 25°C for each 24h period. Temperatures ranged from 20-33°C with a daily mean of 25-26°C (experiment 1), 22-35°C with a daily mean of 25-26°C (experiment 2) and 20-33°C with a daily mean of 23-26°C (experiment 3). The heated glasshouse compartment used was isolated from the outside by other compartments which acted as insulators, such that the temperature never fell below 20°C at night, even in the middle of winter.

When necessary, supplementary illumination from high pressure sodium lamps was used to increase light intensity and duration in the winter months. Daylength was 12 hours during times of year (end of September to end of March) when natural daylength was 12 hours or less. Plants for experiment 1 were grown in 10 cm diameter plastic pots. Susceptible cultivars were harvested 30 days after inoculation while resistant cultivars were transferred to 16 cm plastic pots and harvested 60 days after inoculation. Plants for experiments 2 and 3 were raised

in 16 cm diameter plastic pots throughout the experiments. Plants were again harvested 30 days after inoculation (susceptible cultivars) or 60 days (resistant cultivars). Ten cm diameter pots were placed on plastic saucers 11 cm diameter and 2.5 cm deep and 16 cm diameter pots on saucers 14 cm diameter and 3.5 cm deep. Other cultural operations and nutrient application was as described in the general materials and methods (section 2).

3.2.2 Virus isolates

Virus isolates used for each experiment were:-

Experiment 1 : RYMV-N, -1C and -SL

Experiment 2 : RYMV-N, -K and-Nr

Experiment 3 : RYMV-N, -1C, -SL, -K and -Nr

3.2.3 Inoculation

Inoculum was prepared from frozen leaves of infected cv. ITA 212 (3 weeks after inoculation) as described in the general materials and methods. The tissue:buffer ratio was 1:10 (w/v). Plants were inoculated at the 4-5 leaf stage, leaves 2 and 3 were inoculated. Dates of inoculation were 21 April, 1987 (experiment 1), 30 June, 1987 (experiment 2) and 6 October, 1987 for experiment 3. Leaves were numbered acropetally (basal leaf = leaf 1). Healthy control plants were sham-inoculated with sap from healthy rice leaves (cv. ITA 212).

3.2.4 Leaf length and plant height

To determine the effect of the RYMV isolates on leaf growth and plant height, the length of all unfolded leaves, i.e. leaves more than 1 cm long, and plant height were measured commencing immediately after inoculation. Measurements were taken every 5 days and continued for 30 and 60 days after inoculation for susceptible and resistant cultivers, respectively.

3.2.5 Shoot and root fresh and dry weights

By 30 days after inoculation, susceptible cultivars were severely affected and most of the leaves had at least begun to turn necrotic. The plants were therefore harvested at this date. Shoots were removed at soil level. The roots were washed carefully with running tap water to remove the soil and compost then blotted free of excess water. After determination of shoot and root fresh weight, samples were oven dried at 70°C for 3-4 days in a forced draught oven before taking dry weights. Shoot and root fresh and dry weights were taken as above for resistant cultivars 60 days after inoculation.

3.2.6 Symptom severity and disease incidence

Each inoculated plant was inspected daily for the presence or absence of RYMV symptoms. After the initial appearance of symptoms, plants were inspected and symptoms recorded on alternate days. Symptom severity and disease incidence was recorded and calculated as described in the general materials and methods.

3.2.7 Experimental design and analysis

A randomized complete block design with 20 treatments (5 cultivars x 3 isolates + 5 healthy controls) was used for experiments 1 and 2. There were 30 treatments (5 cultivars x 5 isolates + 5 healthy controls) in experiment 3, again arranged as a randomized complete block design. There were 10 plants (5 replicate pots) per treatment giving 200 plants (experiments 1 and 2) or 300 plants (experiment 3). Treatments were arranged as five blocks, each containing one pot from each treatment randomized within the design immediately after virus inoculation. Data were analysed using a Genstat programme on the University of London Amdahl computer. Each parameter recorded was subjected to analysis of variance. In the analysis of variance, when the variance ratio was significant, the least significant difference (L.S.D.) was calculated, i.e. L.S.D. = S.E.D. x t, where S.E.D. is the standard error of the difference and t is the t statistic of the stated level of probability. L.S.D. was most frequently used to compare treatment means.

3.2.8 ELISA indexing

Sixty days after inoculation, a test sample was collected from every plant of the resistant cultivars in each experiment. The sample consisted of five, 3 mm diameter, disks taken by means of a cork borer from the youngest unfolded leaf in such a way as to be representative of the entire lamina. Samples were kept in small, labelled, polyethylene bags and stored at -20°C.

3.2.9 Reaction of rice cultivars to infection with RYMV at various stages

Two rice cultivars, Lac 23 representative of resistant cultivars and ITA 212 representative of susceptible cultivars, were sown on 28 August, 1987 in vermiculite. Seedlings at the 1-2 leaf stage (7-10 days after sowing) were transplanted into 16 cm diameter plastic pots. Four seedlings were transplanted into each pot. After feeding, the plants were kept under glasshouse conditions (temperature range 22°C to 31°C, mean daily range 24-26°C) and supplementary lighting was provided when natural daylength was 12 hours or less.

Inoculum was prepared by grinding 1 g of fresh RYMV-N infected rice leaves (cv. ITA 212) as before. Inoculations with infective sap were made to susceptible and resistant plants at different ages. The first inoculation was made on 11 September, 1987 when most seedlings had reached the 3-4 leaf stage; leaf 1 and leaf 2 were inoculated. Two batches of plants were sham-inoculated to serve as controls. Subsequent inoculations were made on 18 September (4-5 leaf stage, leaves 2 and 3 inoculated), 25 September (5-6 leaf stage, leaves 3 and 4 inoculated) and finally 30 September (6-7 leaf stage, leaves 4 and 5 inoculated).

Symptom severity and disease incidence records and plant height measurements were recorded starting on the day of inoculation until 30 (ITA 212) or 40 (Lac 23) days after inoculation. Symptom severity and disease incidence were recorded and calculated as before (section 2). At the end of the experiment, the youngest leaf of each resistant plant was collected and kept at -20°C for ELISA indexing.

3.3 RESULTS

3.3.1 Reaction of susceptible rice cultivars

3.3.1.1 Symptom production

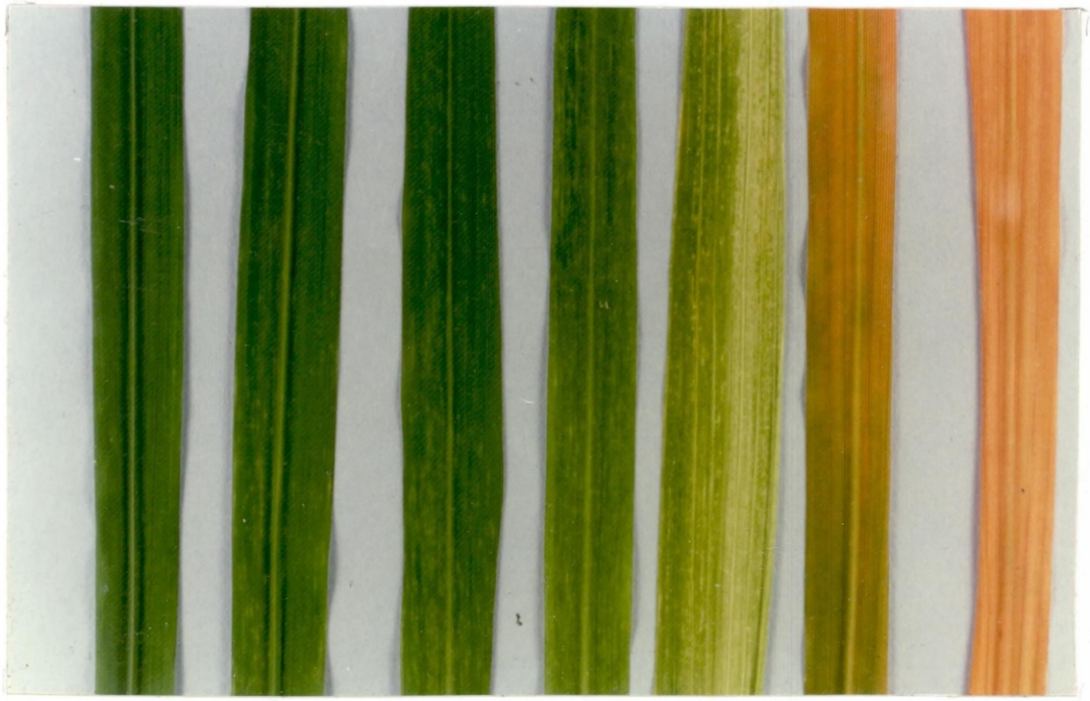
All five RYMV isolates induced similar symptoms on the two susceptible rice cultivars (ITA 212 and BG 90-2) tested. Symptoms were never observed on the inoculated leaves (leaf 2 and leaf 3) of each cultivar inoculated with any of the five isolates tested. Moreover, symptoms were not induced in leaves 4 or 5 which were fully formed at the time of inoculation; symptoms were usually first seen in non-inoculated leaves 6 or 7. Rice seedlings inoculated with RYMV at the 4-5 leaf stage showed initial symptoms 8-13 days after inoculation. Initial symptoms consisted of small (1-3 mm long) chlorotic streaks at the base of the youngest uninoculated leaf. Usually symptoms progressed beyond the chlorotic streak stage but occasionally, in the first leaf to show symptoms, chlorotic streaks did not develop further. As newly-emerging leaves were produced, chlorotic streaks progressively increased in number and size such that they eventually covered the entire leaf lamina. About 10-20 days after the appearance of initial symptoms, leaves tended to turn yellow then orange and eventually they became necrotic (Fig. 3.1A). Orange colouration and necrosis was first seen at the leaf tip and the symptoms then extended basipetally to affect the entire leaf blade. Leaves formed later were completely streaked from emergence and many were spirally twisted (Fig. 3.1B). In severe cases, infected plants did not produce new leaves and were killed. Occasionally symptoms appeared initially on the tillers and only later on the main shoot. Symptom type and progression on tillers were similar to those described for main shoots.

Fig. 3.1 Reaction of susceptible rice cultivar ITA 212 to RYMV-N

A. 1. Initial symptoms, chlorotic streaks (10-20% of lamina area)
2. Chlorotic streaks (21-40% of leaf area)
3. Chlorotic streaks (41-60% of leaf area)
4. Chlorotic streaks (61-80% of leaf area)
5. More general chlorosis
6. Pigmentation starting from the tip of the leaf (top of photograph) and progressing basipetally
7. Uniform orange pigmentation immediately followed by necrosis

B. Twisted young leaves of plant of ITA 212 25 days after inoculation with RYMV-N

A



B



3.3.1.2 Symptom severity

Using the disease severity values detailed in the general materials and methods (section 2), disease severity curves are shown in Figure 3.2 for the three experiments made. Mean symptom severity values for all isolates increased rapidly in both susceptible cultivars and by 30 days after inoculation, when recording stopped, many plants of cv. ITA 212 were dying. Symptom progression was slower in cv. BG 90-2 than cv. ITA 212 in all three experiments. In experiment 1 and to a lesser extent in experiment 3, isolate SL induced the lowest values when cv. ITA 212 plants were inoculated with this isolate.

The values in Table 3.1 are for plants 30 days after inoculation when all plants of cvs. ITA 212 and BG 90-2 had developed symptoms. Symptom severity was consistently less in cv. BG 90-2 than ITA 212 for all RYMV isolates in each experiment; mean values for all isolates combined were significantly ($\underset{\sim}{P}$ = 0.05) less in BG 90-2 than ITA 212 in experiments 2 and 3. In the two experiments which included RYMV-SL, this isolate induced the lowest symptom score irrespective of the host cultivar inoculated (Table 3.1). The remaining isolates did not differ significantly in the severity of symptoms they induced within an experiment. However, isolate Nr gave the highest severity score in both experiments in which it was included.

Fig. 3.2 **Symptom severity progress curves for susceptible rice cultivars 0-30 days after inoculation with RYMV**

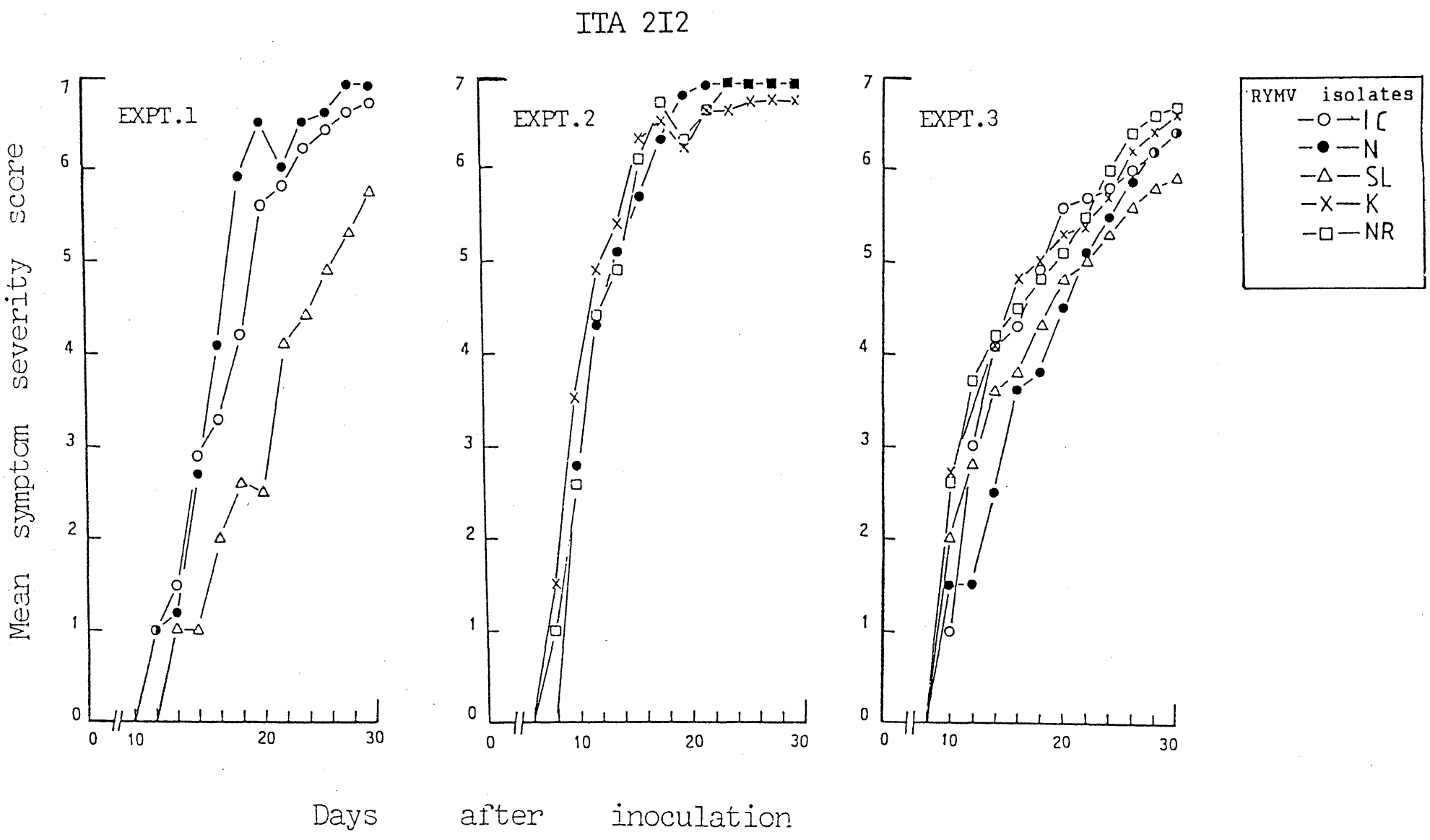


Fig. 3.2 contd.

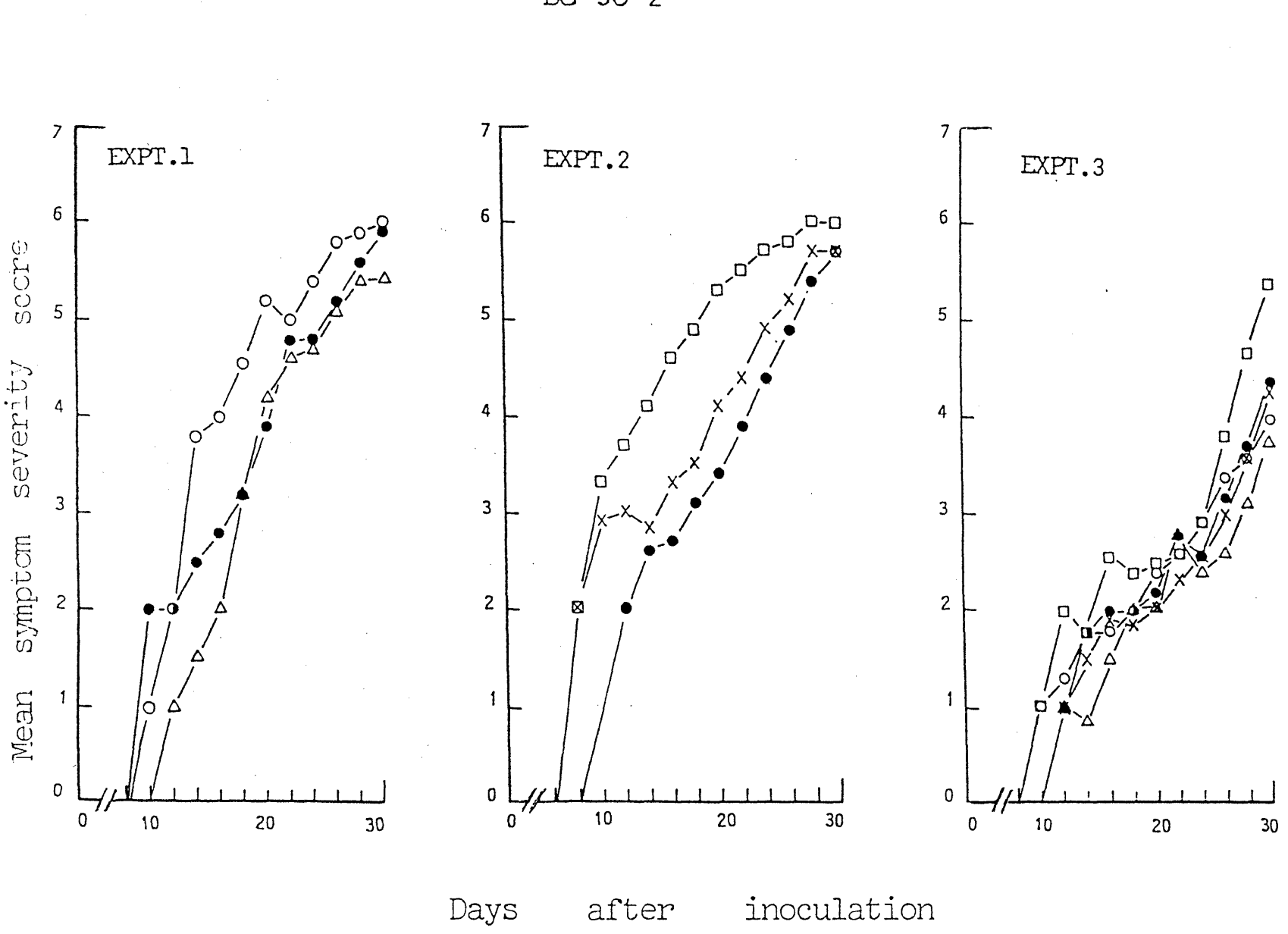

BG 90-2
EXPT.1
EXPT.2
EXPT.3
Mean symptom severity score
Days after inoculation
0
1
2
3
4
5
6
7
0
10
20
30

Table 3.1 Mean symptom severity scores for susceptible rice cvs. ITA 212 and BG 90-2, 30 days after inoculation with isolates of RYMV

Rice cultivar	RYMV isolate	Sympton severity score[a]		
		Expt. 1	Expt. 2	Expt. 3
ITA 212	IC	6.7	-	6.2
	N	6.9	6.9	6.3
	SL	5.7	-	5.8
	Nr	-	6.9	6.8
	K	-	6.7	6.6
All isolates (mean)		6.4	6.8	6.3
BG 90-2	IC	6.0	-	4.8
	N	5.9	5.6	4.9
	SL	5.4	-	4.6
	Nr	-	6.0	5.6
	K	-	5.8	4.7
All isolates (mean)		5.8	5.8	4.9
LSD ($P = 0.05$)[b]		1.1	0.5	0.8
($P = 0.01$)		1.6	0.7	1.1

[a] Symptom severity scale ranged from 0 (no symptoms) to 7.0 (leaves necrotic)

[b] For comparison of individual isolate values within each experiment

3.3.1.3 Disease incidence

Disease incidence increased rapidly and all plants developed symptoms 12-20 days after inoculation (Table 3.2). The incidence of RYMV symptoms in ITA 212 plants increased very rapidly and reached 100% within 4-8 days after the first appearance of symptoms. In experiment 1, the incidence of RYMV-SL on cv. ITA 212 increased more slowly than other isolates tested (Table 3.2), whereas in experiment 3, where all 5 isolates were tested, RYMV-SL incidence was similar to the other isolates.

The incidence of RYMV symptoms in cv. BG 90-2 increased more slowly than RYMV infection of cv. ITA 212 (Table 3.2). All inoculated cv. BG 90-2 plants displayed symptoms within 16-20 days of inoculation. Plants of cv. BG 90-2 inoculated with RYMV-Nr produced symptoms very rapidly and all plants showed symptoms within 2-8 days of initial symptom appearance (Table 3.2).

3.3.1.4 Incubation period

The mean incubation period for cv. ITA 212 infected with RYMV isolates was variable, ranging from 9.4-15.6 days in the three experiments (Table 3.3). Within experiments, the mean incubation period for RYMV-SL was 4.4 days longer than isolates N and IC although, in experiment 3, isolate SL showed a similar incubation period to the other four isolates (Table 3.3). The mean incubation period in cv. BG 90-2 was slightly longer than in cv. ITA 212, ranging from 11.0-15.8 days; this was seen for all isolates in all experiments. The longest incubation periods were encountered in experiment 3. BG 90-2 plants inoculated with RYMV-Nr showed the shortest mean incubation period and plants inoculated with isolate SL the longest (Table 3.3).

Table 3.2 Proportion (%) of rice plants (cvs. ITA 212 and BG 90-2) showing symptoms 0-30 days after inoculation with isolates of RYMV.

Rice cultivar	RYMV isolate	Days after inoculation 6	8	10	12	14	16	18	20
ITA 212	Expt. 1								
	IC	0	0	40[a]	100				
	N	0	0	40	100				
	SL	0	0	10	20	40	60	90	100
	Expt. 2								
	N	0	0	40	70	100			
	Nr	0	30	60	70	100			
	K	0	40	90	100				
	Expt. 3								
	IC	0	0	50	80	100			
	N	0	0	30	70	100			
	SL	0	0	40	70	100			
	Nr	0	0	60	80	100			
	K	0	0	70	100				
BG 90-2	Expt. 1								
	IC	0	0	30	70	90	100		
	N	0	0	10	50	90	100		
	SL	0	0	0	10	30	60	80	100
	Expt. 2								
	N	0	0	0	40	60	90	100	
	Nr	0	0	50	100				
	K	0	20	30	60	90	100		
	Expt. 3								
	IC	0	0	30	40	60	90	90	100
	N	0	0	10	20	50	80	90	100
	SL	0	0	10	40	40	50	70	100
	Nr	0	0	20	30	60	100		
	K	0	0	20	30	50	60	90	100

[a] Incidence calculated from 10 plants per treatment

Table 3.3 Incubation periods for RYMV isolates tested on susceptible rice cultivars ITA 212 and BG 90-2.

Rice cultivar	RYMV isolate	Incubation period (days)					
		Expt. 1		Expt. 2		Expt. 3	
		Mean	Range[a]	Mean	Range	Mean	Range
ITA 212	IC	11.2	10-12	-	-	11.4	10-14
	N	11.2	10-12	11.8	10-14	12.0	10-14
	SL	15.6	10-20	-	-	11.8	10-14
	Nr	-[b]	-	10.8	8-14	11.2	10-14
	K	-	-	9.4	8-12	10.6	10-12
BG 90-2	IC	12.2	10-16	-	-	13.8	10-20
	N	13.0	10-16	14.2	12-20	15.0	10-20
	SL	14.4	12-20	-	-	15.8	10-20
	Nr	-	-	11.0	10-12	13.8	10-16
	K	-	-	12.0	8-16	15.0	10-20

[a] Time interval between inoculation and initial symptom appearance and between inoculation and final disease incidence

[b] Isolates not included

3.3.1.5 Plant height

Changes in plant height with time after inoculation showed similar trends in all three experiments. Results of experiment 3, in which all five isolates were used, are shown in Figure 3.3 (results for experiments 1 and 2 not shown). Irrespective of the virus isolate, extension growth ceased after 15 days in cv. ITA 212 and 20 days in cv. BG 90-2. In both rice cultivars growth cessation preceded maximum symptom severity and infected plants were significantly shorter than mock-inoculated controls after 10 (cv. ITA 212) or 15 (cv. BG 90-2) days irrespective of isolate.

Plant heights 30 days after inoculation (Table 3.4) were very similar for sham-inoculated rice cv. ITA 212 and BG 90-2 in each of the three experiments. Decreases in height caused by RYMV infection relative to controls ranged from 11.3-26.3% (Table 3.4) and were of a similar magnitude in the three experiments. Infection always significantly decreased plant height. Significant differences were not usually obtained between RYMV isolates within a given cultivar although in experiment 3, isolate Nr stunted plants significantly more than isolates K and SL in cv. BG 90-2.

3.3.1.6 Leaf assessment

Leaf symptom severity: Further information on leaf symptom progression was obtained by recording disease severity at each leaf position on alternate days up to 30 days after inoculation. Because disease severity progress curves for individual leaves showed similar trends in all three experiments, typical curves from experiment 3 are presented in Figure 3.4. Symptoms were not produced in inoculated leaves 2 and 3 or in non-inoculated leaves 4 and 5. Leaves 1 to 4

Fig. 3.3 Plant height of susceptible rice cultivars 0-30 days after inoculation with isolates of RYMV. Bars indicate ($\underline{P}$ = 0.05)

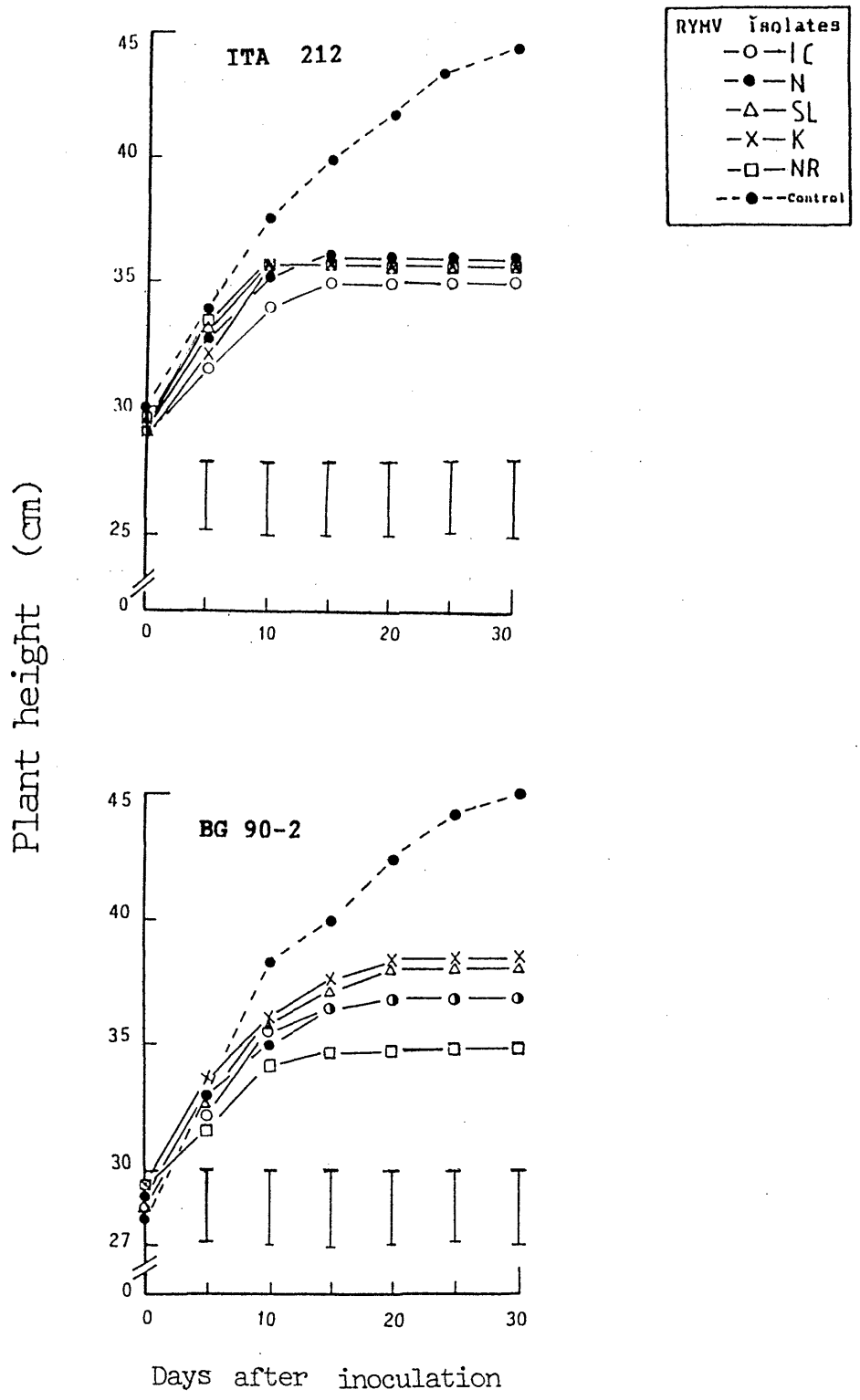


Table 3.4 Plant height of rice (cvs. ITA 212 and BG 90) 30 days after inoculation with isolates of RYMV or sham inoculation.

Rice cultivar	RYMV isolate	Plant height (cm)		
		Expt. 1	Expt. 2	Expt. 3
ITA 212	K	33.4 (17.9)[b]	-	34.0 (23.6)
	N	33.0 (18.9)	38.1 (25.1)	35.8 (19.6)
	SL	32.8 (17.0)	-	35.7 (19.8)
	Nr	-[a]	37.5 (26.3)	35.3 (20.7)
	K	-	37.6 (26.1)	35.6 (20.0)
	Control	40.7	50.9	44.5
BG 90	K	32.6 (19.6)	-	36.8 (16.7)
	N	32.4 (20.8)	43.2 (17.7)	36.6 (17.2)
	SL	33.6 (17.6)	-	38.0 (14.0)
	Nr	-	42.1 (17.8)	34.8 (21.3)
	K	-	43.4 (17.3)	38.3 (11.3)
	Control	40.8	52.5	45.2
L.S.D. (P = 0.05)		1.4	3.4	2.6
(P = 0.01)		1.9	4.4	3.5

[a] Virus isolate not included

[b] Values in parenthesis show height reduction caused by infection as % decrease of inoculated compared with sham inoculated control plants.

had ceased extension growth at the time of inoculation whereas leaf 5 continued growing up to 10 days after inoculation (Fig. 3.5). In cv. ITA 212, symptoms first appeared in leaf 6. Symptoms steadily intensified up to 26 days after inoculation when mean severity values for all isolates tended to plateau up to 30 days after inoculation (Fig. 3.4, leaf 6). In leaf 7, symptom severity values of 1.0 to 4.0 were evident at leaf emergence and these scores progressively increased to values of about 6.0 after 22-26 days thereafter remaining almost constant to 30 days after inoculation, irrespective of virus isolate (Fig. 3.4, leaf 7). The last leaf to emerge, leaf 8, showed severe symptoms (*c.* 5.0) immediately on emergence at about 22 days after inoculation and disease severity scores for all isolates rapidly increased to values above 6.0 (Fig. 3.4, leaf 8). The trends outlined for cv. ITA 212 were also evident in cv. BG 90-2. However, in this cultivar, although initial symptoms increased in severity with leaf position for leaves 6, 7 and 8, mean symptom scores subsequently increased more slowly than in ITA 212 and did not show the levelling off for disease scores in leaves 6 and 7 after 26 days characteristic of cv. ITA 212. There were no obvious consistent differences between the five RYMV isolates. As stated earlier, trends were similar in experiments 1 and 2, the only differences being that symptoms were first produced in leaf 7 rather than leaf 6 as in experiment 3 (Table 3.5). Tabulated values for symptom severity scores at the end of each of the experiments (30 days) reinforced the overall general similarity in disease scores for the various isolates and showed that the combined mean disease score for all isolates in each experiment tended to increase from the first leaf to show symptoms (leaf 7 in experiments 1 and 2; leaf 6 in experiment 3) to the next leaf to emerge and to the final leaf which

Table 3.5 Mean symptom severity scores for specified leaf positions of susceptible rice cultivars 30 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Symptom Severity Score[a]											
		Experiment 1 Leaf position				Experiment 2 Leaf position				Experiment 3 Leaf position			
		6	7	8	9	6	7	8	9	5	6	7	8
ITA 212	IC	0	7.0[c]	6.4	6.7	-	-	-	-	0	6.2	6.3	6.6
	N	0	7.0	6.8	6.9	0	6.9	7.0		0	6.0	6.4	6.8
	SL	0	5.0	6.0	6.0	-	-	-	-	0	4.9	6.0	6.1
	Nr	-[b]	-	-	-	0	6.8	7.0	6.8	0	6.8	6.7	6.9
	K	-	-	-	-	0	7.0	7.0	6.0	0	6.8	6.3	6.6
All isolates (mean)		0.0	6.3	6.4	6.5	0.0	6.9	7.0	6.4	0.0	6.1	6.3	6.6
BG 90-2	IC	0	5.6	6.1	6.2	-	-	-	-	0	4.0	5.3	5.1
	N	0	5.6	6.2	5.8	0	0	5.3	6.0	0	4.4	5.1	5.2
	SL	0	0	5.2	5.5	-	-	-	-	0	3.8	5.0	4.6
	Nr	-	-	-	-	0	4.8	6.5	6.6	0	5.4	5.6	5.7
	K	-	-	-	-	0	4.5	6.3	6.6	0	4.3	4.8	5.0
All isolates (mean)		0.0	3.8	5.8	5.8	0.0	3.1	5.2	6.4	0	4.4	4.8	5.0
LSD ($P = 0.05$)			0.8	1.4	0.9		0.9	0.5	0.5	0	0.9	0.9	0.6
LSD ($\overline{P} = 0.01$)			1.1	1.8	1.2		1.2	0.7	0.7		1.1	1.1	0.7

[a] Mean symptom severity score ranged from 0 (symptomless) to 7.0 (leaf necrosis
[b] Isolates not included
[c] Mean of 10 infected plants per treatment

Fig. 3.4 Mean symptom severity scores for leaves 6, 7 and 8 of susceptible rice cultivars 0-30 days after inoculation with RYMV isolates (experiment 3).

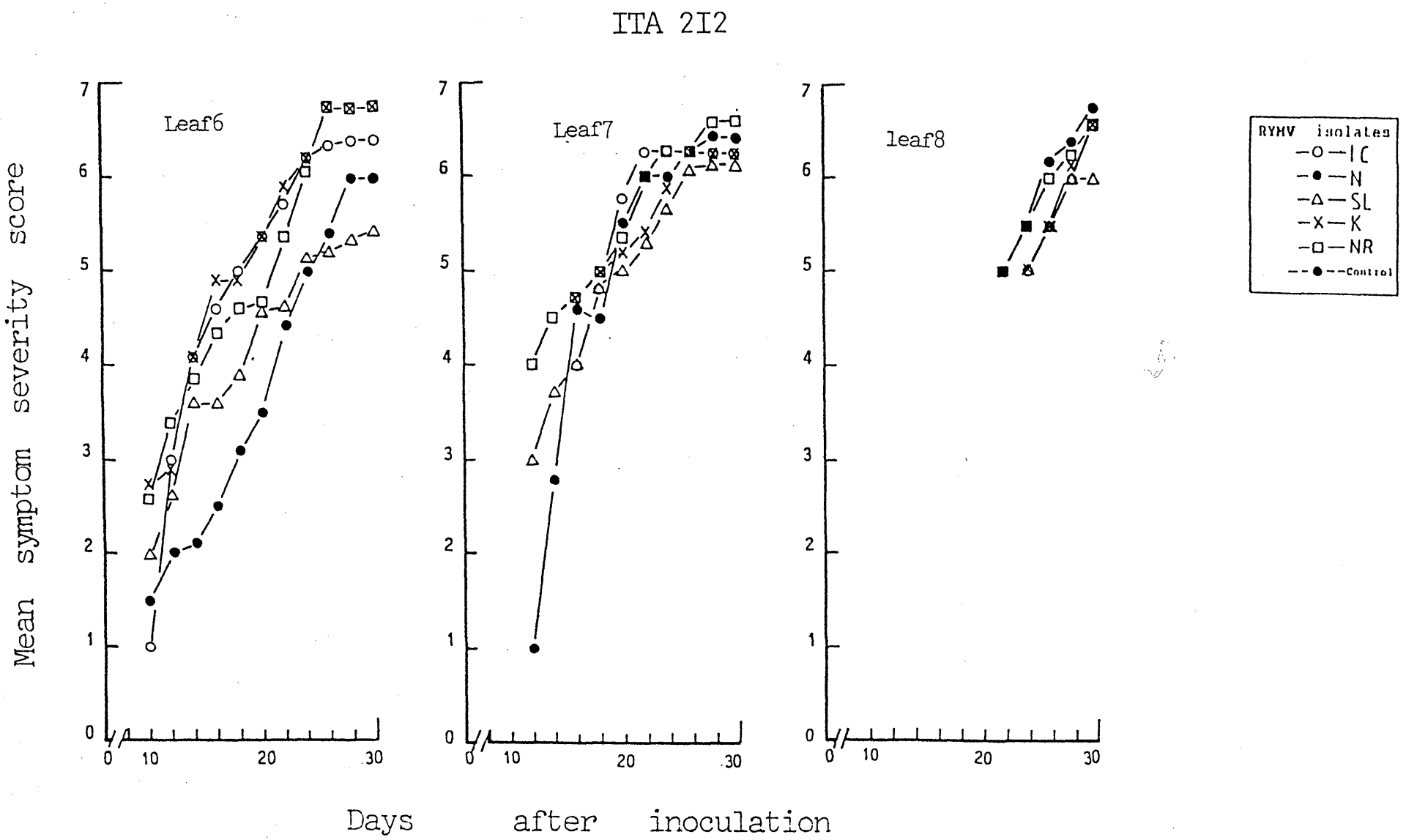
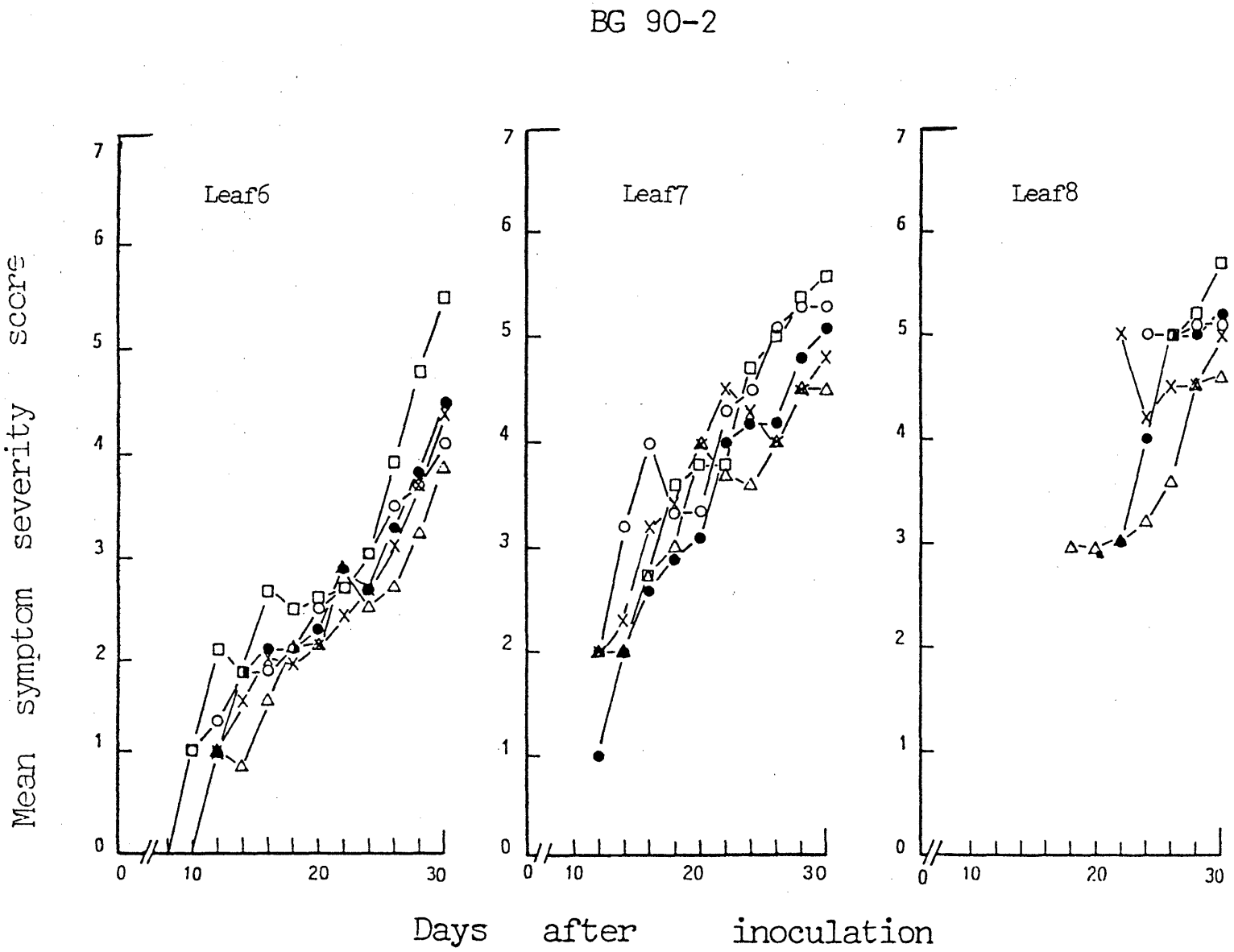


Fig. 3.4 contd.


BG 90-2
Leaf6
Leaf7
Leaf8
Mean symptom severity score
Days after inoculation
0
1
2
3
4
5
6
7
0
10
20
30

emerged during the experiment (Table 3.5). In addition, severity values for all isolates tended to be greater on cv. ITA 212 than cv. BG 90-2; RYMV-SL generally induced slightly less severe symptoms than other isolates in both cultivars and there was some indication that RYMV-Nr induced slightly more severe symptoms than other isolates on cv. BG 90-2, a feature not apparent on cv. ITA 212 (Table 3.5). Overall, differences in disease scores between RYMV isolates were small.

Leaf length: The general effect of RYMV infection on leaf extension growth were similar in the three experiments so, as for plant height (section 3.3.1.5) and leaf symptom severity (section 3.3.1.6), time-course growth curves are presented for experiment 3 only (Fig. 3.5) but data from all three experiments are presented in Table 3.6 for leaf lengths 30 days after inoculation.

Leaf 5, in which disease symptoms were not produced (Table 3.5), ceased extension growth 10-15 days after inoculation in both ITA 212 and BG 90-2. Despite the absence of foliar symptoms, the growth of this non-inoculated leaf was slightly reduced to a similar extent by all five RYMV isolates (Figs. 3.5, leaf 5) whereas leaf 4 was unaffected (Table 3.6). Leaf 6 emerged 5 days after inoculation and increased in length until 20 days after inoculation (Fig. 3.5, leaf 6). Growth was reduced by all RYMV isolates but to a greater extent (21.4 to 23.8 % reduction) than leaf 5 (11.5 to 16.1 % reduction) in cv. ITA 212 and in cv BG 90-2 (22.6 to 25.6 % reduction for leaf 5; 11.4 to 17.1 % reduction for leaf 6). The next leaf, leaf 7, emerged 15 days after inoculation and ceased growing after 20 days (Fig. 3.5, leaf 7); this leaf showed conspicuous symptoms at

Fig. 3.5 Leaf lengths for leaves 5, 6, 7 and 8 of susceptible cultivars 0-30 days after inoculation with RYMV isolates (experiment 3).

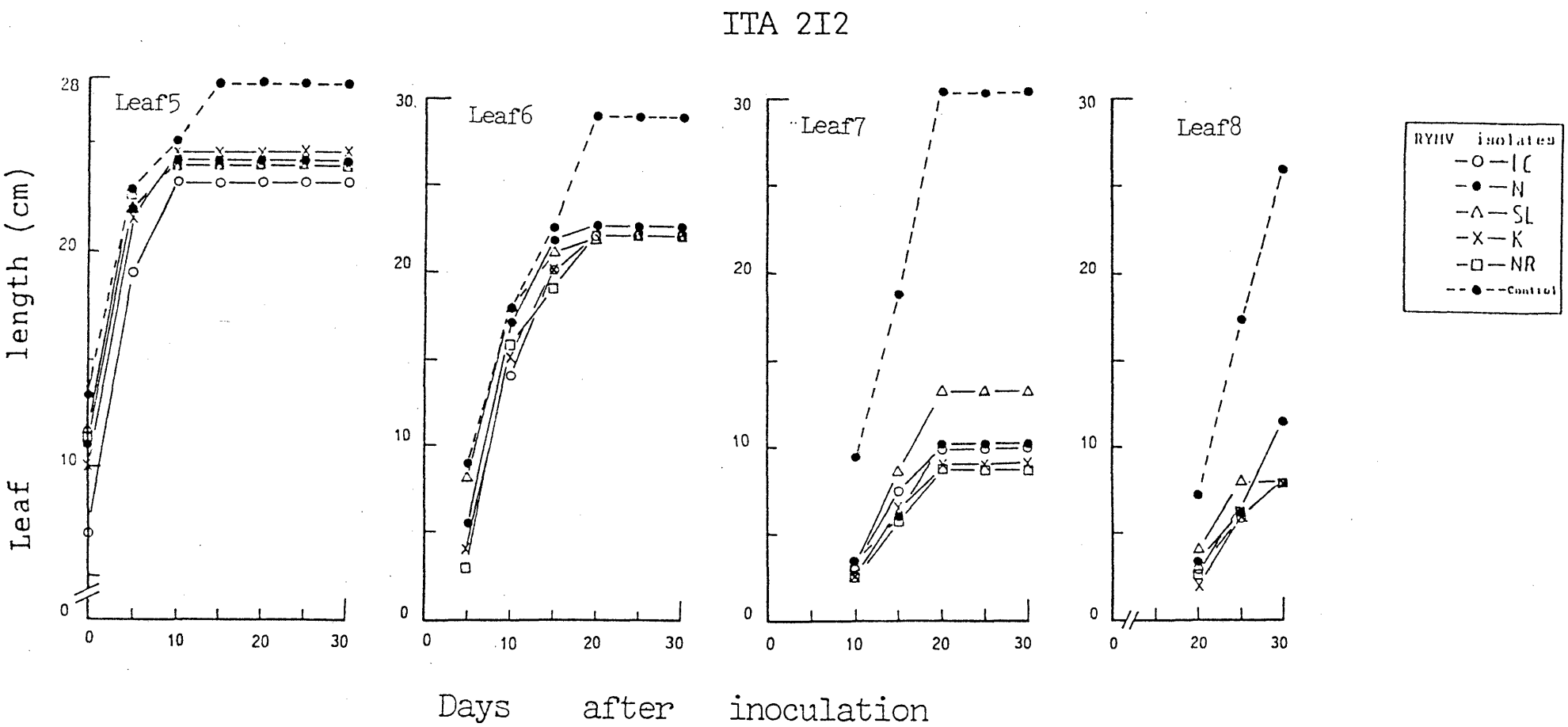


Fig. 3.5 contd.

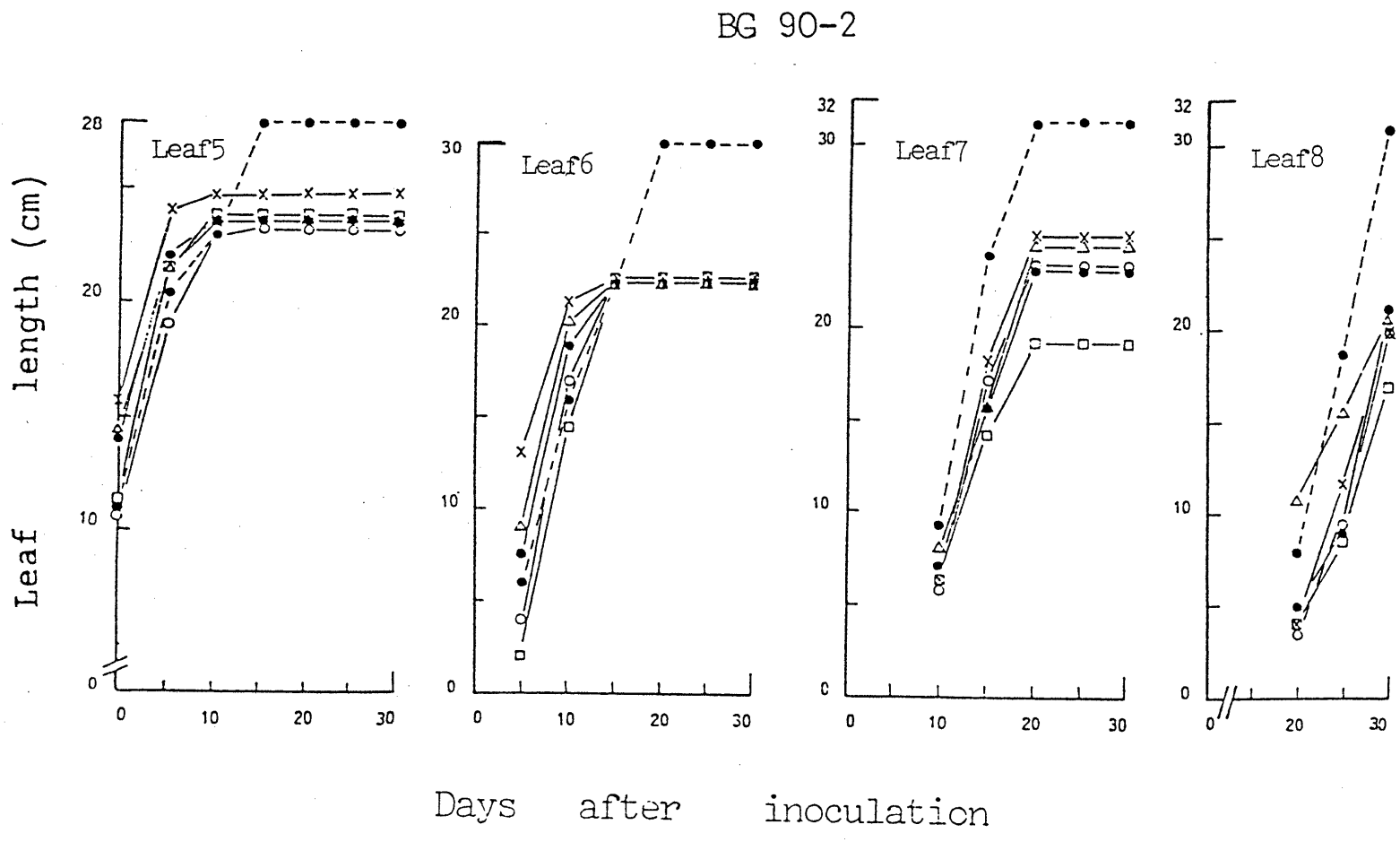

BG 90-2
Leaf5
Leaf6
Leaf7
Leaf8
Leaf length (cm)
Days after inoculation

Table 3.6 Mean leaf length for specified leaf positions of susceptible rice cultivars 30 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Leaf length (cm)											
		Experiment 1 Leaf position				Experiment 2 Leaf position				Experiment 3 Leaf position			
		6	7	8	9	6	7	8	9	5	6	7	8
ITA 212	IC	22.4[a]	18.9	7.5	4.2	-	-	-	-	23.4	22.7	10.4	7.9
	N	22.4	18.2	2.4	0.8	22.0	26.1	13.9	0	24.3	22.1	10.3	7.8
	SL	22.9	21.0	5.5	5.3	-	-	-	-	24.0	22.7	13.4	8.0
	Nr	-[b]	-	-	-	22.4	26.3	17.2	3.3	24.3	22.8	8.8	7.9
	K	-	-	-	-	22.0	24.9	15.3	3.1	24.7	22.3	8.9	8.0
	Control	23.4	24.8	27.4	21.3	23.4	28.7	33.2	32.1	27.9	29.0	30.5	26.0
BG 90-2	IC	22.2	21.1	14.5	10.8	-	-	-	-	23.2	22.3	23.5	20.0
	N	22.0	21.3	16.9	10.9	22.1	27.1	30.8	24.6	23.7	22.7	23.1	22.3
	SL	21.8	22.0	20.5	13.8	-	-	-	-	23.6	23.2	24.6	22.0
	Nr	-	-	-	-	22.0	22.2	29.7	18.9	23.6	22.6	19.2	17.0
	K	-	-	-	-	22.1	27.9	30.5	22.2	24.8	22.6	25.1	20.0
	Control	23.2	24.2	27.3	24.1	22.1	29.0	33.7	33.0	28.0	30.0	31.3	31.0
LSD ($\underline{P}$ = 0.05)		1.4	1.6	2.4	2.9	1.2	3.0	6.3	5.8	1.7	2.2	3.9	2.8
LSD ($\underline{P}$ = 0.01)		1.9	2.2	3.0	3.9	1.7	4.7	8.3	7.7	2.3	3.0	53	3.7

[a] Mean of 10 plants
[b] RYMV isolates not included

emergence (Fig. 3.4, leaf 7). In cv. ITA 212, all RYMV isolates caused a marked decrease in length ranging from a 56.1 % reduction (RYMV-SL) to 71.1 % reduction (RYMV-Nr) (Table 3.6), considerably greater than the growth decreases in leaf 6.

In contrast, in cv. BG 90-2, all RYMV isolates decreased growth, but to a lesser extent - about the same as for leaf 6. The final leaf to emerge, leaf 8, appeared 15 to 20 days after inoculation (Fig. 3.5, leaf 8). This leaf had not ceased extension growth by the end of the experiment. All RYMV isolates reduced its growth markedly in cv. ITA 212 but slightly less so in cv. BG 90-2 (Fig. 3.5, leaf 8; Table 3.6).

Table 3.6 shows that all virus isolates tested had a progressively greater inhibitory effect on leaf growth of cv. ITA 212 with each new leaf that emerged and produced symptoms in the three experiments. This trend was much less pronounced in cv. BG 90-2. No considerable differences were apparent between the five RYMV isolates although RYMV-SL tended to have a slightly less marked effect on leaf growth than the other isolates tested, particularly in cv. ITA 212.

3.3.1.7 Fresh and dry weights of shoots and roots

Infection by all RYMV isolates tested in both susceptible rice cultivars in each of the three experiments caused a marked reduction in fresh and dry weight of both shoots and roots sampled 30 days after inoculation (Tables 3.7, 3.8). The overall percentage reduction in shoot fresh weight for cv. ITA 212 (all isolates combined) for the three experiments was 69.5 % (range 68.3-70.7%) and for root fresh weight 75.8% (range 72.4-79.7).

Table 3.7 Fresh weight of shoots and roots of susceptible rice cultivars 30 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Fresh weight (g)					
		Experiment 1		Experiment 2		Experiment 3	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
ITA 212	IC	1.24[a]	0.65	-	-	0.73	0.47
	N	0.85	0.63	1.19	0.71	0.76	0.43
	SL	1.33	0.91	-	-	0.86	0.59
	Nr	-	-	1.37	0.70	0.74	0.46
	K	-	-	1.45	0.63	0.81	0.56
All isolates (mean)		1.14(69.5)[b]	0.73(75.3)	1.34(70.7)	0.68(72.4)	0.78(68.3)	0.50(79.7)
Control		3.74	2.95	4.58	2.48	2.46	2.43
BG 90-2	IC	1.50	1.28	-	-	0.91	0.76
	N	1.44	1.15	2.38	1.78	0.83	0.71
	SL	1.78	2.15	-	-	0.89	0.88
	Nr	-	-	2.07	1.1	0.74	0.68
	K	-	-	2.30	1.83	0.87	0.83
All isolates (mean)		1.57(53.0)	1.53(38.8)	2.25(50.3)	1.57(42.5)	0.85(54.3)	0.77(42.9)
Control		3.34	2.5	4.53	2.73	2.36	2.87
LSD ($\underline{P}$ = 0.05)		0.24	0.37	0.47	0.65	0.14	0.24
LSD ($\underline{P}$ = 0.01)		0.33	0.5	0.63	0.88	0.18	0.32

[a] Mean of 10 plants per treatment
[b] Values in parentheses are percent decreases caused by virus infection

Table 3.8 Dry weights of shoots and roots of susceptible rice cultivars 30 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Dry weight (g)					
		Experiment 1		Experiment 2		Experiment 3	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
ITA 212	IC	0.42[a]	0.44	-	-	0.29	0.18
	N	0.34	0.43	0.48	0.27	0.31	0.17
	SL	0.49	0.59	-	-	0.32	0.22
	Nr	-[b]	-	0.52	0.25	0.30	0.16
	K	-	-	0.55	0.25	0.30	0.19
All isolates (mean)		0.42(59.6)[c]	0.49(60.8)	0.52(69.9)	0.26(68.3)	0.30(68.4)	0.18(68.4)
Control		1.10	1.25	1.73	0.82	0.95	0.57
BG 90-2	IC	0.58	0.63	-	-	0.34	0.22
	N	0.58	0.61	0.81	0.49	0.31	0.20
	SL	0.71	0.80	-	-	0.33	0.26
	Nr	-	-	0.76	0.32	0.28	0.20
	K	-	-	0.83	0.45	0.37	0.25
All isolates (mean)		0.62(48.3)	0.68(48.1)	0.80(52.7)	0.43(50.0)	0.33(64.5)	0.23(61.7)
Control		1.2	1.31	1.69	0.86	0.93	0.60
LSD ($\underline{P}$ = 0.05)		0.1	0.15	0.14	0.11	0.05	0.56
LSD ($\underline{P}$ = 0.01)		0.14	0.20	0.19	0.14	0.07	0.73

[a] Mean of 10 plants per isolate
[b] RYMV isolates not included
[c] Values in parentheses are percent decreases caused by RMYV infection

Values for cv. BG 90-2 were for shoots 52.5 % reduction (range 50.3-54.3) and for roots 41.8 % reduction (range 38.8-43.6). Thus, RYMV infection of cv. ITA 212 had a slightly greater effect on root than shoot fresh weight, whereas in cv. BG 90-2 shoots were slightly more affected than roots. Furthermore, virus-induced fresh weight decreases were generally greater for cv. ITA 212 than for cv. BG 90-2 (Table 3.7).

Virus-induced decreases in dry weight were generally greater in cv.ITA 212 than in cv. BG 90-2 as was the case for fresh weight, but in contrast to the trend in fresh weight changes, dry weight decreases were essentially similar for shoots and roots. Analysis of the reduction caused by individual virus isolates in each experiment for each cultivar did not reveal any consistent significant differences between the isolates (Table 3.8).

3.3.2 Reaction of resistant cultivars

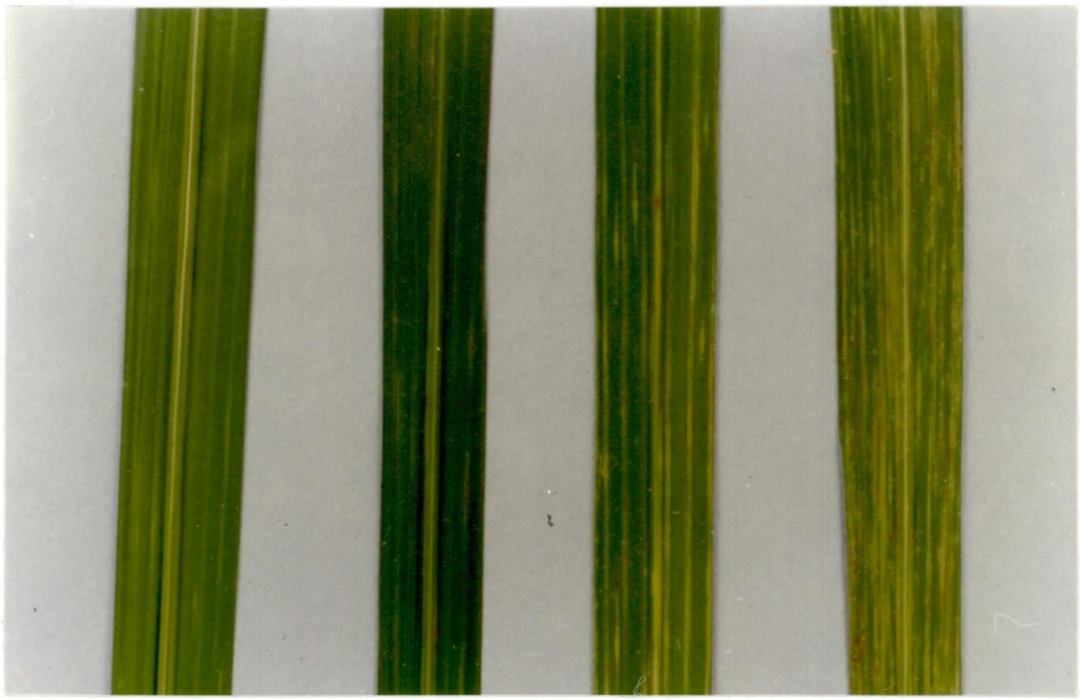
3.3.2.1 Symptom production

All five RYMV isolates induced similar symptoms in the three resistant cultivars tested. Initial symptoms were somewhat similar to those produced on susceptible cultivars consisting of scattered longitudinal chlorotic streaks (5-10 mm long) between the parallel veins of newly emerging non-inoculated leaves. Streaking on the first leaf to show symptoms progressed slowly as their number per unit leaf area increased. Later-formed leaves showed mottle and chlorotic streaks over most of the lamina (Fig. 3.6) and in the latest leaves to emerge mottling became fairly pronounced in

Fig. 3.6 Reaction of resistant cultivar ITA 235 to sap inoculation with RYMV-N.

Left to right: chlorotic streaks in leaves from ITA 235 plants.

Note the increasing length and number of chlorotic streaks with time after inoculation



resistant cultivars. In contrast to susceptible cultivars, conspicuous chlorosis, development of orange pigmentation and necrosis never occurred in the resistant cultivars tested. Thus, the maximum possible disease rating using the 0-7 scale (section 2) for an individual leaf was 5.0, in contrast to the maximum value of 7.0 for leaves of susceptible cultivars. Seedlings of resistant cultivars inoculated with the RYMV isolates at the 4-5 leaf stage showed initial symptoms 15-50 days after inoculation and the timing of initial symptom appearance varied considerably from experiment to experiment. Like susceptible cultivars, symptoms were never produced in inoculated leaves or in non-inoculated leaves fully expanded at inoculation.

3.3.2.2 Symptom severity

Symptom progression curves for the three experiments (Fig. 3.7) show that mean symptom severity values progressively increased about 10-30 days after inoculation but thereafter showed considerable fluctuation up to 60 days. Fluctuations were due firstly to the variable number of plants which produced symptoms and therefore contributed to the mean score for, unlike the situation with susceptible cultivars, not all inoculated resistant plants became systemically infected and produced symptoms (see later sections). In addition, where few plants produced symptoms early, the subsequent appearance of further plants with long incubation periods and an initially low disease severity rating tended to reduce the mean symptom severity score. Such fluctuations tended to obscure all but obvious differences between cultivars and virus isolates. However, irrespective of virus isolate, symptom progression tended to be more rapid and to reach higher values in cv. ITA 235 than in cvs. Lac 23

Fig. 3.7 Symptom severity progress curves for resistant cultivars 0-60 days after inoculation with RYMV isolates (results from 3 experiments).

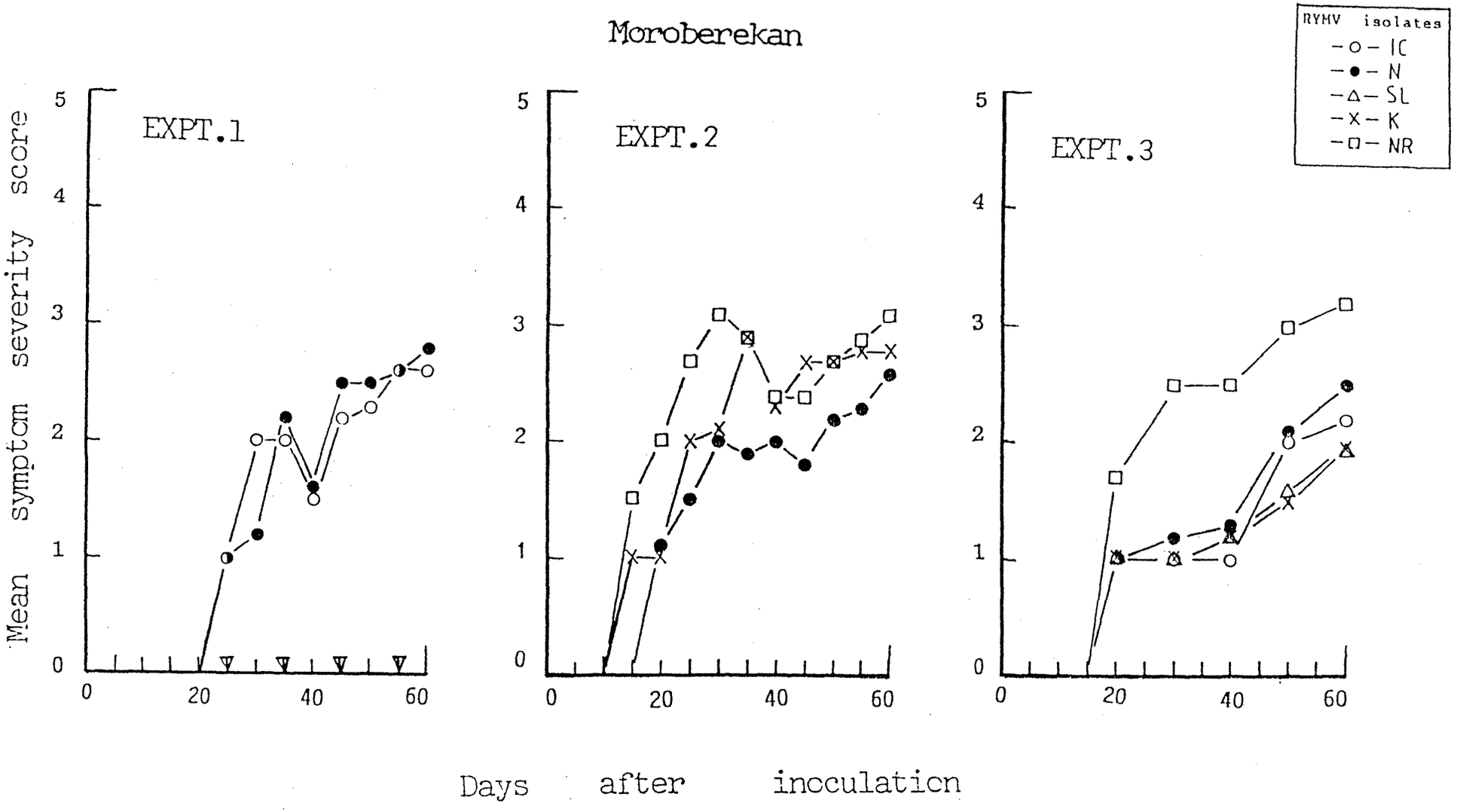


Fig. 3.7 contd.

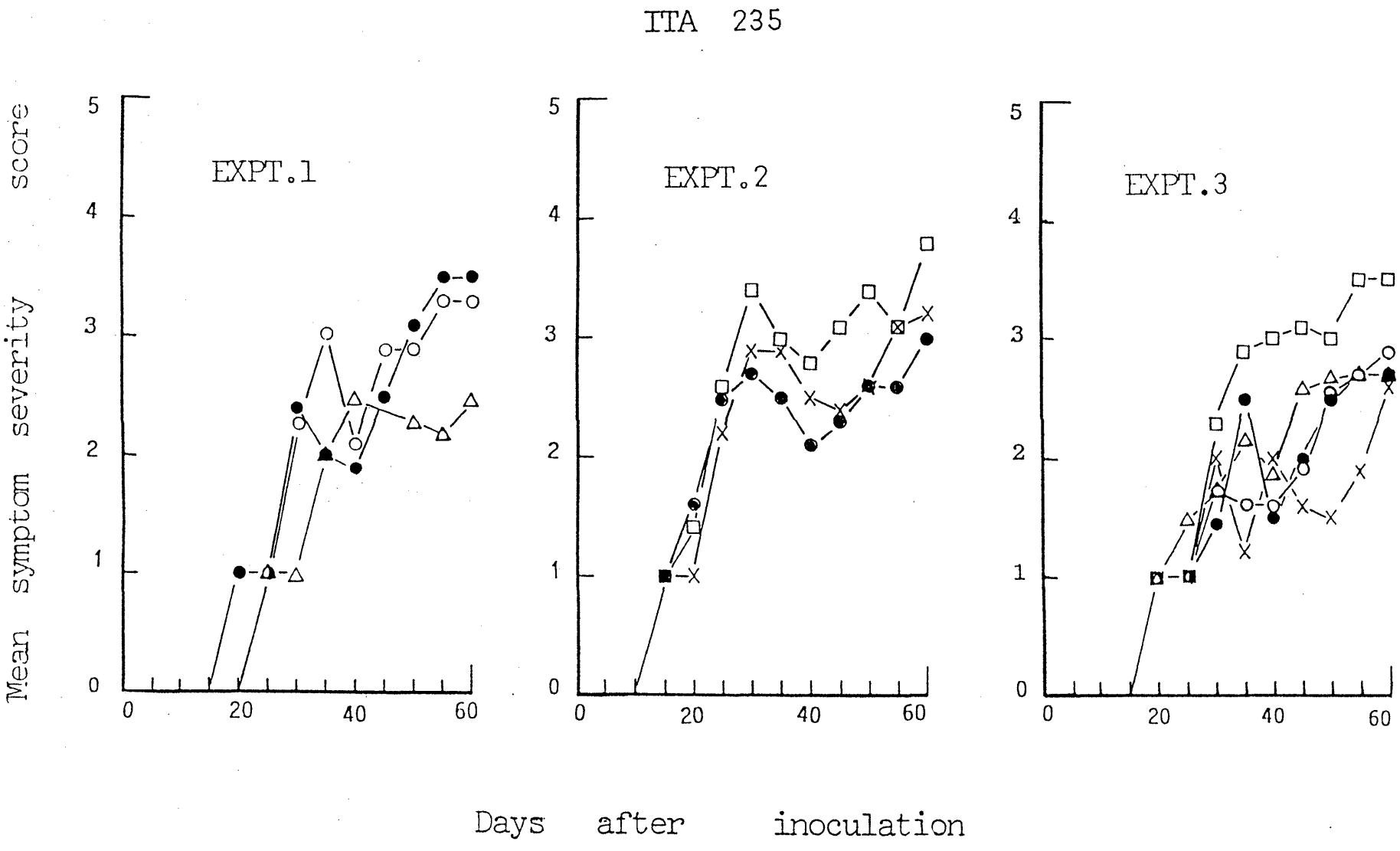
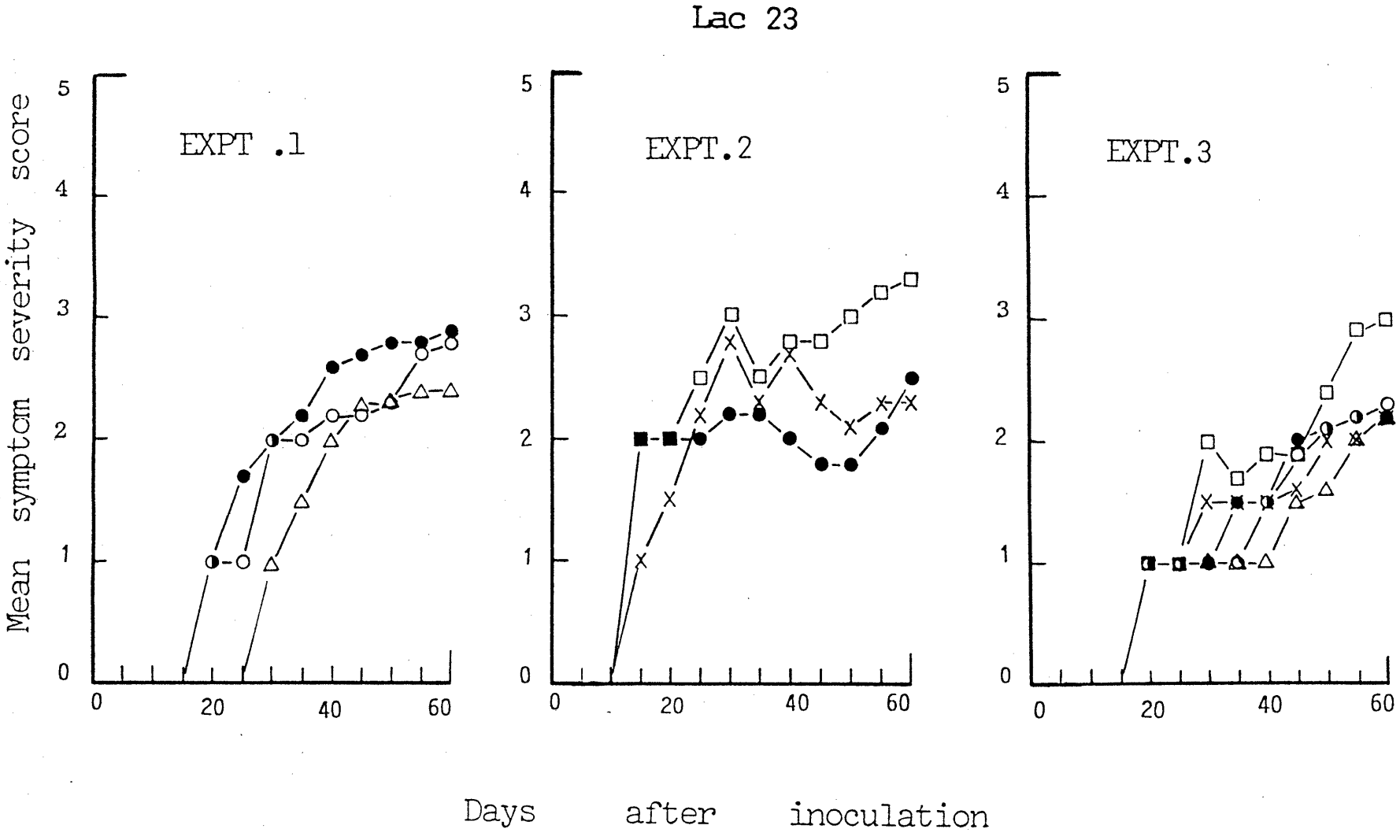

ITA 235
EXPT.1
EXPT.2
EXPT.3
Mean symptom severity score
Days after inoculation
0
1
2
3
4
5
0
20
40
60

Fig. 3. 7 contd.


Lac 23
EXPT .1
EXPT.2
EXPT.3
Mean symptom severity score
0
1
2
3
4
5
0
20
40
60
Days after inoculation

and Moroberekan (Fig. 3.7). RYMV-Nr tended to induce the highest disease rating in all three cultivars in the experiments in which it was included (Fig. 3.7, experiments 2 and 3). RYMV-SL failed to infect any of the 10 Moroberekan plants inoculated in experiment 1.

The symptom severity scores 60 days after inoculation with RYMV isolates shown in Table 3.9 relate only to the variable number (Table 3.10) of plants which produced symptoms; symptomless plants were excluded from the calculations. The mean symptom severity values for resistant cultivars did not exceed 3.8 (Table 3.9) in marked contrast to the values obtained 30 days after inoculation of susceptible cultivars where all values exceeded 3.8 (Table 3.1). The mean scores for the three cultivars tested, irrespective of virus isolate, showed that cv. ITA 235 developed the severest symptoms in all experiments and there was little difference between cvs. Lac 23 and Moroberekan. The low mean symptom severity value for Moroberekan in experiment 1 was due to unsuccessful infection of all plants inoculated with RYMV-SL. RYMV-Nr consistently induced the most severe symptoms in the two experiments in which it was included (Table 3.9).

3.3.2.3 Disease incidence

Of the 330 plants inoculated in the three experiments, 190 developed symptoms within the 60 days before harvest, representing 57.6% of the plants inoculated (Table 3.10); 109 plants produced symptoms within 30 days and 160 plants within 40 days. Thus, most plants that were going to produce symptoms did so within 40 days after inoculation. However, a few plants (30) produced initial symptoms (usually in leaves 6 and 7) only after 40-60 days. Thus in

Table 3.9 Mean symptom severity scores for resistant cultivars Moroberekan, Lac 23 and ITA 235, 60 days after inoculation with isolates of RYMV.

Rice cultivar	RYMV isolate	Symptom severity score[a]		
		Expt. 1	Expt. 2	Expt. 3
Moroberekan	IC	2.6	-	2.7
	N	2.7	2.6	2.5
	SL	0	-	2.3
	Nr	-	3.1	3.2
	K	-	2.7	2.7
	All isolates (mean)	1.8	2.8	2.6
Lac 23	IC	2.8	-	2.3
	N	2.9	2.5	2.2
	SL	2.4	-	2.2
	Nr	-	3.3	3.0
	K	-	2.3	2.2
	All isolates (mean)	2.7	2.7	2.4
ITA 235	IC	3.3	-	2.9
	N	3.5	3.0	2.7
	SL	2.5	-	2.7
	Nr	-	3.8	3.5
	K	-	3.2	2.6
	All isolates (mean)	3.1	3.3	2.9

[a]Symptom severity scale ranged from 0 (no symptoms) to 7.0 (leaves necrotic)

Table 3.10 Proportion (%) of rice plants (cvs. Moroberekan, Lac 23 and ITA 235) showing symptoms 0-60 days after inoculation

Rice cultivar	RYMV isolate	Days after inoculation 15	20	25	30	35	40	45	50	55	60
Moroberekan	Expt. 1										
	IC	0	0	10[a]	10	10	30	30	30	30	30
	N	0	0	10	40	70	90	90	90	90	90
	SL	0	0	0	0	0	0	0	0	0	0
	Expt. 2										
	N	0	30	60	60	60	70	80	80	80	80
	Nr	30	60	70	70	70	100	100	100	100	100
	K	10	50	80	80	90	90	90	90	90	90
	Expt. 3										
	IC	0	10	10	10	10	10	10	10	10	10
	N	0	10	20	30	30	30	30	60	60	60
	SL	0	0	10	20	20	20	20	20	20	20
	Nr	0	40	40	40	40	50	80	80	80	80
	K	0	10	10	10	10	20	20	40	40	40
Lac 23	Expt. 1										
	IC	0	10	10	10	10	20	30	40	40	40
	N	0	10	30	30	60	70	70	70	70	70
	SL	0	0	0	10	10	10	10	10	10	10
	Expt. 2										
	N	0	30	30	30	50	60	60	60	60	60
	Nr	30	70	80	80	100	100	100	100	100	100
	K	10	20	30	40	50	50	60	60	60	60
	Expt. 3										
	IC	0	10	10	10	10	10	30	30	30	30
	N	0	10	20	20	20	30	60	60	60	60
	SL	0	0	10	10	10	20	30	30	30	30
	Nr	0	20	20	30	40	50	70	80	80	80
	K	0	10	10	10	10	10	30	30	30	30
ITA 235	Expt. 1										
	IC	0	0	20	30	30	60	60	60	60	60
	N	0	10	50	50	70	90	90	90	90	90
	SL	0	0	10	10	10	10	10	20	20	20

Table 3.10 contd.

Rice cultivar	RYMV isolate	Days after inoculation 15	20	25	30	35	40	45	50	55	60
	Expt. 2										
	N	10	50	60	60	70	80	80	80	80	80
	Nr	10	70	80	80	80	90	90	90	90	90
	K	10	30	50	60	70	80	80	80	80	80
	Expt. 3										
	IC	0	10	20	20	40	50	50	60	60	60
	N	0	10	20	20	20	40	60	60	60	60
	SL	0	10	20	50	50	50	50	50	50	50
	Nr	0	10	30	50	50	60	80	80	80	80
	K	0	10	10	10	10	40	60	60	60	60

[a] Incidence calculated from 10 plants per treatment

all three experiments and irrespective of virus isolate or resistant host cultivar, symptom incidence usually failed to reach 100% at 60 days after inoculation. The only exception to this was in experiment 2 where RYMV-Nr inoculation caused symptom development in all inoculated plants of cvs. Moroberekan and Lac 23 (Table 3.10). In the two experiments in which it was included, disease incidence caused by RYMV-Nr increased more rapidly and to higher values than any of the other isolates tested.

Examination of disease incidence data for 60 days after inoculation and for the three experiments combined (Table 3.11) shows that about half of the inoculated plants of cvs. Moroberekan and Lac 23 eventually produced symptoms whereas about two thirds of cv. ITA 235 eventually showed symptoms. Most plants (88.3%) inoculated with RYMV-Nr developed symptoms, whereas very few (21.6%) produced symptoms following inoculation with RYMV-SL. The other three isolates tested gave intermediate values (Table 3.11).

Virus incidence was also tested using ELISA (RYMV-N globulin and conjugate) on extracts of the youngest leaves of plants 60 days after inoculation to check particularly for symptomless infection in those plants still not showing symptoms after 60 days. The results (Table 3.12) showed that symptomless plants always indexed ELISA negative whereas all plants with symptoms indexed ELISA positive in the three experiments.

3.3.2.4 Incubation period

The slow and irregular development of symptoms detailed in the previous section was, in part, a reflection of variable and often

Table 3.11. Number and proportion of resistant rice plants with symptoms 60 days after inoculation with isolates of RYMV. (Combined results from experiments 1, 2 and 3)

A. Effect of rice cultivar

	Rice cultivar		
	ITA 235	Moroberekan	Lac 23
No. of plants with symptoms	73	60	57
No. inoculated	110	110	110
Proportion of plants with symptoms (%)	66.4	54.5	51.8

B. Effect of RYMV isolate

	RYMV isolate				
	IC	N	SL	Nr	K
No. of plants with symptoms	23	66	13	53	36
No. inoculated	60	90	60	60	60
Proportion of plants with symptoms (%)	38.3	73.3	21.6	88.3	60.0

Table 3.12. RYMV incidence (%) in resistant rice cultivars 60 days after inoculation based on symptoms and ELISA tests.

Rice cultivars	RYMV isolates	Disease incidence					
		Expt. 1		Expt. 2		Expt. 3	
		ELISA[a]	Symptoms	ELISA	Symptoms	ELISA	Symptoms
Morober-eken	IC	30	30	-	-	10	10
	N	90	90	80	80	60	60
	SL	0	0	-	-	20	20
	Nr	-	-	100	100	80	80
	K	-	-	90	90	40	40
Lac 23	IC	40	40	-	-	30	30
	N	70	70	60	60	60	60
	SL	10	10	-	-	30	30
	Nr	-	-	100	100	80	80
	K	-	-	60	60	30	30
ITA 235	IC	60	60	-	-	60	60
	N	90	90	80	80	60	80
	SL	20	20	-	-	50	50
	Nr	-	-	90	90	80	80
	K	-	-	80	80	60	60

[a] Using youngest leaf of each plant diluted 1:100 (w/v) and RYMV-N antiserum

lengthy incubation periods within and between experiments. Table 3.13 shows that the mean incubation period in the three resistant cultivars, irrespective of virus isolate, varied from experiment to experiment ranging from 22.0-38.8 days (Table 3.13). The minimum incubation period within an isolate/cultivar combination (time from inoculation to initial symptoms) ranged from 15-25 days and the maximum from 30-50 days (Table 3.13). Neither virus isolate nor host cultivar had a clear consistent effect on the length of the incubation period.

3.3.2.5 Plant height

Due, probably, to differing environmental conditions, plants grew progressively less vigorously in experiments 1, 2 and 3, a factor reflected in, for example, decreasing height and fresh and dry weights for inoculated and control plants. However, irrespective of the experiment, control plants of cv. Lac 23 were slightly taller than those of cv. Moroberekan which, in turn, were consistently slightly taller than cv. ITA 235.

Contrasting with infected susceptible cultivars, RYMV-infected resistant cultivars showed continued height increase throughout the 60-day period of measurement in all three experiments: growth curves from experiment 3 are shown in Figure 3.8. Initial slight reductions in plant height caused by infection relative to sham-inoculated controls occurred about 30-40 days after inoculation (Fig. 3.8).

Reductions in height caused by RYMV 60 days after inoculation were usually slight, ranging from a small increase relative to control of 0.5% (RYMK-K, ITA 235, expt. 3) to a decrease of 15.4% (RYMV-Nr, Lac 23, expt. 3) (Table 3.14). Infection with RYMV-Nr consistently

Table 3.13. Incubation periods for RYMV isolates on resistant rice cultivars

Rice cultivar	RYMV isolate	Incubation period (days)					
		Expt. 1		Expt. 2		Expt. 3	
		Mean	Range[a]	Mean	Range	Mean	Range
Morober-ekan	IC	35.0	25-40	-	-	20.0	20
	N	33.0	25-40	26.3	20-45	37.5	20-50
	SL	0	0	-	-	27.5	25-30
	Nr	-[b]	-	25.0	15-40	28.1	20-45
	K	-	-	22.8	15-35	40.0	20-50
Lac 23	IC	38.8	20-50	-	-	36.7	20-45
	N	31.4	20-40	27.9	20-40	36.7	25-45
	SL	30.0	30	-	-	36.7	20-45
	Nr	-	-	22.0	15-35	35.6	20-50
	K	-	-	28.3	15-45	36.7	20-45
ITA 235	IC	33.3	25-40	-	-	34.2	20-40
	N	30.0	20-40	24.4	15-40	35.8	20-45
	SL	37.5	25-50	-	-	27.0	20-30
	Nr	-	-	22.2	15-40	32.5	20-45
	K	-	-	26.3	15-40	38.3	20-45

[a] Time interval between inoculation and initial symptom appearance and inoculation and maximum disease incidence

[b] Isolates not included

Fig. 3.8 Plant height of resistant cultivars 0-60 days after inoculation with isolates of RYMV
Bars indicate ($\underline{P}$ = 0.05)

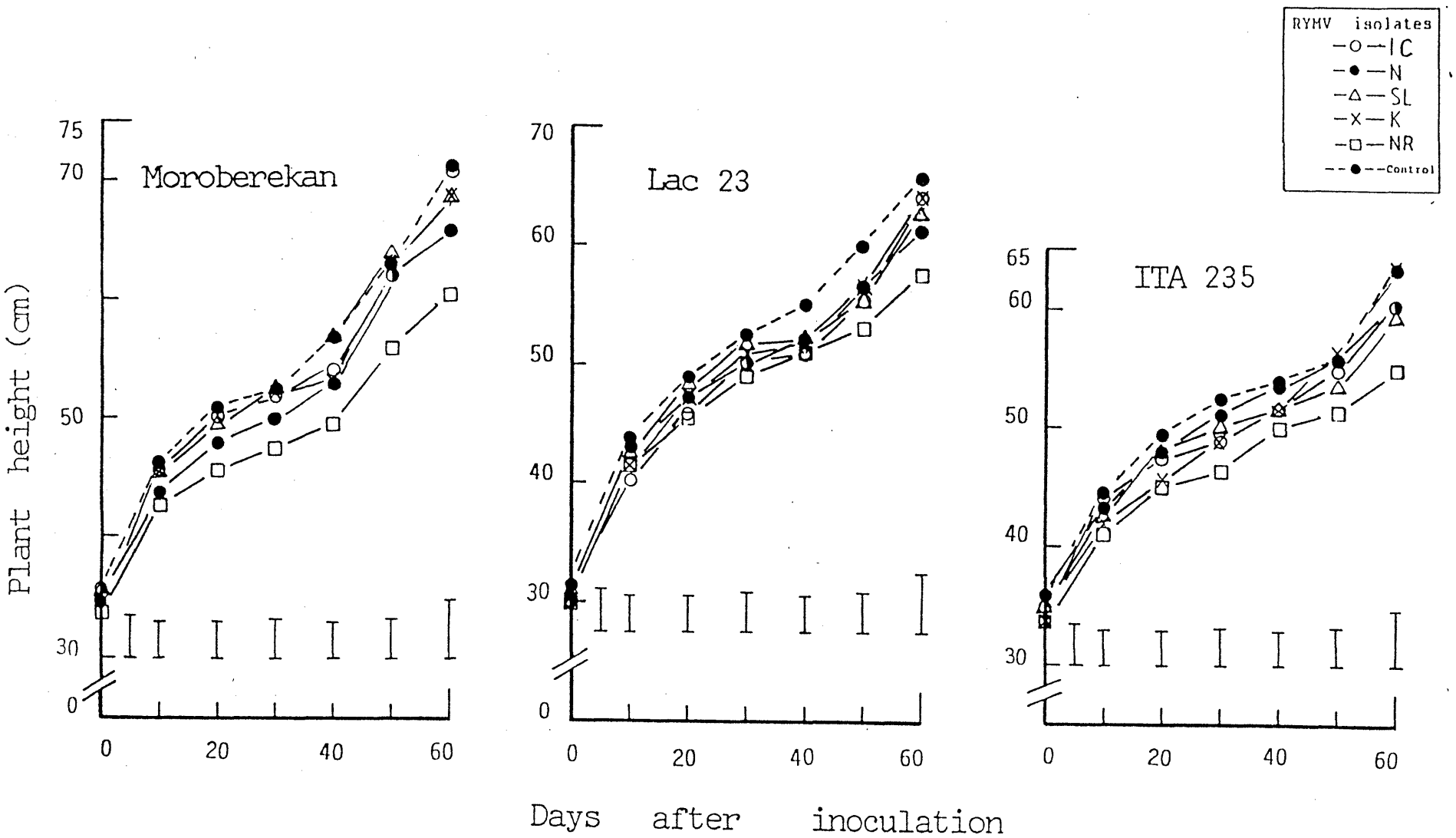


Table 3.14 Plant height of three resistant rice cultivars 60 days after inoculation with isolates of RYMV.

Rice cultivar	RYMV isolate	Plant height (cm)		
		Expt. 1	Expt. 2	Expt. 3
Moroberekan	IC	91.9 (2.2)	-	63.9 (2.6)
	N	83.0 (11.7)	76.8 (4.9)	61.2 (6.7)
	SL	93.0 (1.1)	-	63.2 (3.7)
	Nr	-	72.6 (10.1)	57.4 (12.5)
	K	-	74.9 (7.3)	63.8 (2.7)
	Control	94.0	80.8	65.6
Lac 23	K	100.8 (2.7)	-	70.8 (0.8)
	N	89.5 (13.6)	88.2 (7.6)	66.0 (7.6)
	SL	110.2 (3.3)	-	69.0 (3.4)
	Nr	-	82.3 (13.8)	60.5 (15.4)
	K	-	88.7 (7.1)	68.5 (4.1)
	Control	103.6	95.5	71.4
ITA 235	K	87.5 (1.2)[b]	-	60.4 (4.6)
	N	78.8 (11.1)	71.5 (8.6)	60.4 (4.6)
	SL	85.9 (3.1)	-	59.6 (5.8)
	Nr	-[a]	68.5 (12.4)	55.0 (13.1)
	K	-	77.6 (0.7)	63.6 (-0.5)
	Control	88.6	78.2	63.3
L.S.D.	(P = 0.05)	7.8	5.1	4.9
	(P = 0.01)	10.7	6.9	6.4

[a] Values in parentheses show height reduction caused by infection as % decrease of inoculated compared with sham inoculated controls

[b] Virus isolates not included

significantly reduced height in all three cultivars tested and to a similar extent (10.4-15.4% decrease). RYMV-N usually significantly decreased height (4.6-13.6% decrease) but to a lesser extent than RYMV-Nr. Isolates K, IC and SL had little effect on plant height (Table 3.14).

Results assembled in Table 3.14 relate to all plants of a given cultivar inoculated with a given RYMV isolate. These results probably underestimate the effect of RYMV because, as was shown in section 3.3.2.3 (Tables 3.10 and 3.11), a variable number of plants were successfully systemically infected and plants symptomless after 60 days did not contain detectable amounts of RYMV when young leaves were tested by ELISA using RYMV-N antiserum (Table 3.12) and were therefore possibly healthy. Mean heights calculated for plants with and without symptoms are presented in Table 3.14a which is included for completeness. The results generally show that in all three experiments, the height of plants showing symptoms was markedly less than the height of symptomless plants. On the other hand, heights of symptomless plants were slightly less or more than sham-inoculated plants. However, some data are affected by the number of replicates. For example, in experiment 1 Lac 23 and ITA 235 plants showing symptoms were more stunted when inoculated with RYMV-SL than plants inoculated with isolates IC and N which is contrary to Table 3.14 where RYMV-N was more severe than RYMV-SL. The reason was that the number of infected plants inoculated with SL isolate was very low (1 or 2 plants) which clearly affected mean plant height (Table 3.14a). For the same reason in experiments 2 and 3, heights of symptomless plants were less than heights of Moroberekan and Lac 23 plants showing symptoms when they were inoculated with isolates K and SL respectively.

Table 3.14a Mean plant height of three resistant rice cultivars 60 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Plant height (cm)								
		Experiment 1			Experiment 2			Experiment 3		
		All plants	Symptoms	Symptomless	All plants	Symptoms	Symptomless	All plants	Symptoms	Symptomless
Moroberekan	IC	91.9	78.7(3)[a]	95.3(7)				63.9	50.0(1)	65.4(9)
	N	83.0	75.5(2)	93.5(8)	76.8	76.2(8)	79.0(2)	61.2	58.2(6)	65.7(4)
	SL	93.0	-	93.0(10)				63.2	59.5(2)	64.2(8)
	Nr				72.6	72.6(10)	-	57.4	54.6(2)	66.0(2)
	K				74.9	75.0(9)	74.0(1)	63.8	58.2(4)	67.5(6)
	Control	94.0			80.8			56.6		
Lac 23	IC	100.8	93.4(4)	101.7(6)				70.8	67.7(3)	72.7(7)
	N	89.5	82.7(7)	96.3(3)	88.2	85.2(6)	92.5(4)	66.0	63.8(6)	69.5(4)
	SL	100.2	76.0(1)	100.0(9)				69.0	69.5(2)	68.6(8)
	Nr				82.3	82.3(10)	-	60.5	58.5(8)	68.5(2)
	K				88.7	86.2(6)	95.5(4)	68.5	63.3(3)	70.7(7)
	Control	103.6			95.5			71.4		
ITA 235	IC	87.5	82.5(6)	92.5(4)				60.4	58.0(6)	63.0(4)
	N	78.8	76.8(9)	92.0(1)	71.5	69.4(8)	77.5(2)	60.4	58.3(6)	63.7(4)
	SL	85.9	73.7(2)	88.3(8)				59.6	57.4(5)	61.8(5)
	Nr				68.5	67.7(9)	76.0(1)	55.0	52.4(8)	65.5(2)
	K				77.6	76.2(8)	82.0(2)	63.6	62.0(6)	69.0(4)
	Control	88.6			78.2			63.3		
LSD ($P = 0.05$)		7.8			5.1			4.9		
LSD ($P = 0.01$)		10.7			6.9			6.4		

[a] Values in parentheses show the number of plants, out of 10 inoculated, which produced symptoms or remained symptomless and from which the mean valaues in the body of the table were calculated

3.2.2.6 Leaf assessments

Leaf symptom severity: Progression of disease symptoms was obtained by recording the disease severity at each leaf position every 5 days up to 60 days after inoculation. As with susceptible cultivars, symptom severity progress curves for individual leaves showed similar trends in all three experiments so curves for experiment 3 only are presented in Figure 3.9.

When leaves 2 and 3 of seedlings of resistant cultivars at the 4-5 leaf stage were inoculated with RYMV, symptoms were not produced in inoculated leaves or non-inoculated leaves 4 and 5. Leaf 5 emerged at about the time of inoculation and continued growing for a further 15-20 days (Fig. 3.10). Symptoms first appeared in leaf 6 and in cvs. Moroberekan and Lac 23 symptoms were mild (mean score 1.0) and did not increase with time with all isolates except when these cultivars were inoculated with RYMV-Nr. Isolate Nr induced more obvious symptoms which increased gradually and reached severity values of 1.8 and 2.5 in Lac 23 and Moroberekan respectively. In cv. ITA 235 symptom scores fluctuated but values for leaf 6 were usually less than 2.0 except with isolate Nr which sometimes exceeded this value but did not cause the consistently higher disease symptom scores compared with other isolates as seen in cvs. Moroberekan and Lac 23. In leaf 7, symptom severity values of 1.0-2.5 were evident at leaf emergence (25-30 days after inoculation, Fig. 3.9) and these values gradually increased to about 1.5-3.5 60 days after inoculation. In the three cultivars, overall values were greater than for leaf 6 and isolate Nr clearly induced greatest disease in cvs. Moroberekan and ITA 235 but not in cv. Lac 23.

Fig. 3.9 Mean severity scores for leaves 6, 7 and 8 of resistant cultivars 0-60 days after inoculation with RYMV isolates (experiment 3).

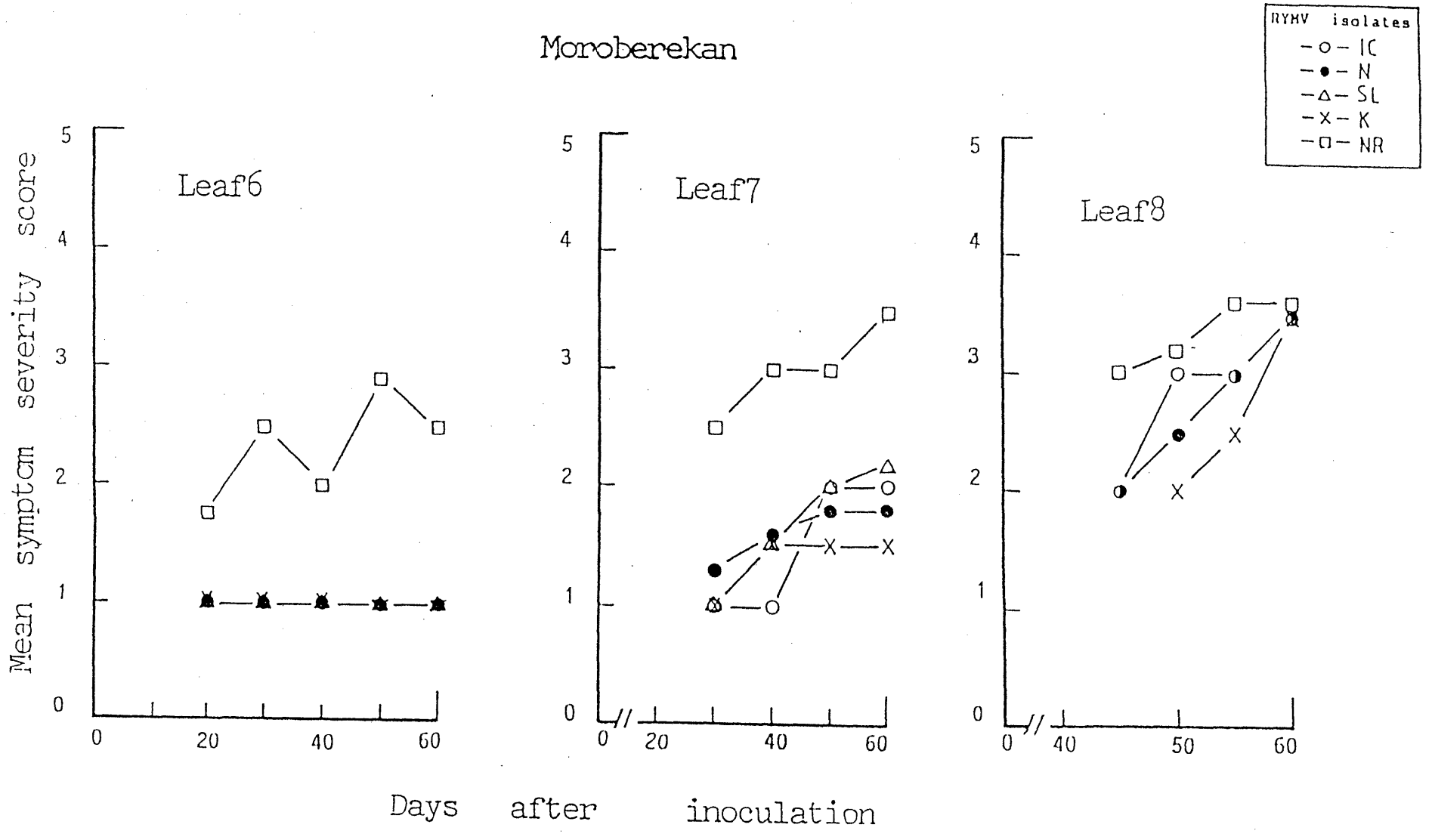


Fig. 3.9 contd.

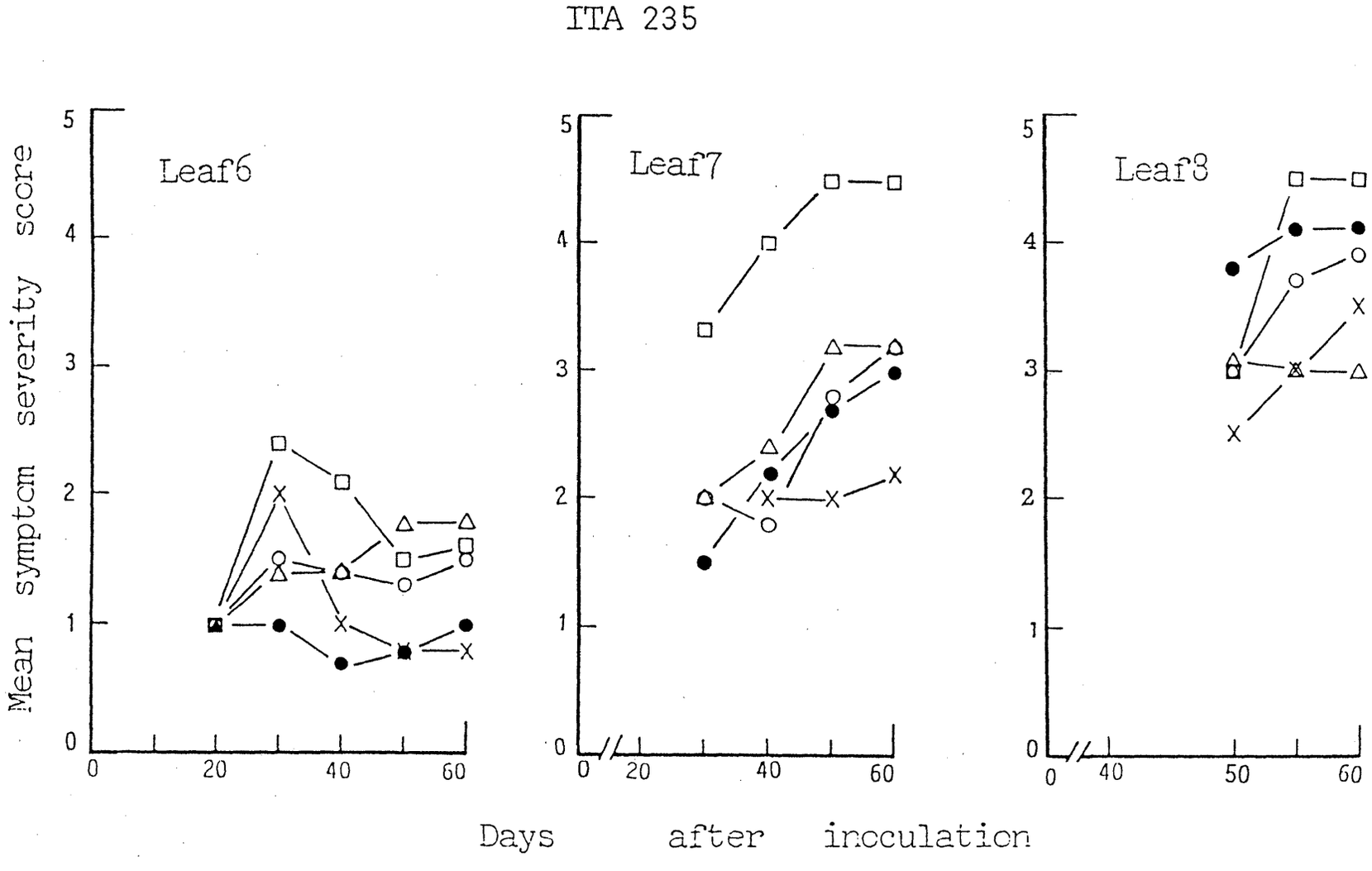
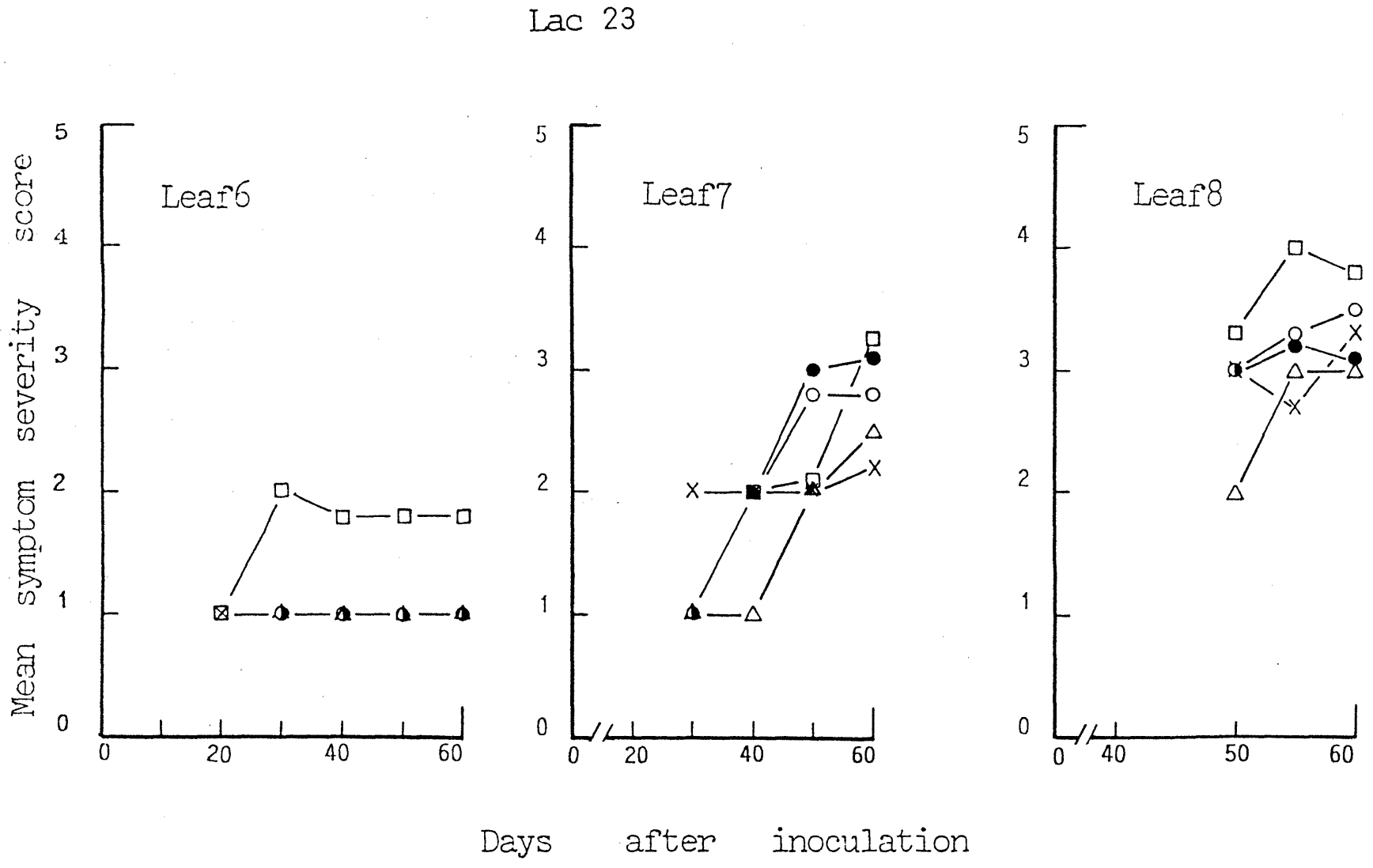

ITA 235
Leaf6
Leaf7
Leaf8
Mean symptom severity score
Days after inoculation
0
1
2
3
4
5
0
20
40
50
60

Fig. 3.9 contd.


Lac 23
Leaf6
Leaf7
Leaf8
Mean symptom severity score
Days after inoculation
0
1
2
3
4
5
0
20
40
50
60

The final leaf to emerge, leaf 8, displayed symptom severity values of 2.0-3.5 immediately on emergence about 45-50 days after inoculation in all cultivars. Disease severity scores for this leaf increased rapidly compared with leaves 6 and 7, and reached values of 3.0-4.5 after 60 days (Fig. 3.9). As for leaves 6 and 7, RYMV-Nr induced the highest values from emergence to 60 days after inoculation (Fig. 3.9).

Table 3.15 details mean symptom severity scores at 60 days and shows that all isolates tended to induce symptoms which increased in severity from the first leaf to show symptoms (leaf 7 in experiments 1 and 2; leaf 6 in experiment 3) to succeeding leaves emerging during the experiment (Table 3.15). Severity values for all isolates in the three experiments tended to be greatest on cv. ITA 235, RYMV-Nr induced more severe symptoms than other isolates and isolate SL often caused the mildest symptoms.

Leaf length: The effect of RYMV infection on leaf growth and development was similar in the three experiments. In experiment 3, leaf 5 emerged at inoculation, disease symptoms were not produced (Table 3.16) and the leaf ceased extension growth 15-20 days after inoculation without being affected by any of the five RYMV isolates (Fig. 3.10; leaf 5).

Leaf 6 emerged 10-15 days after inoculation and continued growing for about 20 days (Fig. 3.10, leaf 6). Slight extension growth changes caused by infection were not significantly different from controls ($P = 0.05$) in any cultivar up to and including 60 days after

Table 3.15 Mean symptom severity scores for individual leaves of resistant rice cultivars (Moroberekan, Lac 23 and ITA 235) 60 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Symptom severity scores: Leaf position (Expt 1) 7	8	9	10	Leaf position (Expt 2) 7	8	9	10	Leaf position (Expt 3) 6	7	8
Moroberekan	IC	1.3	2.8	3.0	3.2	-	-	-	-	1.0	2.0	3.5
	N	1.3	2.9	3.1	3.3	1.0	2.5	3.5	3.7	1.0	1.5	3.5
	SL	0	0	0	0	-	-	-	-	2.0	2.2	2.7
	Nr	-	-	-	-	2.3	2.5	3.7	3.8	2.5	3.5	3.5
	K	-	-	-	-	1.3	3.4	3.0	3.4	1.0	1.5	3.5
All isolates (mean)		0.9	1.9	2.0	2.2	1.5	2.8	3.3	3.6	1.5	2.1	3.3
Lac 23	IC	1.3	2.5	3.5	3.8	-	-	-	-	1.0	2.3	3.3
	N	2.0	3.0	3.0	3.4	1.0	2.0	3.3	3.5	1.0	2.5	3.1
	SL	0.5	2.9	3.0	3.0	-	-	-	-	1.0	2.5	3.0
	Nr	-	-	-	-	1.4	3.8	4.0	4.0	1.8	3.5	3.8
	K	-	-	-	-	1.0	2.4	3.2	3.5	1.0	2.2	3.3
All isolates (mean)		1.3	2.8	3.2	3.3	1.1	2.7	3.5	3.7	1.2	2.6	3.3
ITA 235	IC	2.0	3.2	4.0	4.0	-	-	-	-	1.5	3.2	3.9
	N	2.2	3.6	4.0	4.0	1.0	3.3	3.5	4.0	1.0	3.0	4.1
	SL	2.0	2.0	3.0	3.0	-	-	-	-	1.8	3.0	3.2
	Nr	-	-	-	-	2.3	4.4	4.1	4.2	1.6	4.5	4.5
	K	-	-	-	-	1.6	3.8	3.5	3.7	1.0	3.2	3.5
All isolates (mean)		2.1	2.9	3.7	3.7	1.6	3.8	3.7	4.0	1.4	3.2	3.8

Fig. 3.10 Leaf length for leaves 5, 6, 7 and 8 of resistant cultivars 0-60 days after inoculation with RYMV isolates (experiment 3)

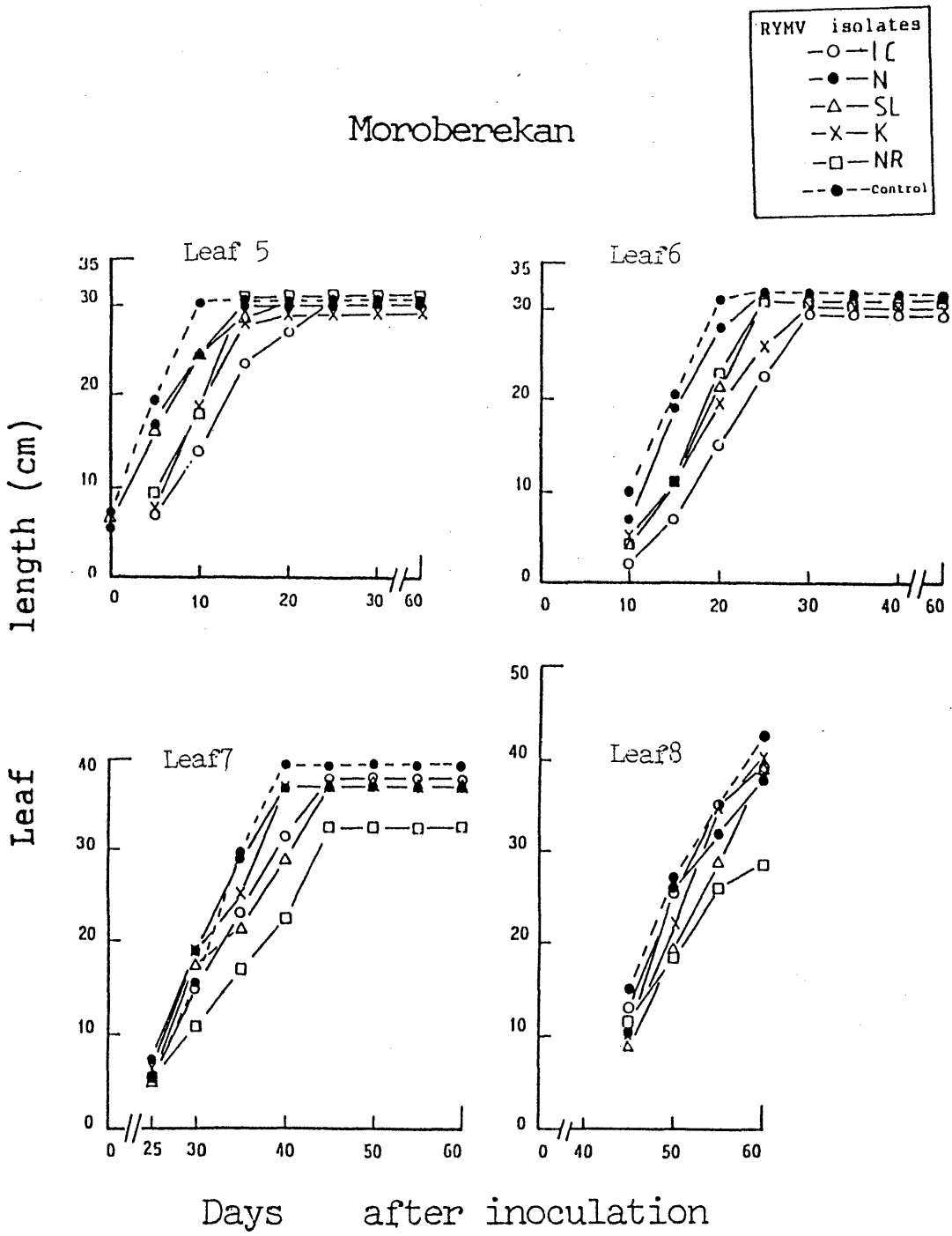


Fig. 3.10 contd.

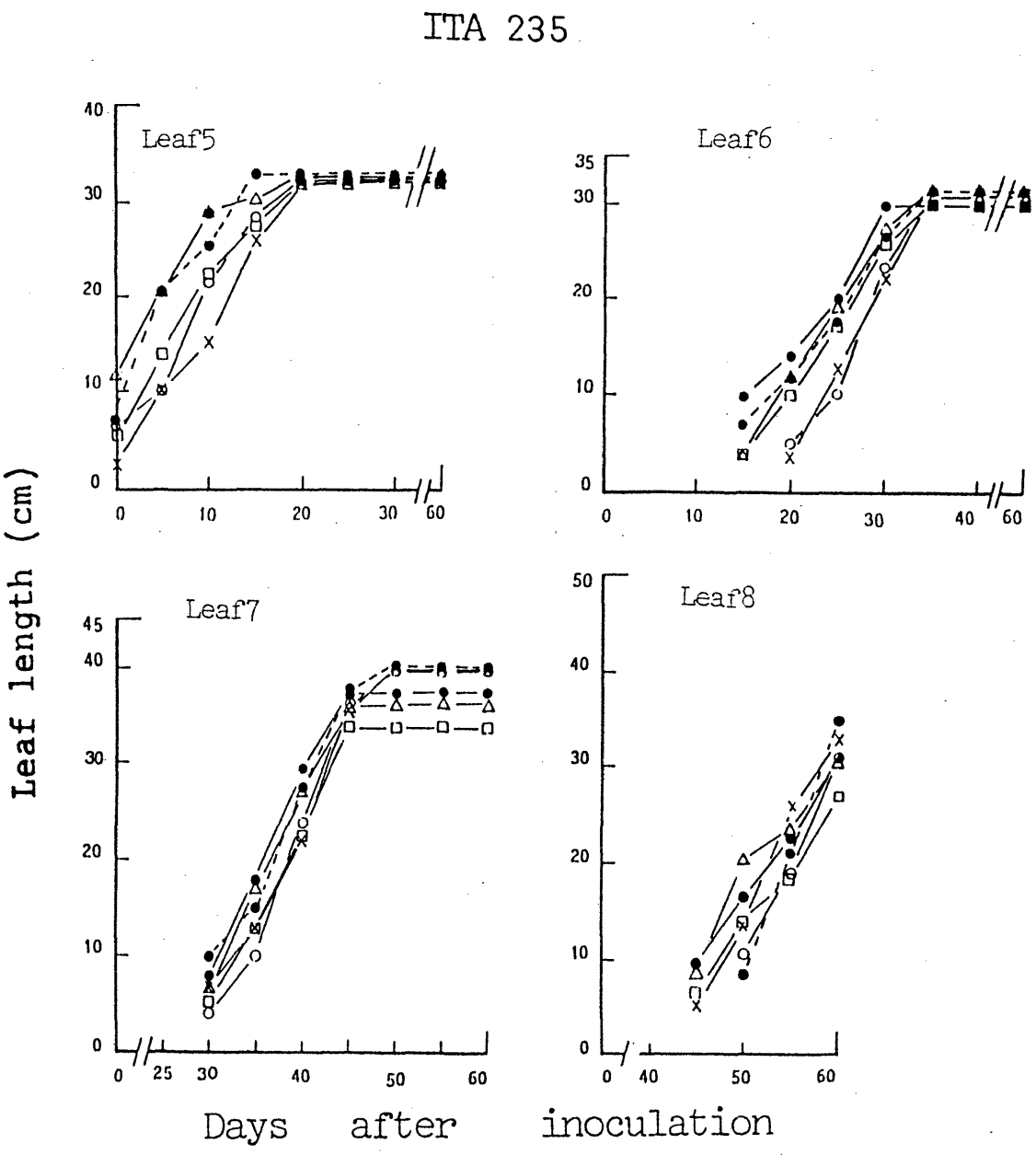
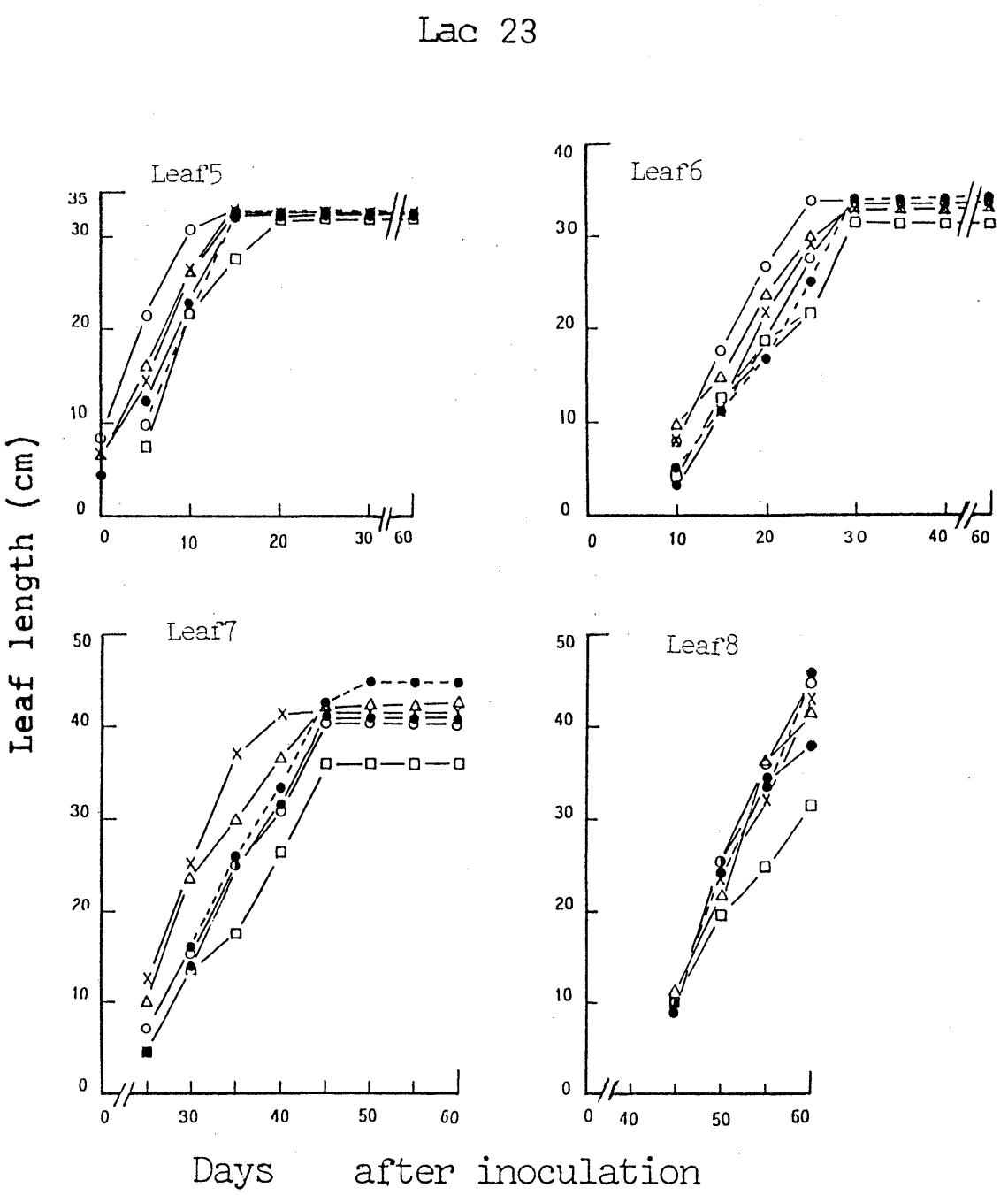


Fig. 3.10 contd.



inoculation (Table 3.16). The next leaf, leaf 7, emerged 25-30 days after inoculation and ceased growing 15-20 days later (Fig. 3.10, leaf 7). This leaf produced symptoms about 30 days after inoculation. Leaf lengths of plants inoculated with RYMV-Nr were significantly different from controls ($P = 0.05$) in all three cultivars. Percentage decreases relative to control were 17.4, 20.4 and 17.2% in cvs. Moroberekan, Lac 23 and ITA 235, respectively. Growth changes induced by other RYMV isolates were not significantly different from the controls. The final leaf, leaf 8, appeared 45 days after inoculation and the experiment was terminated before this leaf was fully extended (Fig. 3.10, leaf 8). Growth was significantly reduced in Moroberekan and Lac 23 inoculated with isolate Nr but although growth was also reduced in cv. ITA 235, it was not significant ($P = 0.05$). Other isolates did not significantly affect the growth of leaf 8. Table 3.16 shows that virtually all cultivars, when inoculated with RYMV-Nr, showed pronounced growth reductions in leaves 7 and 8 compared with other isolates. No significant differences were apparent between other isolates and this was the case in all three experiments.

As for plant height (section 3.3.2.5), results for mean leaf lengths in Table 3.16 take into account all 10 plants per virus/cultivar combination inoculated with a given RYMV isolate. Results for leaf lengths calculated separately for the variable number of plants which did or did not develop symptoms and were found to react positively or negatively respectively in ELISA tests are presented in Table 3.16a. These results show that in all three experiments and for all resistant cultivars where the number of successfully infected plants was not less than 40%, the mean leaf length of the youngest fully-expanded leaves was considerably reduced compared with

Table 3.16 Effect of infection by RYMV isolates on the length of rice leaves (cvs. Moroberekan, Lac 23 and ITA 235) 60 days after inoculation

Rice cultivar	RYMV isolate	Leaf length cm: Leaf position (Expt 1) 6	7	8	9	10	Leaf position (Expt 2) 6	7	8	9	10	Leaf position (Expt 3) 5	6	7	8
Moroberekan	IC	29.3[a]	32.6	40.4	62.6	29.8	-	-	-	-	-	30.6	29.5	38.0	39.6
	N	30.0	32.1	39.2	56.9	32.5	37.2	42.5	47.4	46.8	30.8	30.1	31.8	37.2	38.0
	SL	29.3	31.6	39.5	64.7	31.7	-	-	-	-	-	30.8	31.4	36.8	39.2
	Nr	-	-[b]	-	-	-	36.1	41.7	46.8	44.2	29.8	31.0	30.1	32.6	28.6
	K	-	-	-	-	-	37.2	44.2	46.2	45.5	23.4	29.5	30.9	37.0	40.6
	Control	29.2	32.4	41.1	64.4	35.6	38.3	44.8	52.1	50.9	24.9	30.4	31.9	39.6	42.8
Lac 23	IC	31.8	35.5	39.3	62.3	45.9	-	-	-	-		32.6	33.8	40.6	45.0
	N	31.8	33.9	38.0	59.9	56.9	41.8	48.8	56.5	57.7	43.0	32.7	32.9	41.0	38.2
	SL	31.4	33.1	39.8	65.9	50.2	-	-	-	-		33.7	33.0	42.6	41.4
	Nr	-	-	-	-	-	39.2	48.1	54.2	53.4	45.5	32.2	31.4	35.8	31.4
	K	-	-	-	-	-	41.3	50.1	58.7	58.3	43.1	32.9	32.8	41.4	42.8
	Control	32.5	34.8	39.7	65.2	64.1	40.6	50.0	59.0	61.7	51.1	32.7	33.7	45.0	43.2
ITA 235	IC	28.7	29.5	36.3	61.1	55.6	-	-	-	-		32.4	31.5	40.0	31.0
	N	27.9	29.5	37.6	56.3	43.9	37.1	43.4	45.6	40.6	21.4	33.1	34.3	37.6	31.1
	SL	28.0	29.4	37.0	61.7	33.6	-	-	-	-		32.9	33.0	36.1	30.6
	Nr	-	-	-	-	-	37.7	42.6	45.2	35.7	17.0	32.3	30.0	33.6	27.0
	K	-	-	-	-	-	38.8	46.3	52.3	41.0	17.3	32.2	31.3	40.0	32.8
	Control	29.0	30.8	39.3	60.6	51.1	38.3	46.1	50.9	43.7	22.1	32.8	33.9	40.6	34.8
LSD ($P = 0.05$)		1.9	2.5	6.1	7.8	14.7	2.9	3.2	4.1	6.4	10.3	2.8	2.8	4.2	9.6
LSD ($\overline{P} = 0.01$)		2.5	3.3	8.2	10.5	19.7	3.9	4.3	5.5	8.6	13.8	3.7	3.7	5.5	12.7

[a] Mean of 10 plants per treatment
[b] Isolates of RYMV not included

Table 3.16a Mean leaf length of plant showing symptoms and symptomless plants of resistant cultivars 60 days after inoculation with RYMV isolates

Rice cultivar	RYMV isolate	Leaf length cm: Leaf position (Expt 1) 6	7	8	9	10	Leaf position (Expt 2) 6	7	8	9	10	Leaf position (Expt 3) 5	6	7	8
Moroberekan															
Infected	IC	31.5(3)	31.5	33.7	54.7	37.7						26.5(1)	28.0	30.0	37.0
	N	29.2(9)	31.9	37.3	55.4	32.8	38.0(8)	43.4	48.0	46.5	29.8	30.0(6)	30.7	36.0	34.2
	SL	-	-	-	-	-						32.8(2)	32.2	35.0	34.0
	Nr						36.1(10)	41.7	46.8	44.2	29.8	30.9(8)	30.9	32.4	24.9
	K						37.2(9)	44.2	48.5	41.4	21.3	30.0(4)	29.5	35.5	37.5
Symptomless	IC	29.6(7)	32.9	37.4	63.8	26.5						27.7(9)	29.8	38.9	39.9
	N	30.5(1)	34.0	40.0	65.0	32.0	34.0(2)	39.0	45.0	50.0	35.0(2)	29.5(4)	31.2	39.0	43.7
	SL	29.3(10)	31.6	37.0	61.7	31.7						35.9(8)	31.1	37.5	40.5
	Nr						-	-	-	-	-	31.8(2)	31.0	37.5	44.0
	K						37.0(1)	43.0(1)	46.0(1)	49.0(1)	42.0(1)	29.3(6)	30.3	38.0	42.7
Control		29.2(10)	32.4	41.1	64.4	35.6	38.3	44.8	52.1	50.9	24.9	30.4	31.9	39.6	42.8
Lac 23															
Infected	IC	31.4(4)	35.9	36.5	56.7	53.0						32.2(3)	35.8	39.3	38.7
	N	31.6(7)	39.3	36.6	58.4	54.3	42.5(6)	48.1	53.7	53.7	35.8	33.0(6)	35.6	40.7	31.2
	SL	26.0(1)	27.5	30.0	54.5	40.5						33.0(3)	37.5	45.5	37.0
	Nr						39.2(10)	48.1	54.2	53.4	45.5	32.0(8)	32.1	34.7	31.3
	K						41.6(6)	49.6	57.8	53.8	36.5	33.2(3)	31.3	36.0	31.7
Symptomless	IC	32.1(6)	35.2	41.2	66.1	41.2						32.8(7)	33.7	41.1	47.8
	N	31.7(3)	34.8	41.2	63.7	62.8	40.2(4)	49.8	59.8	63.8	53.8	32.2(4)	34.5	43.5	48.7
	SL	21.3(9)	33.7	40.9	67.2	51.2						34.0(7)	35.6	42.4	42.7
	Nr											33.0(2)	31.0	40.0	47.0
	K						41.8(4)	51.5	59.0	65.0	53.0	32.8(7)	35.2	43.3	47.6
Control		32.5	34.8	39.7	65.2	64.1	40.6	50.0	59.0	61.7	51.1	32.7	33.7	45.0	43.2
ITA 235															
Infected	IC	29.5(6)	29.7	32.8	57.2	40.2						32.3(6)	31.1	38.0	28.2
	N	28.5(9)	28.3	36.8	55.2	43.0	36.2(8)	43.1	44.2	37.9	17.1	32.4(6)	34.5	36.5	30.6
	SL	28.0(2)	28.0	33.2	52.5	30.5						32.6(5)	31.4	33.0	26.8
	Nr						37.5(9)	42.7	44.9	35.3	17.4	30.2(8)	29.7	32.0	27.0
	K						38.8(8)	46.9	51.9	44.4	16.8	31.8(6)	31.1	37.8	31.4
Symptomless	IC	27.8(4)	29.0	40.5	67.0	42.2						32.2(4)	32.1	43.0	35.2
	N	27.0(1)	30.0	43.0	66.0	52.0	35.0(2)	45.0	50.5	51.5	38.0	34.1(4)	34.0	39.2	39.5
	SL	28.8(8)	29.8	38.0	64.0	33.4						33.7(5)	32.8	36.4	34.2
	Nr						41.5(1)	47.0	48.0	43.0	13.0	30.5(2)	30.0	38.0	41.0
	K						39.5(2)	50.5	57.0	47.5	19.5	32.9(4)	31.2	43.2	35.5
Control		29.0	30.8	39.1	60.1	51.1	38.3	46.1	50.9	43.7	22.1	32.8	33.9	40.6	34.8

Values in parentheses show the number of replicate plants from which the mean leaf length was calculated

uninoculated controls, whereas in symptomless plants this was not so. For example, in experiment 3, for Moroberekan plants inoculated with RYMV-N (disease incidence 60%), leaves 7 and 8 of plants showing symptoms were severely affected compared with symptomless or control plants. However, in experiment 2, the length of leaf 8 in symptomless plants was less than that of plants showing symptoms when Moroberekan was inoculated with RYMV-N (disease incidence 80%).

3.3.2.7 Fresh and dry weight of shoots and roots

The progressive decrease in fresh weights of healthy control plants (Table 3.17) in experiments 1 to 3 may be due to the differing environmental conditions (see Materials and Methods). However, the effect of the five RYMV isolates on shoot and root growth was essentially similar in the three experiments. In experiment 1, where resistant cultivars were inoculated with isolates IC, N and SL, decreases in shoot and root fresh weight of cv. ITA 235 and in root fresh weights of cv. Lac 23 plants inoculated with RYMV-N were apparent (Table 3.17). In experiments 2 and 3, fresh weights of shoots and roots of all three resistant cultivars were markedly reduced by RYMV-Nr. Other isolates occasionally slightly affected fresh weight but there were no obvious consistent trends (Table 3.17).

Dry weight data also showed that RYMV-Nr caused a marked decrease in roots and shoots in all cultivars whereas other isolates had minor effects or none at all (Table 3.18).

There were indications that fresh weights were adversely

Table 3.17. Fresh weights of shoots and roots of three resistant cultivars 60 days after inoculation with isolates of RYMV.

Rice cultivar	RYMV isolate	Fresh weight (g)					
		Expt. 1		Expt. 2		Expt. 3	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
Moroberekan	IC	13.5	12.6	-	-	2.9	2.0
	N	12.7	11.5	8.6	7.5	2.9	2.0
	SL	14.8	12.9	-	-	3.3	2.2
	Nr	-	-	7.8	6.6	2.4	1.7
	K	-	-	8.1	7.4	3.0	1.9
All isolates (mean)		13.7 (10.4%)	12.3 (10.2%)	8.2 (16.3%)	7.2 (12.2%)	2.9 (17.1%)	2.0 (13.0%)
Control		15.3	13.7	9.8	8.2	3.5	2.3
Lac 23	IC	12.6	11.1	-	-	3.1	2.1
	N	12.1	9.3	9.4	7.9	2.8	1.9
	SL	12.9	11.6	-	-	3.1	2.3
	Nr	-	-	8.0	6.1	2.2	1.7
	K	-	-	9.6	7.6	2.9	2.0
All isolates (mean)		11.9 (13.1%)	10.7 (14.4%)	9.0 (14.3%)	7.2 (15.3%)	2.8 (17.6%)	2.0 (13.0%)
Control		13.7	12.5	10.5	8.5	3.4	2.3
ITA 235	IC	11.1	9.9	-	-	2.7	1.8
	N	9.5	8.4	8.6	7.4	2.7	1.8
	SL	11.9	11.1	-	-	2.6	2.1
	Nr	-	-	6.6	5.8	2.1	1.6
	K	-	-	8.5	7.6	2.6	1.7
All isolates (mean)		10.5 (21.1%)	9.8 (14.9%)	7.9 (20.2%)	7.4 (11.9%)	2.5 (21.8%)	1.8 (18.2%)
Control		13.3	11.4	9.9	8.4	3.2	2.2
L.S.D. ($P = 0.05$)		2.95	2.07	1.27	0.94	0.42	0.34
($P = 0.01$)		3.97	2.78	1.70	1.27	0.55	0.45

[a] Values in parentheses show weight reduction caused by infection as % decrease of inoculated compared with sham-inoculated controls.

Table 3.18. Dry weight of shoots and roots of three resistant cultivars 60 days after inoculation with isolates of RYMV.

Rice cultivar	RYMV isolate	Dry weight (g)					
		Expt. 1		Expt. 2		Expt. 3	
		Shoots	Roots	Shoots	Roots	Shoots	Roots
Moroberekan	IC	3.04	2.64	-	-	0.79	0.58
	N	2.83	2.57	2.17	1.92	0.80	0.60
	SL	3.12	2.87	-	-	0.80	0.65
	Nr	-	-	2.00	1.84	0.80	0.53
	K	-	-	1.10	1.90	0.81	1.60
All isolates (mean)		3.0 (9.9%)	2.69 (8.8%)	2.09 (12.5%)	1.89 (14.1%)	0.78 (7.1%)	0.59 (10.6%)
Control		3.32	2.95	2.39	2.2	0.84	0.66
Lac 23	IC	2.87	2.53	-	-	0.81	0.60
	N	2.67	2.20	2.36	2.09	0.80	0.58
	SL	3.08	2.65	-	-	0.82	0.62
	Nr	-	-	2.05	1.81	0.67	0.52
	K	-	-	2.42	2.18	0.80	0.60
All isolates (mean)		2.87 (9.7%)	2.46 (12.1%)	2.28 (14.9%)	2.03 (12.1%)	0.78 (7.1%)	0.58 (9.4%)
Control		3.18	2.8	2.68	2.31	0.84	0.64
ITA 235	IC	2.45	2.32	-	-	0.64	0.53
	N	2.12	1.98	2.18	1.93	0.65	0.52
	SL	2.54	2.46	-	-	0.68	0.56
	Nr	-	-	1.75	1.50	0.57	0.46
	K	-	-	2.18	1.93	0.63	0.52
All isolates (mean)		2.37 (19.7%)	2.25 (14.4%)	2.04 (18.7%)	1.79 (20.1%)	0.63 (22.82)	0.52 (14.7%)
Control		2.95	2.63	2.51	2.24	0.81	0.61
L.S.D.($P = 0.05$)		0.54	0.42	0.30	0.26	0.08	0.08
($P = 0.01$)		0.70	0.57	0.40	0.35	0.10	0.10

[a] Values in parentheses show weight reduction caused by infection as % decrease of inoculated compared with sham-inoculated controls.

affected slightly more in cv. ITA 235 than in cvs. Moroberekan and Lac 23. Reductions in shoot fresh weight for ITA 235 ranged from 20.2-21.8% and for root fresh weight 11.9-18.2% relative to controls. Reductions for cv. Moroberekan were, for shoot fresh weight, 10.4-14.1% and for roots 10.2 to 13.0% and in Lac 23 reductions ranged from 13.1-17.6% (shoots) and 13.0-15.3% (roots).

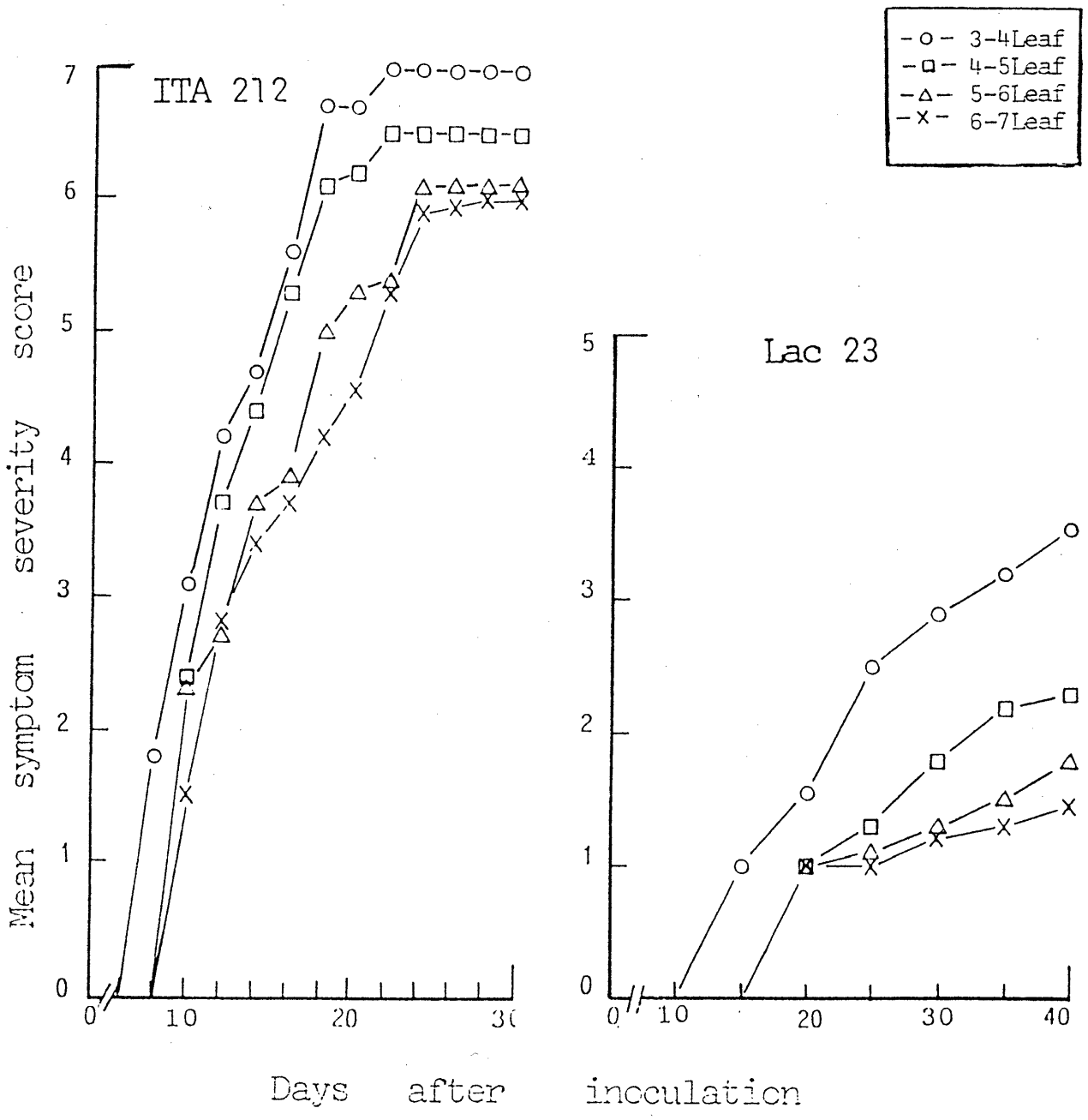
3.3.3 Reaction of susceptible and resistant rice cultivars to infection at various growth stages

3.3.3.1 Symptom severity

When susceptible ITA 212 plants were inoculated at the 3-4 leaf stage with RYMV-N inoculum applied to leaves 1 and 2, initial symptoms appeared 7 days after inoculation on leaf 4 or 5. Symptom severity increased quickly and reached the necrotic stage 22 days after inoculation; all infected plants were killed (Fig. 3.11). Plants inoculated at the latest stage tested (6-7 leaf stage) showed a slower development of symptoms with lower severity values compared with plants inoculated at the 3-4 leaf stage (Fig. 3.11). Overall, the rate and intensity of symptom development progressively decreased with advance in plant age at inoculation. Nevertheless, after 30 days, inoculated plants of all treatments produced mean scores of 6.0 or above (orange foliar colouration) (Fig. 3.11).

In resistant Lac 23 plants inoculated at the 3-4 leaf stage initial symptoms occurred in leaf 5, 14 days after inoculation. Mean symptom scores increased fairly rapidly and reached 3.6 after 40 days, whereas plants inoculated at the 6-7 leaf stage showed very slow symptom development and low symptom scores after 40 days (Fig. 3.11). As with the susceptible cultivar, disease severity scores progressively decreased with increased plant age at inoculation (Fig. 3.11).

Fig. 3.11 Mean disease severity scores for rice cultivars ITA 212 and Lac 23 inoculated with RYMV-N at different stages of growth.



3.3.3.2 Disease incidence

All plants of cv. ITA 212 showed symptoms when inoculated with RYMV-N regardless of the age at which the plants were inoculated. Symptoms first appeared after 7 days in plants inoculated at the 3-4 leaf stage, whereas initial symptoms took 10 days to appear in the other treatments (Table 3.19). The time required for symptom expression to be total (100%) increased the older the plants at inoculation (Table 3.19).

Lac 23 plants inoculated at the 3-4 leaf stage developed symptoms earlier than plants inoculated at later stages (Table 3.19). Irrespective of plant age at inoculation, total infection (100%) was never obtained and the number of plants with symptoms declined with increasing plant age at inoculation (Table 3.19) such that 90% of inoculated plants showed symptoms when inoculated at the 3-4 leaf stage but only 40% did so when inoculated at the 6-7 leaf stage.

When Lac 23 plants were harvested 40 days after inoculation, samples of young leaves were obtained from all plants in the experiment, and tested for the presence of RYMV-N by ELISA using extracts diluted 1:100 (w/v). Results showed that 90, 70, 70 and 55% of plants inoculated at the 3-4, 4-5, 5-6 and 6-7 leaf stage respectively, were systemically infected. These values were slightly in excess of the proportion of plants showing symptoms for the last three treatments (Table 3.19) thus demonstrating the presence of some symptomless but infected plants.

Table 3.19 Proportion (%) of rice plants showing symptoms after inoculation at different stages of development with RYMV-N

Rice cultivar	Growth stage at inoculation	Leaves inoculated	Days after inoculation																		
			6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	25	30[b]	35	40[c]
ITA 212	3-4 leaf	1+2	0	90[a]	90	100															
	4-5 leaf	2+3				0	70	85	90	100											
	5-6 leaf	3+4				0	60	75	85	100											
	6-7 leaf	4-5				0	40	65	75	80	80	80	80	80	80	80	90	100			
Lac 23	3-4 leaf	1+2								0	10	10	45	45			70	75	90	90	90
	4-5 leaf	2+3											0	10			20	40	45	60	60
	5-6 leaf	3+4											0	10			35	40	40	50	60
	6-7 leaf	4-5												0			20	20	25	40	40

[a] Proportion (%) of plant showing symptoms (20 plants per treatment)
[b] Cv. ITA 212 harvested
[c] Cv. LAC 23 harvested

3.3.3.3 Incubation period

Overall, incubation periods were more than twice as long for resistant Lac 23 than susceptible ITA 212 irrespective of the growth stage at inoculation with RYMV-N. In both cultivars, the average incubation periods tended to increase the older the plants at inoculation (Table 3.20). Thus, ITA 212 plants inoculated at the 3-4 leaf stage had an average incubation period of 7.2 days which increased to 13.1 days when inoculated at the 6-7 leaf stage. Mean incubation periods were similar for plants inoculated at the 4-5 or 5-6 leaf stage although differing in susceptible and resistant cultivars (Table 3.20).

3.3.3.4 Plant height

The mean height reduction caused by RYMV-N inoculation at all inoculation dates for ITA 212 and Lac 23 was 27.1% and 4.6% respectively. Greatest reductions were for ITA 212 (48.%) and Lac 23 (11.6%) when plants were inoculated at the 3-4 leaf stage. The extent of virus-induced stunting decreased with increasing age of the plant at inoculation. Significant differences ($\underline{P}$ = 0.05) were obtained between healthy controls and infected ITA 212 plants irrespective of the time of inoculation (Fig. 3.12). Lac 23 plants inoculated at the 3-4 leaf stage were slightly but significantly shorter ($\underline{P}$ = 0.05) than uninoculated controls whereas inoculation at later growth stages did not exert a significant effect on height.

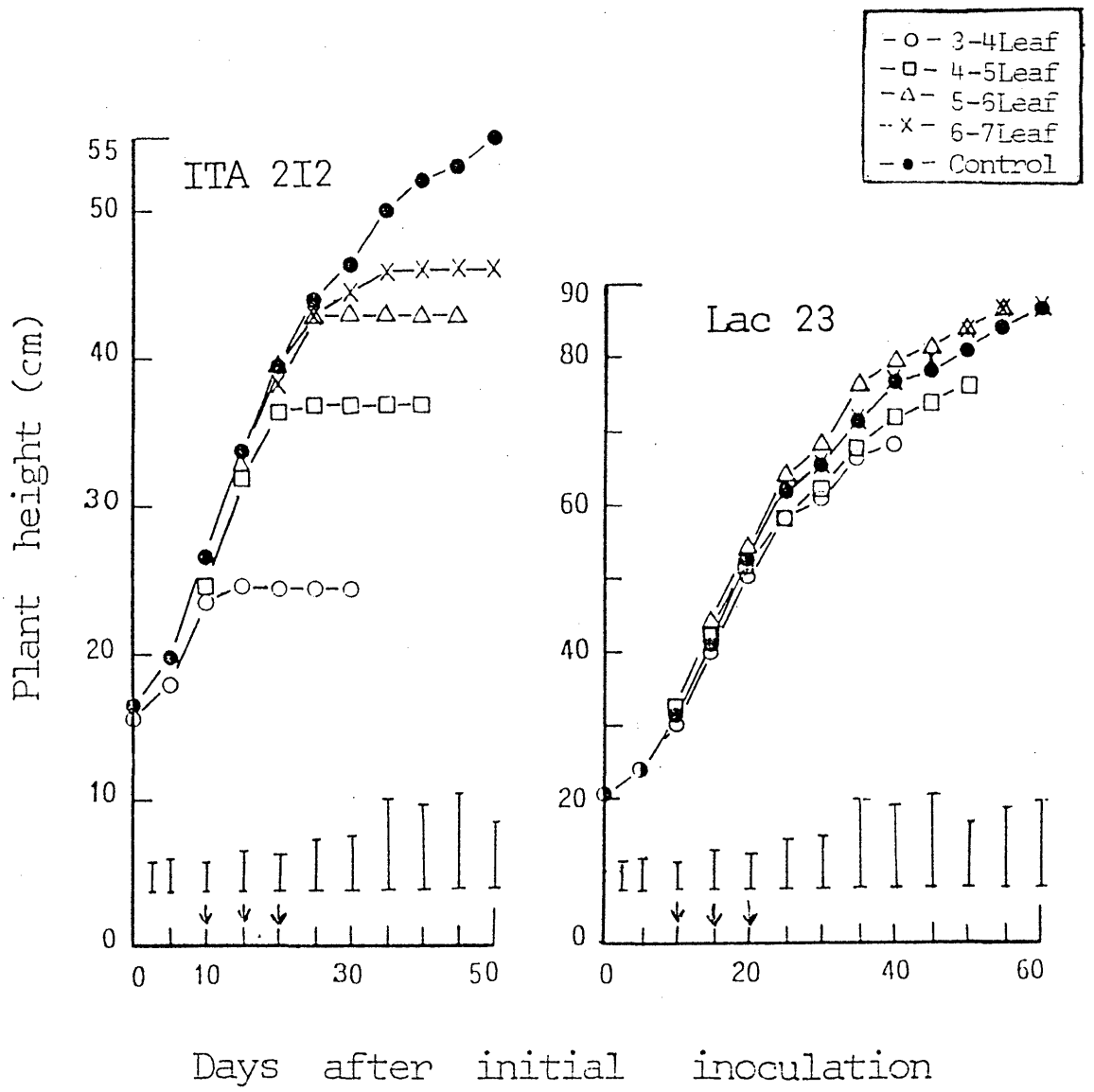
Table 3.20 Incubation periods for RYMV-N on susceptible ITA 212 and resistant Lac 23 rice inoculated at different stages of development

Growth stage at inoculation	Leaves inoculated	Incubation period (days)			
		ITA 212[a]		Lac 23[b]	
		Mean	Range	Mean	Range
3-4 leaf	1 + 2	7.2	7-9	20.1	14-30
4-5 leaf	2 + 3	10.5	10-13	25.8	17-35
5-6 leaf	3 + 4	10.8	10-13	25.3	17-40
6-7 leaf	4 + 5	13.1	10-25	26.9	20-35

[a] Harvested 30 days after inoculation

[b] Harvested 40 days after inoculation

Fig. 3.12 Plant height for rice cultivars ITA 212 and Lac 23 inoculated with RYMV-N at different stages of growth.



Arrows indicate second, third and fourth inoculations.
Bars indicate L.S.D. ($\underline{P}$ = 0.05)

3.3.3.5 Virus content

When Lac 23 plants were ELISA indexed after 40 days for the presence or absence of RYMV-N in the two youngest leaves, interesting differences in absorbance values were noticed. Mean readings for those plants where extracts yielded positive ELISA values are shown in Table 3.21. The amount of virus as indicated by the A_{410} x 100 readings in the youngest leaf was consistently slightly higher than in the older leaf tested irrespective of the stage at which plants were inoculated. Amount of virus antigen progressively decreased the older the plant at inoculation (Table 3.21).

Table 3.21. Mean absorbance (A_{410}nm x 100) readings for extracts from the two youngest leaves of Lac 23 plants 40 days after inoculation with RYMV-N.

Growth stage at inoculation	A_{410} x 100			
	Leaf position			
	7	8	9	Mean
3-4 leaf	139±8.13[a](18)[b]	164±4.0 (18)		151.5
4-5 leaf	90±12.7 (14)	102±12.1 (14)		96.0
5-6 leaf		69±8.9 (14)	78±9.1 (14)	73.0
6-7 leaf		56±5.9 (11)	67±11.9 (11)	62.5

[a] Only extracts which gave values greater than 2x healthy sap controls included. Extracts diluted 1:100 (w/v) and tested in duplicate wells. The reaction was measured 30 minutes after the addition of substrate.

[b] Values in parentheses are the number of extracts from individual plants giving a positive ELISA value and from which the tabulated mean values + S.E. were calculated

Mean background reading (healthy sap 1:100 w/v) was 6.5 + 0.15

3.4 Discussion

Because of spatial constraints, it was not possible initially to include five isolates in a single experiment. Therefore, three experiments were made; in experiments 1 and 2 three isolates were tested with RYMV-N common to both and in experiment 3 all five isolates were used.

Certain considerations were taken into account throughout the work to minimize variability. Considerations included avoidance of contamination between isolates, the use of uniform-sized plants at inoculation and a constant virus dilution and inoculum procedure for the three experiments. Within an experiment environmental conditions of temperature, daylength and light intensity were, of course, constant for all rice genotypes and RYMV isolates included.

Symptom expression, disease incidence, plant height, leaf length and fresh and dry weight of shoots and roots were used in studying the interactions between rice cultivars and isolates of RYMV. It was not possible, due to limitation of time and space, to allow plants to occupy the glasshouse compartments for the lengthy period required for flowering and seed maturation. Therefore reproductive parameters were not included in the three interaction experiments to provide yield assessments. Several workers have found that symptom expression and virus content (e.g. Huger and Sherwood, 1985; Stoddard *et al.*, 1987a,b) or symptom severity and disease incidence (e.g. Kuhn and Smith, 1967; Scott and Rosenkranz, 1981; Hibino *et al.*, 1987) or disease severity and plant height or yield reduction (e.g. Smith, 1967; Jones and Catherall, 1970; Baltenberger

et al., 1987; Fomba, 1988) were useful methods for studying the host-virus relationships.

To avoid confusion, it is worthwhile to expand briefly on some terms associated with virus resistance. A cultivar is resistant to a particular virus if it possesses qualities that suppress or retard the multiplication or movement of the particular virus or the development of virus symptom is reduced; while a cultivar is susceptible if the virus readily infects and multiplies within the host and usually induces conspicuous symptoms. Therefore, resistance and susceptibility may be considered as opposite ends of a 'graded' scale of host response to infection. Resistance and susceptibility may both be categorized as high, moderate and low. High susceptibility and low resistance are sometimes considered synonymous (Cooper and Jones, 1983; Walkey, 1985). The term tolerance is, with respect to plant virus diseases, used when the host response to infection by a given virus is minimal in that yield is little affected and symptoms mild or absent even though the rate of virus multiplication and movement within the host is often similar to the situation in susceptible cultivars (Cooper and Jones, 1983; Walkey, 1985).

In the present study, sap transmission of RYMV isolates to rice cvs. ITA 212 and BG 90-2 induced the production of symptoms in new leaves formed after inoculation, whereas symptoms did not appear in inoculated leaves or those leaves fully expanded at the time of inoculation. Symptoms induced by all five RYMV isolates on both susceptible cultivars consisted of chlorotic streaking leading to general foliar chlorotic mottle followed by the appearance of orange

pigment. Ultimately, leaves turned necrotic and, in severe interactions, the plants were killed. Symptoms were essentially similar to earlier studies using RYMV isolates from Kenya (Bakker, 1974), Ivory Coast (Fauquet and Thouenel, 1977) and Sierra Leone (Fomba, 1988) on susceptible rice genotypes. The symptom severity values for all isolates increased with time and reached scores of 5.0 or more at 30 days after inoculation and in severe cases plants were killed at this time. The nature and progress of symptoms observed in the three experiments generally agreed with previous findings obtained at IITA (Nigeria) when cvs. ITA 212 and BG 90-2 were inoculated with RYMV-N. On the basis of their reaction to RYMV-N infection, the two cultivars were considered to be highly susceptible to the Nigerian isolate by John *et al*. (1985).

A rapid increase in disease incidence, short incubation period and marked reductions in plant height, leaf length and fresh and dry weights of shoots and roots were characteristic of all isolates of RYMV on both cultivars by 30 days after inoculation.

Comparison between the cultivars showed that although both ITA 212 and BG 90-2 were severely affected when inoculated with all five RYMV isolates, in the three experiments made, ITA 212 was generally the more affected of the two. Thus, disease severity scores for ITA 212 ranged from 6.3-6.8, whereas values for BG 90-2 ranged from 4.9-5.8. The trend was confirmed and extended by results for incubation period lengths (9.4-15.6 days for ITA 212; 11.0-15.8 days for BG 90-2) and reduction in plant height (17.0-26.3% decrease for ITA 212; 11.3-20.8% for BG 90-2), and leaf length and fresh and dry weights changes for shoots and roots in all three experiments. The

percentage reduction in shoot fresh weight caused by RYMV infection for cv. ITA 212 ranged from 68.3-70.7% and for root fresh weight 72.4-79.7%. Values for BG 90-2 were, for shoots, 50.3-54.3% decrease and for roots 38.8-43.6%.

Irrespective of susceptible rice cultivar, the summarised data for experiment 3 presented in Table 3.22 shows that for the parameters listed all plants inoculated with RYMV isolates were much affected compared with healthy control plants. Only small differences between isolates were evident, the most obvious being relatively severe effects associated with RYMV-Nr whereas isolate SL tended to have the least effect on rice growth, an effect which was still considerable when compared with healthy rice plants. It was not possible to differentiate between isolates using rice cvs. ITA 212 and BG 90-2, so it would seem that susceptible plants are poor differential hosts.

To the best of the writer's knowledge, there have been no previous extensive comparative studies of several geographically separated RYMV isolates. However, in a comparative study at Wageningen (The Netherlands) between Kenyan and Nigerian isolates results showed that generally the reaction of RYMV-K on susceptible cultivars was slightly more severe than the Nigerian isolate (IITA, 1986). A similar comparative study in the U.K. (Wye College) with plants grown under glasshouse conditions and using three RYMV isolates (IC, N and SL), Kumwenda (1988) found that isolate IC had a more severe effect on rice (cvs. ITA 212 and IR 9729-67-3) than the other two isolates. This conflicts with the present results when the same three isolates were simultaneously tested in experiment 1.

Table 3.22 Mean[a] effects of RYMV isolates on susceptible rice cultivars 30 days after inoculation (experiment 3)

RYMV isolate	Length of leaf 7 (cm)	Plant height (cm)	Dry weight (g)		Fresh weight (g)		Symptom severity[b]	Disease incidence (%)	Incubation period (days)
			Shoots	Roots	Shoots	Roots			
IC	16.9	35.4	0.31	0.2	0.82	0.62	5.5	100	12.6
K	17.0	37.2	0.33	0.22	0.84	0.69	5.6	100	12.8
N	16.8	36.0	0.31	0.18	0.80	0.57	5.6	100	13.5
Nr	14.0	35.0	0.29	0.18	0.74	0.57	6.2	100	12.5
SL	19.0	36.9	0.33	0.24	0.87	0.73	5.2	100	13.6
Control	30.9	44.8	0.94	0.58	2.27	0.67			
LSD(P=0.05)	2.8	1.8	0.04	0.04	0.09	0.16	0.56		

[a] Means for ITA 212 and BG 90-2 results combined
[b] Scale of 0.0 (symptomless) to 7.0 (severe symptoms)

Results from this experiment indicated that isolate N had slightly more severe effects than isolates IC and SL. These trends were confirmed by the results of experiment 3, where all five isolates were tested and isolate Nr (not included in experiment 1) was, overall, the most aggressive. However, both studies concluded that isolate SL had the least effect.

The results in this section clearly show that on susceptible rice cultivars ITA 212 and BG 90-2, all RYMV isolates tested induced severe symptoms (severity score above 5.0), 100% infection, short incubation periods (about 12.5 days) and stunted growth (Table 3.22). The designation of both cultivars as susceptible to RYMV-N (John *et al*., 1985) can therefore be extended to include RYMV isolates IC, SL, K and Nr. Although, as stated above, all isolates had a marked effect on both cultivars, isolate Nr tended to induce the severest effect and isolate SL the least severe but it was still not possible to distinguish confidently between isolates on the basis of the reaction of susceptible rice cultivars to infection by them.

When cultivars known to be resistant in previous studies with a Nigerian RYMV isolate (IITA, 1984, 1985; John *et al*., 1985) were inoculated with the five isolates in the present work, all isolates induced similar symptoms. Like susceptible cultivars, symptoms were not produced in inoculated leaves or in non-inoculated leaves fully expanded at inoculation. For example, symptoms first appeared in leaf 6 (experiment 3) or leaf 7 (experiments 1 and 2) in plants inoculated at the 4-5 leaf stage. In experiment 3, leaf 5 emerged at about the time of inoculation and ceased extension growth 15-20 days after inoculation and leaf 6, which showed symptoms, emerged 10-15

days after inoculation (Fig. 3.10). Unlike the susceptible cultivars, in the resistant cultivars tested mottling was the most conspicuous symptom on those plants which showed symptoms and leaves showing symptoms never turned orange or necrotic (Table 3.9) even by 60 days after inoculation. Disease severity scores for an individual leaf increased slowly with time but symptom progression for a population of inoculated plants showed considerable fluctuations up to 60 days after inoculation (Fig. 3.7). This was proably due to the variable number of inoculated plants which showed symptoms and to the late appearance of further plants with long incubation periods and initially low disease severity ratings which tended to reduce mean symptom severity scores. The overall mean disease severity score for the three cultivars was 3.2, in marked contrast to susceptible cultivars where all exceeded 3.2 (Table 3.23). These findings confirmed the results of John *et al*. (1985) who found that 6 weeks after inoculation, the mean disease severity score for resistant cultivars (e.g. IR 42, TOS 415, Lac 23 and Kulu) did not exceed 3.0 using a disease severity scale of 0 to 6.0, whereas susceptible cultivars (e.g. ITA 212) showed a severity rating of 5.0. Kumwenda (1988) found that for cultivars Lac 23, Moroberekan and ITA 235 inoculated with RYMV isolates IC, N and SL and kept under glasshouse conditions, symptom severity scores 33 days after inoculation ranged from 2.5-4.5, whereas values for ITA 212 ranged from 7.0-8.0 using a symptom severity scale of 0 (symptomless) to 9.0 (severe symptoms). Fauquet and Thouvenel (1977) found that Moroberekan plants inoculated with an Ivory Coast isolate developed a foliar mottle and the virus was recovered when plants were back-indexed on a susceptible rice cultivar. Similarly, Fomba (1988) found that cultivars such as Lac 23, IRAT 13 and IRAT 110 showed faint mottling and survived to flower

and produce seeds after inoculation with an Ivory Coast isolate. In contrast to these findings, John *et al*. (1985) found that Moroberekan plants inoculated with RYMV-N and grown under Nigerian conditions remained symptomless but agar-gel diffusion tests revealed the presence of RYMV-N in symptomless plants. Present experience showed that it was difficult to assess symptom severity on resistant cultivars under direct sunshine and perhaps Moroberekan may develop faint symptoms under field conditions, but these are difficult to see.

Symptom recovery or remission was not encountered in the present study with either susceptible or resistant cultivars. The recovery phenomenon was first reported by John *et al*. (1985) who found that some resistant cultivars (e.g. Kulu) inoculated with a Nigerian isolate of RYMV at 30 days old, initially showed symptoms but later appeared healthy and in agar-gel diffusion tests the virus was recovered from such symptomless leaves. Further work done in Nigeria by Kumwenda (1988) reported symptom recovery in resistant cultivars (e.g. Lac 23) and also mentioned reduction in disease severity of susceptible cultivars (e.g. ITA 212) when plants were kept for flowering. The absence of symptom recovery in the present study could be due to severe effect of RYMV, particularly on susceptible cultivars, in plants grown under glasshouse conditions. Bakker (1974) reported that the effect of RYMV was more pronounced under glass than in the field. The length of the experimental period may also be a factor as, in the present experiments, plants were harvested 30 and 60 days after inoculation for susceptible and resistant cultivars respectively, which may have been insufficient for some plants to show recovery.

The proportion of inoculated plants showing symptoms in all resistant cultivars used and irrespective of RYMV isolate was never 100%, a situation in marked contrast to susceptible cultivars where sap inoculation always gave total infection. Summarized results for the three resistant cultivars combined (Table 3.23) show that disease incidence ranged from 33.3-80%. ELISA indexing of the youngest leaves of inoculated plants 60 days after inoculation confirmed the absence of systemic RYMV antigen in tip leaves of symptomless plants whereas young leaves of plants showing symptoms contained RYMV antigen.

Although rice is self-pollinated, variation among plants of a given resistant rice cultivar to virus infection has been reported previously. Hibino *et al*. (1987, 1988) found disease incidence was low in resistant cultivars when vector inoculated with rice tungro bacilliform and rice tungro spherical viruses. Scott and Rosenkranz (1987) found that variability in the occurrence of maize dwarf mosaic virus (MDMV) symptoms among maize (self-pollinated line) was not conditioned by genetic variability within the inbred line. Nevertheless, failure to obtain complete infection was unexpected in the present study and the phenomena is examined further in the next section.

Another clear feature evident in resistant plants was the time required for the appearance of systemic symptoms for those plants in which systemic symptoms were produced. The average incubation period for susceptible cultivars of 12.5-13.6 days contrasted markedly with the lengthy period (30.4-38.3 days) encountered for resistant

Table 3.23 Mean [a] effects of RYMV isolates on resistant cultivars 60 days after inoculation (experiment 3)

RYMV isolate	Length of leaf 7 (cm)	Plant height (cm)	Dry weight (g)		Fresh weight (g)		Symptom severity [b]	Disease incidence %	Incubation period (days)
			Shoots	Roots	Shoots	Roots			
IC	39.5	65.0	0.74	0.57	2.9	1.98	2.6	33.3	30.6
K	39.5	65.3	0.75	0.57	2.87	1.87	2.4	43.3	38.3
N	38.6	62.5	0.75	0.57	2.78	1.89	2.5	60	36.6
Nr	34.0	57.6	0.64	0.50	2.22	1.68	3.2	80	32.0
SL	38.1	63.9	0.77	0.61	2.99	2.21	2.4	33.3	30.4
Control	41.7	66.8	0.83	0.64	3.37	2.29			
LSD(P=0.05)	2.4	2.8	0.5	0.05	0.24	2.0			

[a] Means for ITA 235, Lac 23 and Moroberekan (30 plants) (results combined)
[b] Scale of 0 (symptomless) to 7 (severe symptoms)

cultivars (Table 3.23). Several studies have also shown that resistant cultivars may show a delay in the appearance of symptoms. For example, Scott *et al*. (1969); Kuhn and Smith (1977) and Anzola *et al*. (1982) found that resistance in maize to maize dwarf mosaic virus (MDMV) was associated with delayed symptom appearance. Bijaisoradat and Kuhn (1985) found that the incubation period of soybean cultivar Jackson resistant to cowpea chlorotic mottle virus was longer than in the susceptible cultivar Davis.

Thus, the increased latent period characteristic of RYMV infection of resistant compared with susceptible rice cultivars has parallels in several virus graminaceous/host combinations. Measurement of rice growth showed, irrespective of virus isolate or resistant cultivar, that plant height of resistant cultivars was only slightly reduced (Table 3.14) and infected rice continued extension growth during the 60 day experimental period (Fig. 3.8), unlike susceptible cultivars where height was significantly reduced 30 days after inoculation (Table 3.4) and extension growth ceased 2 weeks after inoculation (Fig. 3.3). In view of the similarity in plant height between infected and healthy resistant plants it was not surprising that shoot and root fresh and dry weights were generally little affected by any of the five RYMV isolates (Table 3.23). The effect of RYMV isolates on the length of leaf 6 (first leaf to show symptoms) was not significant but the length of the next leaf (leaf 7) was significantly reduced in all resistant cultivars inoculated with the Niger isolate of RYMV. The final leaf, leaf 8 (although the experiment was terminated before this leaf fully expanded) was also significantly reduced in Moroberekan and Lac 23 inoculated (Fig. 3.10) with isolate Nr. Unlike susceptible cultivars, despite the

absence of symptoms on leaf 5 of susceptible cultivars, the growth of this leaf was reduced by all isolates (Fig. 3.5, leaf 5), whereas leaf 5 and 6 of resistant cultivars were not affected (Fig. 3.10).

Concerning differential cultivar effects, the mean scores for the three cultivars tested, irrespective of RYMV isolate, showed that ITA 235 developed the highest symptom severity values in all experiments and there was little difference between cultivars Moroberekan and Lac 23 (Table 3.9).

Disease incidence 60 days after inoculation for the three experiments combined (Table 3.11) shows that half of the inoculated plants of cvs. Moroberekan and Lac 23 eventually showed symptoms, whereas about two-thirds of cv. ITA 235 eventually showed symptoms (Table 3.11).

Leaf severity values for all isolates in the three experiments tended to be greatest on cv. ITA 235 (Table 3.15). Measurements of plant growth revealed that fresh weights of shoots and roots in cv. ITA 235 were more affected than cvs. Moroberekan and Lac 23 (Table 3.17).

When RYMV isolates are compared, RYMV-Nr induced more severe symptoms in the two experiments in which it was included (Table 3.9). For resistant cultivars inoculated with RYMV isolates, most plants (88%) inoculated with RYMV-Nr developed symptoms, whereas very few (21.6%) produced symptoms following inoculation with RYMV-SL (Table 3.11). As far as leaf severity is concerned, RYMV-Nr induced the highest values on leaf 6 and leaf 7 (Fig. 3.10) and RYMV-SL often

tended to cause the mildest symptoms. All cultivars inoculated with RYMV-Nr showed pronounced reductions in the lengths of leaf 7 and 8 compared with other isolates. In experiments 2 and 3 fresh and dry weights of shoots and roots of all resistant cultivars were markedly affected by RYMV-Nr. Summarised data (Table 3.23) shows that infection with RYMV-Nr significantly reduced rice growth and caused the greatest symptom severity and disease incidence. Thus the severe effects of the Niger isolate found in the present study could be related to severe effects seen in the Niger Republic under field conditions. There was an epidemic of RYMV in 1984 and 1985 and the virus continues to limit rice production there (IITA, 1987).

From the results obtained here it is possible that the Niger isolate of RYMV may be more damaging than other isolates and this isolate should be among those included in any breeding and/or screening programme in Africa.

Results suggested that the five cultivars examined could be categorized as follows:

Cultivar	Reaction to RYMV
ITA 212	highly susceptible
BG 90-2	highly susceptible
Moroberekan	highly resistant
Lac 23	highly resistant
ITA 235	moderately resistant

The designation of ITA 235 as moderately resistant and Lac 23 and Moroberekan as highly resistant is not perhaps surprising when it

is recalled that ITA 235 is a hybrid between a lowland Asian cultivar (susceptible) and an African upland cultivar (resistant) whereas Lac 23 and Moroberekan are traditional African upland cultivars. Both susceptible cultivars (ITA 212 and BG 90-2) are lowland Asian cultivars introduced into Africa as part of the continuing replacement of African rice (*O. glaberrima*) by *O. sativa*.

There are many examples which illustrate that resistance is often associated with a delay in the appearance of symptoms, mild symptoms, lower disease incidence and frequently reduced virus content including work on maize and maize dwarf mosaic virus (MDMV) (Kuhn and Smith, 1977), barley and barley yellow dwarf (Jedinski, 1977; Skaria *et al*., 1985) and rice to RYMV (IITA, 1985). Resistance may also involve reduced systemic virus movement as in maize and MDMV (Tu and Ford, 1970; Jones and Tolin, 1972). Therefore, it was considered important to study the nature and possible mechanisms of resistance to RYMV and such work constitutes most of the next section. However, resistance and susceptibility may also be affected or modified by the stage of development when plants are initially infected (e.g. Smith, 1967; Rosenkranz and Scott, 1978; Gregory and Ayers, 1982) and this was examined here as part of the general investigation on interactions between RYMV isolates and rice cultivars using RYMV-N and rice cultivars Lac 23 and ITA 212.

Despite the fact that all susceptible plants tested were infected and produced high symptom severity scores 30 days after inoculation, plants inoculated when very young (3-4 leaf stage) were the most severely affected. Plant height was reduced by 48% when plants were inoculated at the 3-4 leaf stage but only by 13.8% when

inoculation was done at the 6-7 leaf stage. Similarly, the mean incubation periods tended to increase the older the plant at inoculation.

For the resistant cultivar, Lac 23, disease severity and incidence 40 days after inoculation decreased gradually with plant age at inoculation. Although plant height was reduced in plants inoculated at the 3-4 leaf stage, the reduction was very slight compared with the control.

Results are in general agreement with previous work on rice viruses. Ling and Palomar (1967) and Narayanasamy (1972) found that as the age of rice at inoculation with tungro disease was increased, percentage infection and yield losses were progressively reduced. Similarly, Bakker (1974) found that when the susceptible rice cultivar 'Sindano' was inoculated with a Kenyan isolate of RYMV, the adverse effects of infection progressively decreased with increase in plant maturity, whereas in the resistant cultivar Basmati 217, this effect was restricted to plants infected at very young stages.

Plant age at inoculation affected virus content in Lac 23 with more virus being produced in plants inoculated when very young. Similar results have been found for cowpea genotypes inoculated with southern bean mosaic virus at different growth stages (Kuhn *et al.*, 1986; Hobbs and Kuhn, 1987). The increase in virus production in young plants could be associated with symptom severity in those young plants (Fig. 3.11). Association of virus content with symptom severity had been reported earlier. For example, in oat to barley yellow dwarf virus (Jedlinski *et al.*, 1977; Skaria *et al.*, 1985) and

in wheat to wheat streak mosaic virus (Seifers and Martin, 1987).

It has been found that ITA 212 plants inoculated at later stages did not overcome RYMV infection as well as Lac 23 plants inoculated at early stages (3-4 leaf stage) and did not break down resistance to RYMV. Therefore, it is suggested that the best age of rice plants for evaluation and screening to RYMV infection is the 3-4 leaf stage.

S E C T I O N F O U R

THE NATURE AND SOME POSSIBLE MECHANISMS OF RESISTANCE TO RICE YELLOW MOTTLE VIRUS

4.0 THE NATURE AND SOME POSSIBLE MECHANISMS OF RESISTANCE TO RICE YELLOW MOTTLE VIRUS

4.1 INTRODUCTION

Classification of rice cultivars as resistant or susceptible to RYMV-N is based primarily on symptom expression although the extent of virus-induced decreases in plant height and yield have also been taken into account (IITA, 1984; John *et al*., 1985; Fomba, 1988).

The results of the interaction experiments (Section 3) clearly showed that inoculation with five RYMV isolates almost always resulted in the rapid appearance of severe symptoms and total infection of susceptible cultivars. In contrast, using the same inocula and inoculating the same leaf positions, infection of resistant cultivars was usually associated with the slow appearance and development of mild symptoms and a variable proportion of plants which failed to produce symptoms and which indexed RYMV-negative in ELISA tests. Failure of the virus to produce symptoms and antigen in non-inoculated leaves of only some resistant plants could be a reflection of failure to successfully infect a proportion of the inoculated leaves. Equally, it could be that although inoculated leaves were successfully infected, the virus was unable to replicate and move to an extent sufficient to permit transport to and infection of non-inoculated leaves.

It has been shown that resistance in some cereals may be associated with reduced virus titre as for oats to barley yellow dwarf virus (Jedlinski *et al*., 1977), wheat to wheat soil-borne

mosaic virus (Hunger and Sherwood, 1985; Skaria *et al*., 1985) and wheat to wheat streak mosaic virus (Seifers and Martin, 1988). That restricted virus movement may also be involved is illustrated by the work of Tu and Ford (1970), Jones and Tolen (1972) and Lei and Agrios (1986) on maize to maize dwarf virus (MDMV). Resistance to virus infection also has been found in some cereals such as ryegrass to ryegrass mosaic virus (RMV) (Salehuzzaman and Wilkins, 1984), in autumn-sown oat to oat mosaic virus (Catherall and Valentine, 1987) and in rice to rice tungro virus (Hibino *et al*., 1987).

The nature and mechanisms involved in the resistance of rice cultivars to RYMV infection has not been studied. In addition to factors related to RYMV replication and transport, resistance may be associated with inhibitory substances similar to those found by Salehuzzaman and Wilkins (1983) for ryegrass resistant to ryegrass mosaic virus. A further possibility is that sap transmission is less efficient in resistant cultivars due to differences in the morphology of the leaf surface of resistant and suceptible cultivars. Experiments detailed in this section sought to examine these possibilities.

4.2 NATURE OF RESISTANCE TO RYMV IN RESISTANT CULTIVARS

4.2.1 Experiment 1 : Infection and virus antigen distribution in resistant and susceptible plants at various times after inoculation

This experiment was designed to determine firstly, how many plants were truly not infected by testing all leaves individually using ELISA (at various times after inoculation). Secondly, to determine the presence or absence of virus in individual inoculated and non-inoculated leaves 20-40 days after inoculation. Such information may be of value in the efficient diagnosis of infected resistant plants when ELISA serology is used in screening experiments. The third objective was to obtain some information on the relative virus content (as RYMV antigen) in susceptible and resistant plants.

4.2.1.1 MATERIALS AND METHODS

Plant material: Being a typical old upland cultivar, Lac 23 was chosen as representative of resistant cultivars. For comparative purposes, cultivar ITA 212 was used to represent a very susceptible lowland cultivar. After soaking, seeds were sown in vermiculite on 26th July, 1987. After germination, seedlings were transplanted into 10 cm diameter pots with one plant per pot. Glasshouse temperatures ranged from 21-30°C with a daily average of 24-26°C. Supplementary lighting (12 hours) was provided.

Virus isolate: The Nigerian isolate (RYMV-N) was chosen as homologous antiserum was available.

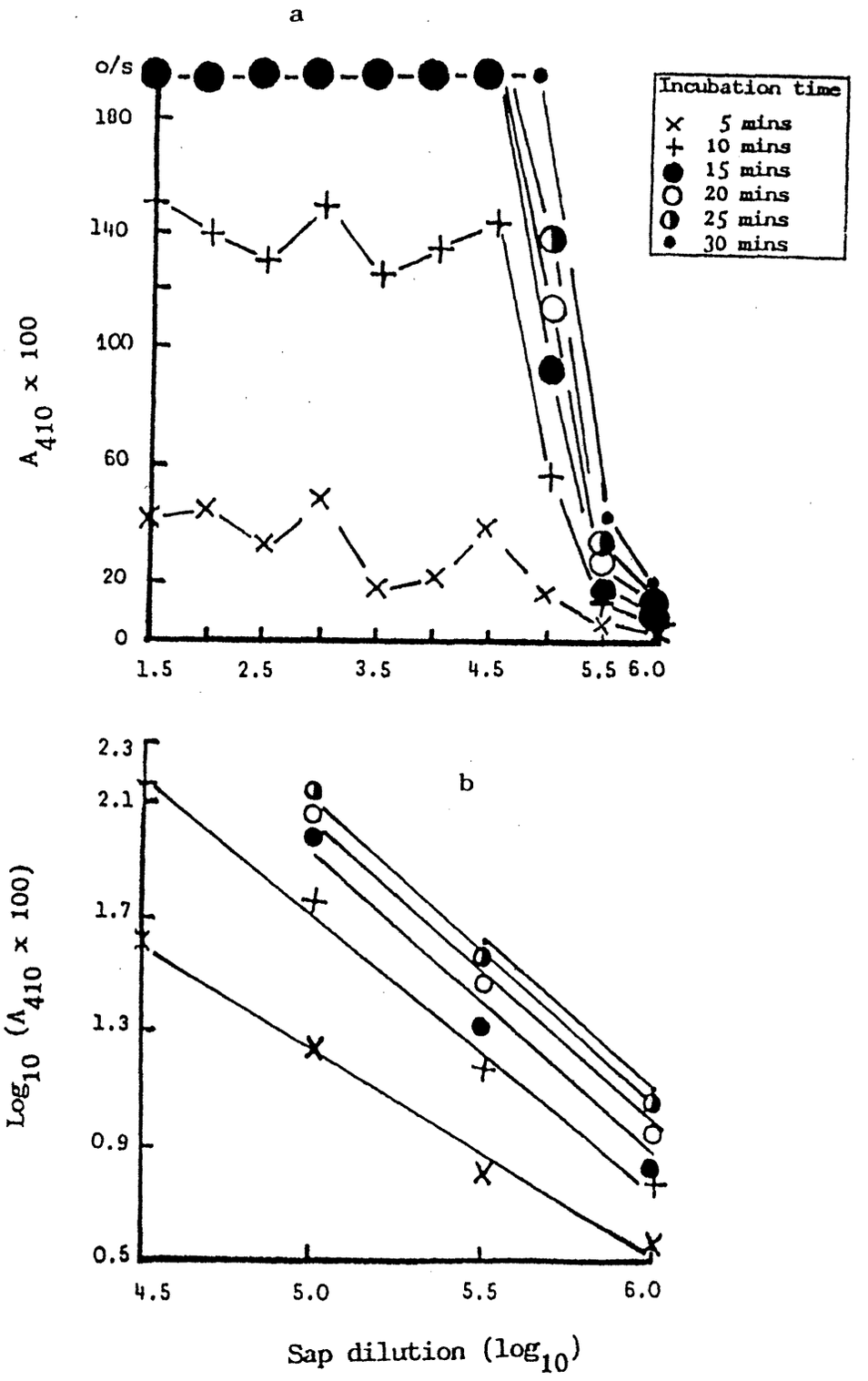
Inoculation procedure: Leaves 1, 2 and 3 of plants at the 4-5 leaf stage were inoculated with RYMV-N by stroking both leaf surfaces with a cotton wool swab dipped in the inoculum. Inoculum (1:10 w/v) was prepared as before. Inoculated leaves were rinsed with distilled water immediately after inoculation.

Harvesting dates: Leaves were harvested 20, 30 and 40 days after inoculation for Lac 23 and 20 days after inoculation for ITA 212. Each leaf of each plant was labelled and stored at -20°C prior to ELISA testing. The three inoculated leaves of each plant were bulked and treated as one sample.

There were 24 replicate plants for each harvest (three harvests for Lac 23, one for ITA 212). The four treatments (plots) were randomly allocated to each of three replicate blocks with 8 replicate plants of each treatment in each block giving a total of 32 plants per block.

Determination of virus concentration: Figure 4.1 shows a representative dose-response curve, typical of the many obtained in the study, where sap from systemically RYMV-N infected leaves of cv. ITA 212 (3 weeks after inoculation) was diluted as serial half-$\log_{10}$ dilution steps and tested using direct ELISA against an alkaline phosphatase conjugate of RYMV antiserum (1:1000 v/v dilution) following coating of the plates with 1 µg/ml globulin. With the ELISA reader used, absorbance (as A_{410} x 100) was linearly related to concentration up to A_{410} x 100 values of *c.* 180; values greater than 200 were off scale (O/S; Fig. 4.1a). A_{410} values plotted in Figure

Fig. 4.1 Absorbance values (A_{410} x 100 nm) at 5 minute intervals obtained by direct ELISA of extracts serially diluted in half $\log_{10}$ steps from RYMV-N infected ITA 212 leaves.



4.1b are for test results 30 minutes after the addition of substrate and for three replicate wells per dilution.

It was repeatedly found that because of the high concentrations of RYMV characteristic of the virus in susceptible rice cultivars (Bakker, 1974), sap dilutions less than about 1:10,000 to 1:100,000 (w/v) for systemically infected leaves 2-3 weeks after inoculation usually resulted in a very rapid production of nitrophenol causing absorbance values to exceed the upper limit detectable by the machine within the usual 30 minute incubation period.

Typical values after 5 and 10 minutes incubation (Fig. 4.1a) showed that absorbance values for $\log_{10}$ sap dilutions of 1.5 to 4.5 were very similar and suggest that at these high virus concentrations (relatively low sap dilutions) the system was 'saturated', i.e. the maximum possible number of enzyme molecules had been trapped by the system resulting, in the presence of excess substrate, in the absence of a quantitative relationship between virus content and absorbance.

For sap dilutions from log 4.5 to log 6.0 (i.e. 1:31622 to 1:1,000,000 w/v dilution) A_{410} values declined with increasing extract dilution. It was not until a sap dilution of $\log_{10}$ 6.0 (1:1,000,000 w/v) or beyond that absorbance values approached those obtained for healthy control sap. Absorbance values "on scale" and significantly greater than control sap background values usually spanned about 3-4 half-$\log_{10}$ dilution steps.

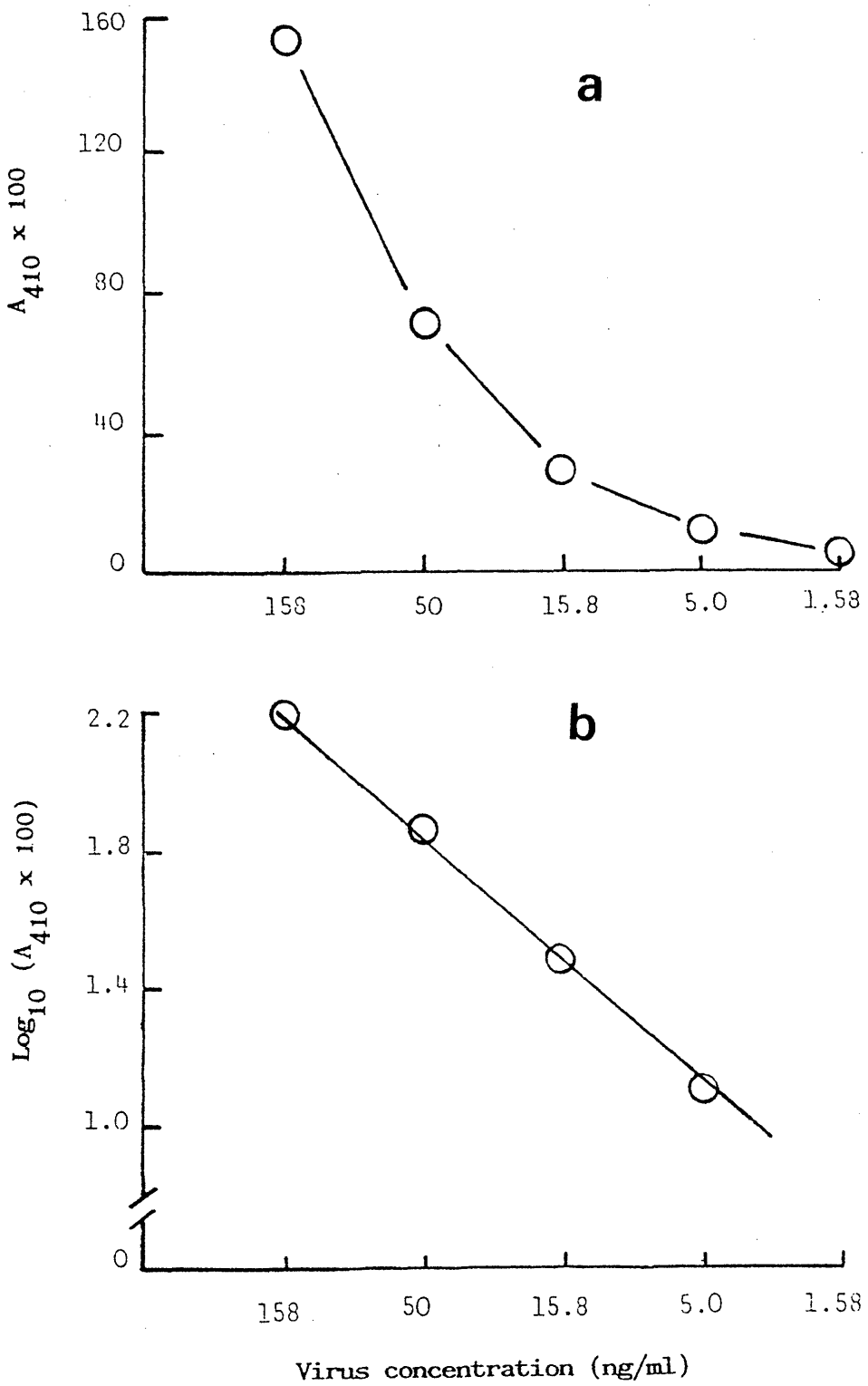
As many points as possible on that part of a curve with values

'on scale' (i.e. below 180) and significantly greater than the control values (the sigmoidal portion of the curve) were then plotted as $\log_{10} A_{410} \times 100$ against $\log_{10}$ sap dilutions to linearise the relationship between absorbance and sap dilutions (virus concentration). These curves from the results in Figure 4.1a are shown in Figure 4.1b, where straight lines have been fitted by linear regression. With the exception of the 5 minute curve, all curves were linear and parallel.

Results emphasized firstly that, at least for sap from susceptible rice cultivars, several dilutions would usually have to be tested and that most of these would have to exceed *c.* 1:10,000 (w/v) unless suceptible rice leaves were tested soon after inoculation, if absorbance values on that part of the dose-response curve proportional to virus concentration were to be used for calculation of virus content. Secondly, it was evident that the relationship between virus content and absorbance could be linearised by plotting $\log_{10} \times A_{410} \times 100$.

Although the relative amounts of virus in different leaf extracts could be determined from the relative dilutions of the sap extract required to give A_{410} values on scale or to a predetermined absorbance value (e.g. Ward *et al.*, 1987) in order to express concentrations in absolute terms e.g. µg/g tissue, calibration curves were needed using known amounts of purified virus. A typical curve for purified RYMV-N is illustrated in Figure 4.2; the method used to purify RYMV-N is detailed in Section 5.2.1. The curves obtained for experiments done on different occasions with various preparations of RYMV-N and with different conjugates were essentially similar to

Fig. 4.2 Dose response curves for ELISA tests on a half-log dilution series of partially purified RYMV-N plotted against absorbance (top) and $\log_{10}$ absorbance (bottom).



those depicted in Figure 4.2.

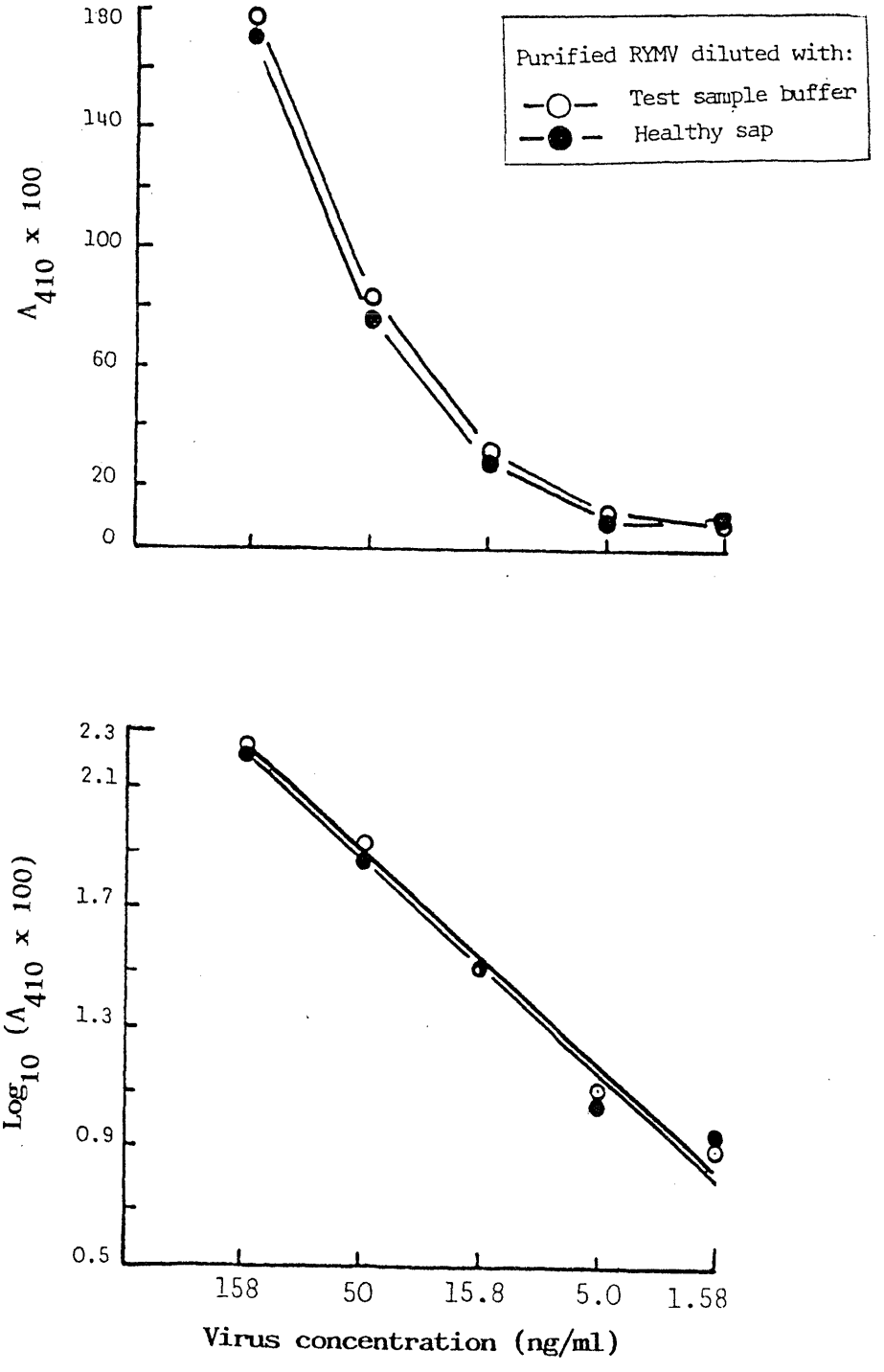
The general shape of the curve was similar to those obtained with rice sap dilutions with values off scale at virus concentration greater than *c.* 500 ng/ml. At 158 ng/ml and below, a sigmoid shaped curve was produced which eventually flattened out as background (buffer only wells) values were approached. The limit of detection was usually 1 to 5 ng/ml. Using half $\log_{10}$ dilution steps, four or five points (spanning a virus concentration range from 158 to 1-5 ng/ml) were usually 'on scale'. Using 'on scale' values as before (Fig. 4.1b) and plotting absorbance as $\log_{10}$ (A_{410} x 100) linear dose response curves were produced (Fig. 4.2b).

As it was necessary to determine virus concentration from crude sap dilutions in test samples, the effect of rice sap on the absorbance values for purified RYMV-N was investigated. The results (Fig. 4.3) show that diluting the stock purified RYMV-N preparation with sap from healthy rice (cv. ITA 212) diluted 1:10 (w/v) with test sample buffer had little effect on the absorbance values for a range of concentrations of purified virus, readings being essentially the same at all virus dilutions tested whether or not sap was added.

This result together with the fact that rice sap was usually diluted well in excess of 1:100 (w/v), led to the use of purified virus diluted in test sample buffer without the addition of healthy sap for the construction of RYMV-N calibration curves.

As a result of the foregoing, the procedure adopted for determination of virus (antigen) concentration in this and other

Fig. 4.3 ELISA dose response curves of a half-$\log_{10}$ dilution series of purified RYMV-N diluted with TSB or healthy (cv. ITA 212) rice sap plotted using absorbance (top) or $\log_{10}$ absorbance (bottom)



succeeding experiments was as follows. In most instances three replicate microtitre plate wells were used per rice sap test sample. This usually meant that treatments (including dilutions) exceeded the wells available on a single plate. Therefore, each of three plates was regarded as a block and each treatment (test sap extract) was allocated to one well on each of the three plates. The three replicate wells (one in each block) were separately randomly allocated to a given treatment within each block. On each plate at least three wells were allocated to the negative control (healthy sap) and one well to each of usually five concentrations of purified RYMV (usually 1.58, 5.0, 15.8, 50 and 158.0 ng/ml, i.e. a half $\log_{10}$ dilution series). A calibration curve was then constructed from the mean for the three replicate wells for each virus concentration and plotted as $\log_{10}$ (A_{410} x 100) against $\log_{10}$ RYMV concentration. The line of best fit was calculated by the method of least squares. Occasionally, intermediate concentrations were included to ensure that a minimum of five points were used in the construction of the standard calibration curve.

Test extracts were tested at an appropriate range of dilutions (usually as consecutive 10-fold steps) and the dilution giving an absorbance value nearest the mid-point of the linear dose-response curve was used to calculate the virus concentration of the diluted sample from the calibration curve using linear regression to estimate x (virus concentration for the experimental value determined) from y ($\log_{10}$ absorbance). After adjustment for sap dilution, final values were expressed as amount of virus/gram fresh weight of leaf tissue. Within an experiment, incubation times were standardized as either 30 or 60 minutes.

4.2.1.2 RESULTS

Detection of RYMV-N: Although inoculated plants of cv. ITA 212 showed symptoms in leaves 6 and 7 only at harvest (20 days after inoculation), all inoculated and non-inoculated leaves were RYMV-N positive in ELISA tests (Table 4.1). In the resistant cultivar, Lac 23, the situation was quite different for plants harvested 20 days after inoculation. RYMV-N was detected in only 45.8% of the plants inoculated, in 45.8% of inoculated leaves, 29.1% of the non-inoculated leaves 4, 5 and 6, and in 35.9% of leaf 7 (Table 4.1). In no instance was a non-inoculated leaf RYMV positive and the corresponding bulked inoculated leaf sample RYMV negative. Similarly, in plants harvested after 30 days, only half of the inoculated leaves were successfully infected and only 37.5% of the non-inoculated leaves tested RYMV positive. For Lac 23 plants tested 40 days after inoculation, inoculated leaves and leaf 4 had senesced and again, only just over half of the non-inoculated leaf extracts reacted positively in ELISA tests. Results presented in Table 4.1 show clearly that for Lac 23 the proportion of inoculated leaves successfully infected was consistently more than the proportion of non-inoculated leaves in which the virus had moved systemically when tested 20 and 30 days after inoculation. RYMV-N was detected with increased frequency at all leaf positions tested in the separate batches of Lac 23 plants harvested at 20, 30 and 40 days after inoculation (Table 4.1). Thus, for example, in Lac 23 leaf 6 the proportion of plants in which RYMV was detected increased from 29.1% after 20 days to 37.5% after 30 days and reached 58.3% 40 days after inoculation. The results also showed that in the resistant cultivar, Lac 23, the presence of virus in the inoculated leaves was not always

Table 4.1 Proportion (%) of inoculated and non-inoculated leaves of cv. ITA 212 (20 days after inoculation) and cv. Lac 23 (20, 30 and 40 days after inoculation) which indexed RYMV positive by ELISA.

Cultivar/ Days after inoculation	Leaf position[a]					
	Inoculated	Non-inoculated				
	Leaves 1, 2 & 3	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
ITA 212 : 20	100	100	100	100	100	[d]
Lac 23 : 20	45.8	29.1	29.1	29.1	31.6[c]	[d]
Lac 23 : 30	50	37.5	37.5	37.5	37.5	37.5
Lac 23 : 40	[b]	[b]	58.3	58.3	58.3	58.3

[a] Each treatment consisted of 24 plants

[b] Leaves senesced before harvest

[c] Nineteen plants in which leaf 7 had emerged

[d] Leaf had not emerged

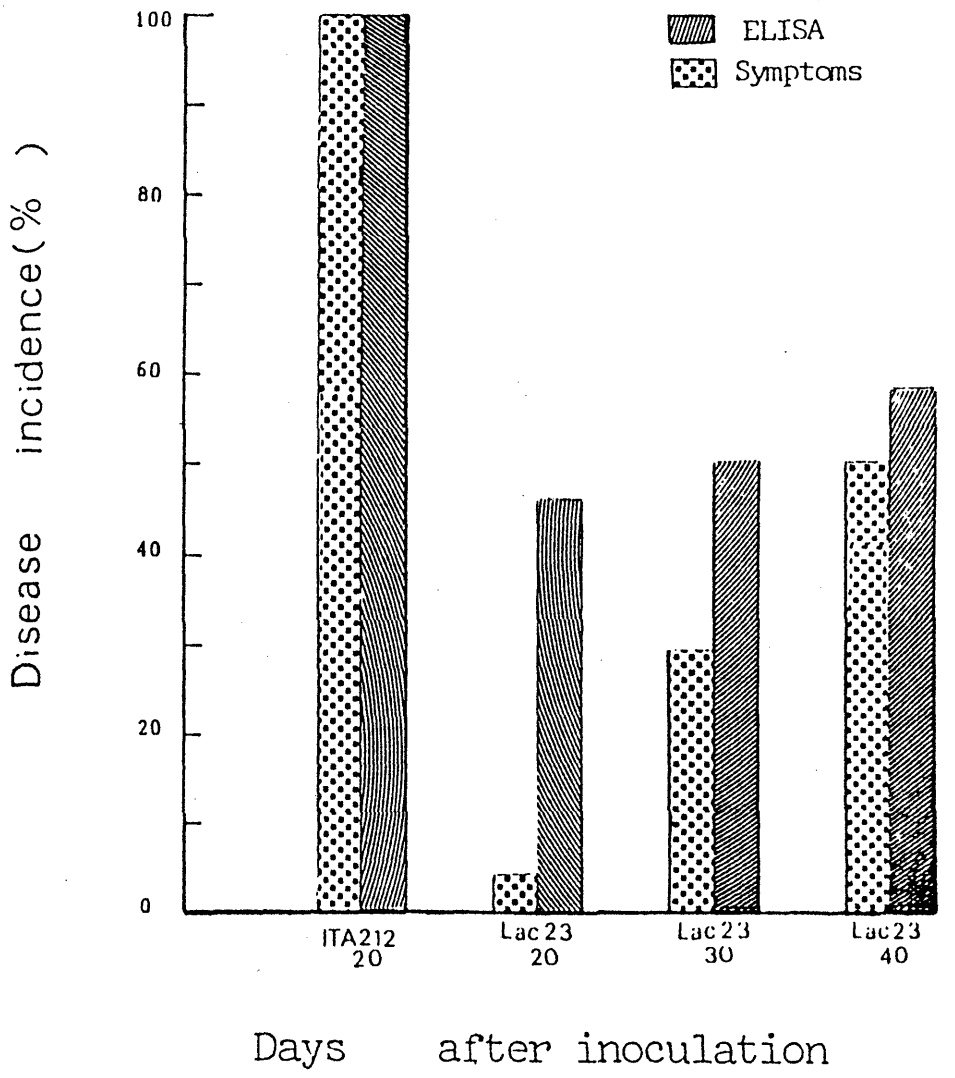
associated with subsequent systemic invasion whereas the existence of RYMV in the young leaves was always associated with the presence of RYMV in all inoculated and non-inoculated leaves (Table 4.1). The high proportion of RYMV antigen in leaf 7 of Lac 23 plants after 20 days (Table 4.1) was probably influenced by the absence of leaf 7 in some of the inoculated plants at this time.

Disease and antigen incidence: All 24 inoculated cv. ITA 212 plants showed conspicuous symptoms 20 days after inoculation. In Lac 23 the number of plants showing symptoms increased with time, being 4% after 20 days and increasing to 50% after 40 days (Fig. 4.4).

Based on ELISA tests, the proportion of Lac 23 plants containing RYMV antigen was 45.8% after 20 days increasing slightly to 58.3% after 40 days; values considerably in excess of those based on symptoms only after 20 and 30 days (Fig. 4.4). This showed that some symptomless plants, even at 40 days after inoculation, were RYMV positive. It is possible that these plants may have developed symptoms later. Clearly, even at 40 days, some inoculated Lac 23 plants did not contain RYMV antigen in inoculated and non-inoculated leaves or there was insufficient antigen to cause a positive response with ELISA. All plants with symptoms in the susceptible cultivar (ITA 212) indexed RYMV positive.

Virus concentration: After RYMV-positive plants had been identified by preliminary ELISA tests, 0.2 ml of each RYMV-positive extract from each leaf for each treatment was pooled and tested at several dilutions to determine the average amount of viral antigen in each infected leaf using the method detailed in the materials and methods

Fig. 4.4 Incidence of RYMV-N as evidenced by ELISA tests and the presence of symptoms in inoculated susceptible (cv. ITA 212) and resistant (cv. Lac 23) rice cultivars.



of this section. Results (Table 4.2) show the absorbance means from pooled leaf extracts which reacted RYMV positive only. The highest absorbance values were obtained with ITA 212 leaf extracts 20 days after inoculation, whereas the lowest values were encountered with Lac 23 leaves 20 days after inoculation. Absorbance was greater in young than in old leaves; for example, in ITA 212 absorbances for extracts from leaves 6 and 7 at 10^{-4} dilution were high and off-scale, whereas in leaves 4 and 5 absorbances were on-scale (Table 4.2). Virus amounts were calculated using calibration values from an appropriate test extract dilution indicated in Table 4.2.

Results (Table 4.3) showed that the amount of RYMV in inoculated leaves of ITA 212 harvested 20 days after inoculation was greater than in non-inoculated symptomless leaves at position 4, slightly less than in the symptomless leaf 5 but substantially less than in those non-inoculated leaves which showed symptoms (leaves 6 and 7).

In Lac 23 plants, the amount of RYMV-N in inoculated leaves tested 20 days after inoculation was very low and only just detectable in the leaf extract diluted 1:100 (Table 4.2). The amount present was less than in inoculated leaves tested after 30 days. Similarly, in non-inoculated leaves, irrespective of leaf position, highest amounts of RYMV antigen were found in leaves harvested 40 days after inoculation with low amounts present in non-inoculated leaves harvested after 20 days (Table 4.3).

Although, as stated above, RYMV antigen was detected in Lac 23 plants tested 20 days after inoculation, the amount of virus in

Table 4.2 Mean absorbance values (A_{410} x 100) from ELISA tests on extracts from inoculated and non-inoculated leaves of RYMV inoculated rice cv. ITA 212 (20 days) and cv. Lac 23 (20, 30 and 40 days after inoculation)

Cultivar	Harvest (Days after inoculation)	Reciprocal of sample dilution(s)	Absorbance A_{410} x 100 — Leaf tested[a]					
			Inoculated	Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
ITA 212	20	10^{-2}	O/S (21)[c]	O/S (24)	O/S (24)	O/S (24)	O/S (24)	NP
		10^{-3}	O/S	160±1.7	O/S	O/S	O/S	
		10^{-4}	136±0.6[b]*	25±0.3	142±4.1*	O/S	O/S	
		10^{-5}	22±0.9	7±0.6	22±0.9	47±1.4*	66±0.6*	
Lac 23	20	10^{-2}	40±0.6*	16±0.6	26±0.3(7)*	22±0.6(7)*	16±0.3(6)*	NP
Lac 23	30	10^{-2}	160±1.1	133±2.9(8)*	O/S (8)	O/S (8)	O/S (8)	O/S (7)
		10^{-3}	30±0.6*	19±0.9	65±1.7*	70±1.7*	140±1.7*	159±2.9*
Lac 23	40	10^{-2}	NP	NP	O/S (6)	O/S (14)	O/S (14)	O/S (14)
		10^{-3}	NP	NP	79±3.5*	93±2.9*	144±1.1*	169±2.3*
Healthy sap ITA 212		10^{-2}	5.6±0.1					

* Value from which virus amount was calculated
a RYMV-positive leaf extracts of each treatment were pooled
b Absorbance measured 30 minutes after the addition of substrate; mean and standard error of the mean
c Values in parentheses show number of leaf extracts pooled
NP Leaf not present
O/S Off scale

Table 4.3 RYMV (antigen) content of inoculated and non-inoculated rice leaves (cvs. ITA 212 and Lac 23) at various times after inoculation

Rice cultivar	Harvest (days after inoculation)	Virus content µg/g fresh weight)[a]					
		Inoculated leaves	Non-inoculated leaves				
			Leaf 4	Leaf 5	Leaf 6	Leaf 7	Leaf 8
ITA 212	20	1259±60b	153±1.1	1329±45.0	2880±115.0	4649±58.8	NP
Lac 23	20	2±0.0	0.6±0.01	1±0.01	1±0.01	0.6±0.0	NP
Lac 23	30	16±0.1	12±0.1	44±1.7	51±1.7	128±2.6	154±3.5
Lac 23	40	NP	NP	68±3.5	74±3.5	132±1.1	168±3.0

[a] [b] Values calculated from results summarized in Table 4.2
Calculated virus content ± standard error of the mean
NP Leaf not present

inoculated and non-inoculated leaves was very low compared with virus content 30 and 40 days after inoculation (Table 4.3).

In Lac 23 plants harvested 30 days after inoculation where inoculated and non-inoculated leaves were tested, the amount of RYMV in inoculated leaves was slightly greater than in the non-inoculated leaf 4 but less than non-inoculated leaves 5, 6, 7 and 8, where amounts progressively decreased with leaf age such that highest amounts were found in the youngest leaf (leaf 8). A similar trend was apparent for Lac 23 plants harvested 40 days after inoculation.

Comparing susceptible and resistant cultivars, the amount of RYMV at 1259 µg/g tissue in inoculated leaves of ITA 212 after 20 days was 19.7 times higher than the 15.8 µg/g tissue calculated for inoculated leaves of Lac 23 after 30 days. Similarly, in non-inoculated leaves, ITA 212 had consistently substantially higher amounts of RYMV-N than Lac 23. For example, after 20 days leaf positions 5 and 7 of ITA 212 contained 20 and 35 times more RYMV antigen than the same leaf positions of Lac 23 harvested after 40 days.

4.2.2 Experiments 2 and 3. RYMV movement and accumulation in resistant and susceptible rice plants

The results of the previous experiment (experiment 1, section 4) showed that ELISA tests failed to detect RYMV antigen in a proportion of inoculated resistant plants and that the presence of virus in inoculated leaves was not always associated with subsequent systemic invasion with or without symptoms. Results also showed that after 20 days some symptomless non-inoculated leaves of Lac 23 plants indexed positive for RYMV and that low amounts of RYMV antigen were detected in such plants. It was considered important to investigate further the relationship between infection of inoculated leaves and any subsequent systemic spread of the virus in resistant compared with susceptible cultivars. Two experiments were therefore made with the following interrelated objectives. Firstly, to determine the amount of RYMV antigen accumulating in infected inoculated leaves in relation to the time after inoculation. Secondly, to relate this to subsequent virus movement into non-inoculated leaves and, eventually, symptom production. Thirdly, to determine the amount of virus produced in inoculated and non-inoculated tip leaves in resistant and susceptible cultivars at different times after inoculation, and lastly to establish the time required for RYMV-N to move out of inoculated leaves into non-inoculated leaves.

5.2.2.1 MATERIALS AND METHODS

Plant materials: Seeds of the susceptible cultivar ITA 212 and resistant cultivars Lac 23, Moroberekan and ITA 235 were sown on 23rd January, 1988 (experiment 2) and seeds of ITA 212, Lac 23 and ITA 235

on 18th August, 1988 (experiment 3). Seedlings (1-2 leaf stage) were transplanted on 5th February, 1988 (experiment 2) or 26th August, 1988 (experiment 3) into 10 cm diameter plastic pots with two plants per pot (experiment 2) or one plant per pot (experiment 3). Supplementary lighting (12 hours) was supplied in experiment 2 but not experiment 3. Glasshouse temperatures ranged from 22-30°C (experiment 2) or 23-33°C (experiment 3) with a daily mean of 24-26°C throughout the two experiments.

Virus isolate: The Nigerian isolate of RYMV was used.

Mechanical inoculation: Frozen leaves of cv. ITA 212 infected with RYMV-N and harvested 3 weeks after inoculation were used for inoculum preparation. Inoculum was made as before (1:10 tissue:buffer ratio) and applied to leaves 2 and 3 (experiment 2) or leaf 3 (experiment 3) of uniform 4-5 leaf stage plants by wiping three times on both sides of the lamina using a cotton wool swab dipped into inoculum. Plants were rinsed with distilled water immediately after inoculation.

Experimental design: In experiment 2, there were twenty treatments (4 cultivars x 5 harvests) with five replicated pots containing two plants per pot, thus giving 10 plants per treatment. In experiment 3, there were fifteen treatments (3 cultivars x 5 harvests) and 20 replicate plants per treatment (1 plant per pot). In both experiments, treatments (plots) were arranged according to a randomized complete block design using five blocks. Blocks were arranged on the glasshouse benches immediately after inoculation.

Leaf collection: For virus accumulation and movement purposes all

inoculated and the youngest non-inoculated exerted tip leaf of each plant were collected and stored at -20°C. Leaves were harvested 5, 10, 15, 20 and 30 days after inoculation experiment 2) or 1, 2, 3, 5 and 12 days after inoculation (experiment 3). Plants from which leaf material had been removed were allowed to continue growing and those which developed symptoms were recorded. Additional samples from the youngest leaf of all plants were collected after 30, 45 and 90 days in experiment 2. Before terminating the experiment, some inoculated plants of experiment 2 showing symptoms and other symptomless plants of the resistant cultivars were kept, allowed to flower and produce seed and the seed collected (see experiment 5, section 4).

ELISA tests: The entire inoculated leaves were harvested separately from each plant as was the youngest non-inoculated leaf. ELISA indexing and virus concentration determinations were essentially as detailed earlier for experiment 1 (section 4.2.2.1).

4..2.2.2 RESULTS (Experiment 2)

Virus antigen levels were measured using ELISA. Inoculated and the youngest leaves of resistant and susceptible cultivars were collected at 5 day intervals from 5-30 days after inoculation and also 30, 45 and 90 days after inoculation for the youngest leaf only. On each occasion, each harvested leaf from each plant was individually diluted 1:100 (w/v) in test sample buffer (TSB). Leaves of 'mock' inoculated controls were treated similarly.

Determination of RYMV-N in inoculated leaves: Table 4.4 shows that both inoculated leaf 2 (L2) and inoculated leaf 3(L3) of susceptible

Table 4.4 Detection of RYMV by ELISA in inoculated leaves of resistant and susceptible cultivars at intervals after inoculation.

Sampling time (Days after inoculation)	Cultivar	Inoculated leaves tested				
		L2 only[a]	L3 only[a]	Both L2 and L3	L2,L3 or both	RYMV not detected
5	Lac 23	3[b]	0	0	3	7
	Moroberekan	3	0	1	4	6
	ITA 235	0	0	7	7	3
	All resistant	6	0	8	14	16
	ITA 212	0	0	10	10	0
10	Lac 23	1	3	2	6	4
	Moroberekan	2	1	2	5	5
	ITA 235	0	1	8	9	1
	All resistant	3	5	12	20	10
	ITA 212	0	0	10	10	0
15	Lac 23	1	4	4	9	1
	Moroberekan	1	3	5	9	1
	ITA 235	0	0	10	10	0
	All resistant	2	7	19	28	2
	ITA 212	0	0	10	10	0
20	Lac 23	2	1	6	9	1
	Moroberekan	1	5	3	9	1
	ITA 235	1	0	9	10	0
	All resistant	4	6	18	28	2
	ITA 212	-[d]	-	-	-	-
30	Lac 23	-	9	-	9	1
	Moroberekan	-	8	-	8	2
	ITA 235	-	10	-	10	0
	All resistant	-	27	-	27	3
	ITA 212	-	-	-	-	-

[a] L2 = leaf 2; L3 = leaf 3

[b] Number of plants out of 10 (or 30 for all resistant cultivars combined) in which RYMV was detected in the leaf or leaves indicated.

[c] Number of plants out of 10 (or 30 plants) in which RYMV was not detected in both inoculated leaves.

[d] Leaves not present

cv. ITA 212 plants were successfully infected by RYMV-N as evidenced by a positive reaction in ELISA tests for leaves tested 5, 10 and 15 days after inoculation; inoculated leaves had senesced after 20 and 30 days. A more complex picture emerged for the three resistant cultivars tested. A variable number of inoculated leaves contained sufficient RYMV antigen to evoke a positive reaction in ELISA tests, the number being influenced mainly by the cultivar and the time after inoculation when inoculated leaves were tested. Considering all three resistant cultivars together and results for 5 to 20 days after inoculation inclusive, 120 leaves at position 2 and position 3 were inoculated and of these, virus was detected in 15 inoculated leaf 2 only and 18 inoculated leaf 3 only, so there was no clear effect of leaf position on susceptibility to infection when only one of the two leaves inoculated became infected. Both leaf 2 and 3 were infected in 57 plants and the likelihood of both inoculated leaves being infected increased with time after inoculation. If it is considered that, within the limits of detection by ELISA, a plant was successfully infected if RYMV antigen was detected in one or both inoculated leaves, then the number of such infected resistant plants increased with time after inoculation, presumably reflecting a progressive increase in virus accumulation and therefore the likelihood of detection; values (out of 30 inoculated plants per harvest) were 14, 20, 28, 28 and 27 for 5, 10, 15, 20 and 30 days after inoculation. Thus based on the later, and possibly more reliable samples, almost all resistant plants were successfully infected in that RYMV-N had accumulated to levels detectable in the inoculated leaf or leaves.

Considering differences between the three resistant cultivars

used, it appears from Table 4.4 that for cv. ITA 235 it is likely that all plants were successfully infected but that after 5 and 10 days there was insufficient virus accumulation to elicit a positive response in ELISA in 3 plants and 1 plant respectively. Similar trends were apparent for cvs. Lac 23 and Moroberekan but here total infection was not detected even at 20 or 30 days after inoculation, and infection was detected on fewer occasions in both inoculated leaves than found for cv. ITA 235.

Detection of RYMV-N in non-inoculated young leaves: Results for ELISA tests with extracts of the young tip leaf from resistant cultivars showed that the virus was undetectable in the youngest non-inoculated leaf 5 days after inoculation whereas after 10 and 15 days 2 and 4 plants respectively (out of 30) were RYMV positive (Table 4.5). However, even after 30 days less than half of the inoculated plants (12 out of 30) contained a detectable amount of virus. For cvs. Lac 23 and Moroberekan, virus was not detected in any samples after 10 days and in only two plants (one of each cultivar) after 20 days. In contrast, for cv. ITA 235 some samples induced an RYMV positive response after 10 days and numbers increased with time reaching 8 out of 10 plants 30 days after inoculation (Table 4.5). With the susceptible cv. ITA 212, one plant was RYMV positive after 5 days then, as expected, the number increased rapidly reaching 8 out of 10 after 10 days and thereafter all plants were systemically infected.

Virus content of inoculated leaves: In the resistant cultivars three particular considerations were taken into account when estimating virus content from ELISA absorbance readings. Firstly, in samples tested soon after inoculation both inoculated leaves of some plants

Table 4.5 Detection of RYMV-N by ELISA in the youngest leaf of resistant and susceptible cultivars at intervals after inoculation

Cultivar		Number of plants (out of 10) reacting RYMV positive[a]				
		Days after inoculation				
		5	10	15	20	30
Resistant	Lac 23	0	0	1	1	1
	Moroberekan	0	0	1	1	3
	ITA 235	0	2	2	6	8
	All resistant cvs.	0	2	4	8	12
Susceptible	ITA 212	1	8	10	10	10

[a] Extract dilution 1:100 (w/v)

indexed RYMV negative but some of these plants later produced symptoms and were therefore clearly infected. Secondly, one or both inoculated leaves of many plants indexed RYMV positive but some plants were not subsequently systemically invaded and failed to develop symptoms. Lastly, both inoculated leaves of some plants indexed RYMV negative and did not produce symptoms later.

Clearly, ELISA readings from all inoculated leaves cannot be used to obtain a mean value for virus content because a variable, although small, number of plants although inoculated were not infected and readings from extracts of such plants would have a variable influence on the mean value if the mean is calculated from all inoculated plants. Thus, readings for those plants where both inoculated leaves indexed RYMV negative and the plants failed to produce symptoms within 60 days after inoculation were excluded, where one inoculated leaf indexed RYMV positive and the other RYMV negative, for convenience the leaf which indexed negative was considered infected and the A_{410} value obtained was included in the calculation of mean absorbance. Thus, absorbance readings were taken from extracts from all plants considered infected (i.e. a positive ELISA value for at least one of the two inoculated leaves) in arriving at a mean absorbance value for each or both inoculated leaves. In ITA 212 extracts of both leaves of all inoculated plants were RYMV positive and subsequently all plants showed systemic symptoms so the calculation of mean absorbance values was straightforward.

Amounts of RYMV-N in inoculated leaves of resistance cultivars Lac 23, Moroberekan and susceptible ITA 212 are shown in Table 4.6.

Table 4.6 Accumulation of RYMV-N (µg/g tissue) in successfully infected inoculated leaves 2 and 3 and the youngest non-inoculated leaf of RYMV resistant and susceptible rice cultivars at various times after inoculation

Cultivar and leaf tested	Time sampled (days after inoculation)						
	5	10	15	20	30*	45	90
Lac 23							
Leaf 2	ND (8)[a]	0.5±0.01[b](7)	1.0±0.1(10)	8.0±0.1(9)	-		
Leaf 3	ND (8)	0.5±0.01(7)	3.0±0.2(10)	5.0±0.6(9)	24.0±1.7(9)		
Youngest	ND (7)	ND (5)	ND (7)	0.3±0.0(3)	25.0±1.6(26)	328±13(26)	292±21(26)
Moroberkan							
Leaf 2	ND (7)	0.6±0.05(7)	1.0±0.0(10)	3.5±0.03(9)	-		
Leaf 3	ND (7)	0.8±0.05(7)	2.0±0.05(10)	6.0±0.05(9)	24.0±3.0(8)		
Youngest	ND (5)	ND (4)	ND (6)	0.3±0.0(5)	28.0±4.0(25)	645±58(25)	360±10(25)
ITA 235							
Leaf 2	ND (9)	2.5±0.02(10)	10.0±0.8(10)	12.0±0.3(10)	-		
Leaf 3	ND (9)	7.0±0.05(10)	12.0±0.05(10)	56.0±1.3(10)	175.0±2.0(10)		
Youngest	ND (7)	ND (9)	ND (8)	16.0±0.2(7)	152.0±1.0(41)	1128±86(41)	761±51(41)
ITA 212							
Leaf 2	1.0±0.05(10)	15.0±1.0(10)	42.0±0.3(10)				
Leaf 3	1.0±0.03(10)	24.0±0.6(10)	269.0±12.9(10)				
Youngest	ND (10)	81.0±7.0(10)	587±37.0(10)	4220.0±312(10)	6410±182(10)	2850±294(5)	1590±147(5)

[a] Values in parentheses show the number of leaf extract samples pooled. For leaves 2 and 3 extracts pooled from plants showing symptoms or RYMV antigen in one or both inoculated leaves. For tip leaves, extracts pooled from plants showing symptoms 60 days after inoculation

[b] Mean virus content (µg/g tissue) ± standard error of the mean; Absorbance measured 30 minutes after the additional substrate

\- Leaf 2 senesced

ND Not detected; absorbance value less than twice that of control sap

* Note : There were 10 replicate inoculated plants per cultivar for each of the five harvests from 5 to 30 days after inoculation. For samples of tip leaves there was a maximum of 10 leaves/cultivar available for harvest at 5 to 20 days after inoculation. For harvests 30, 45 and 90 days youngest leaves were taken from each plant at the batches of 10 allocated to the five harvests (5 to 30 days) giving a maximum total of 50 replicate samples for the last three harvests of tip leaves

RYMV-N was not detected in either of the two inoculated leaves after 5 days in all three resistant cultivars. There was a gradual increase in virus content in both inoculated leaves of all cultivars with time after inoculation. The amount of RYMV-N in leaves of ITA 235 was consistently higher than in Lac 23 or Moroberekan irrespective of time after inoculation but less than the amount detected in ITA 212 (Table 4.6). By 15 days, an appreciable amount of RYMV had accumulated in cv. ITA 235 and particularly in ITA 212, whereas amounts were negligible and only just detectable in Lac 23 and Moroberekan. Even after 30 days, the amounts of RYMV in the three resistant cultivars were substantially less than in susceptible ITA 212 by 15 days. There was a tendency for amounts to be greater in leaf 3 than leaf 2 for all cultivars.

Virus content of the youngest non-inoculated leaf: In determining the virus content of non-inoculated leaves, similar considerations were taken into account as detailed above for inoculated leaves in that readings from extracts from all plants which failed to produce systemic symptoms within 60 days after inoculation were excluded in obtaining mean absorbance readings for virus content calculation. Leaf extracts from those plants which showed symptoms were pooled and tested by ELISA as in experiment 1 (section 4.2.1).

RYMV was not detected in the youngest leaves tested after 5 to 15 days in Lac 23, Moroberekan and ITA 235 (Table 4.6). As for inoculated leaves, Lac 23 and Moroberekan had similar amounts of RYMV in the youngest leaf also. Virus concentrations in the tip leaves of ITA 235 were consistently greater than in Lac 23 and Moroberekan at 20, 30, 45 and 90 days after inoculation. With ITA 235 extracts

yielded up to *c.* 700-1100µg virus/g tissue, about twice the amounts found in Moroberekan and Lac 23. RYMV content progressively increased in tip leaves of all three resistant cultivars from first detection at 20 days to a peak at 45 days, contents then declined after 90 days. Virus content with time showed a similar trend in susceptible ITA 212 with a maximum value at 30 days. Values were exceedingly high in ITA 212 tip leaves with virus contents in excess of 1.5 mg/g tissue 20 to 90 days after inoculation and a peak value of over 6 mg/g tissue 30 days after inoculation. Such values were considerably in excess of those found in resistant cultivars after 20 and 30 days but differences were reduced to a factor of 2 to 5 after 45 and 90 days. The highest virus contents encountered in resistant plants after 45 and 90 days were usually within the range of 0.3 to 1.0 mg/g tissue which, although less than in susceptible ITA 212, still represent substantial amounts of virus.

Relationship between virus detection in inoculated leaves and systemic infection

Irrespective of sampling date, some inoculated resistant plants in which virus was detected in the inoculated leaves failed to become systemically infected (Table 4.7). This phenomenon was observed in all resistant cultivars even when inoculated leaves were removed and indexed 20 or 30 days after inoculation when virus detection in inoculated leaves was probably most reliable (Table 4.7). In contrast, for inoculated leaf samples taken 5, 10 or 15 days after inoculation, some plants in which virus was not detected in the inoculated leaves produced new leaves which eventually developed systemic symptoms. The number of such plants progressively

Table 4.7 Development of systemic symptoms by 60 days after inoculation in RYMV-N inoculated resistance cultivars whose inoculated leaves were removed and ELISA indexed at various times after inoculation

Removal of inoculated leaves (days after inoculation)	Cultivar	Virus detected in inoculated leaves: No. of plants (out of 10) in which virus detected	Virus detected in inoculated leaves: No. of plants showing subsequent systemic symptoms	Virus detected in inoculated leaves: No systemic symptoms developed	Virus not detected in inoculated leaves: No. (out of of 10) in which virus not detected	Virus not detected in inoculated leaves: Plants showing subsequent systemic symptoms	Virus not detected in inoculated leaves: No systemic symptoms developed
5	Lac 23	3	1	2	7	6	1
	Moroberekan	4	2	2	6	3	3
	ITA 235	7	5	2	3	2	1
	Combined resistant	14	8	6	16	11	5
10	Lac 23	7	4	3	3	1	2
	Moroberekan	5	2	3	5	2	3
	ITA 235	9	8	1	1	1	0
	Combined resistant	21	14	7	9	4	5
15	Lac 23	9	6	3	1	1	0
	Moroberekan	9	5	4	1	1	0
	ITA 235	10	8	2	0	0	0
	Combined resistant	28	19	9	2	2	0
20	Lac 23	9	3	6	1	0	1
	Moroberekan	9	4	5	1	0	1
	ITA 235	10	7	3	0	0	0
	Combined resistant	28	14	14	2	0	2
30	Lac 23	9	4	5	1	0	1
	Moroberekan	9	5	4	1	0	1
	ITA 235	10	10	0	0	0	0
	Combined resistant	28	19	9	2	0	2

decreased as inoculated leaves were removed from 5 to 15 days after inoculation and when inoculated leaves were removed after 20 and 30 days, all plants considered not infected (both inoculated leaves indexed RYMV negative), as expected, did not produce systemic symptoms. The number of plants in which both inoculated leaves were RYMV negative progressively decreased with sample age (days after inoculation) reflecting the increased reliability of detection. The number of plants with infected inoculated leaves and which gave rise to systemic infection was consistently greater in cv. ITA 235 than in cv. Moroberekan and Lac 23, irrespective of the time of removal of inoculated leaves. Thus, for example 20 days after inoculation, the number of infected plants (inoculated leaves gave positive ELISA values) for cvs. Lac 23, Moroberekan and ITA 235 was 9, 9 and 10 plants respectively, and the number of systemically infected plants was 3, 4 and 7 respectively (Table 4.7).

Relationship between leaf removal and subsequent systemic symptoms: Irrespective of the time of removal of inoculated leaves of susceptible cv. ITA 212 all plants developed systemic symptoms by 30 days after inoculation (Table 4.8). In the resistant cultivars Lac 23 and Moroberekan, there was no consistent effect of removal date of inoculated leaves on the number of plants which produced systemic symptoms irrespective of the time of inoculated leaf removed. The situation was similar for cv. ITA 235 except that slightly more (*c.* 80%) inoculated plants developed systemic symptoms (Table 4.8).

Disease development: Symptoms were first seen in a few plants of each resistant cultivar 27 days after inoculation. Most inoculated plants which were going to develop systemic symptoms had done so 25-35 days

Table 4.8 Numbers of plants which developed systemic symptoms after inoculation with RYMV-N and subsequent removal of inoculated leaves at different times after inoculation.

Rice cultivar	Number of plants showing systemic symptoms (out of 10)[a]					
	Days between inoculation and removal of inoculated leaves					
	5	10	15	20	30	Mean
Lac 23	7	5	7	3	4	5.2
Moroberekan	5	4	6	5	5	5.0
ITA 235	7	9	8	7	10	8.2
Mean (resistant)	6.3	6.0	7.0	5.0	6.3	6.1
ITA 212	10	10	10	10	10	

[a] Systemic symptoms 30 and 60 days after inoculation for susceptible and resistant cultivars, respectively.

after inoculation (Fig. 4.5); thus after 35 days 40%, 36% and 64% of cvs. Lac 23, Moroberekan and ITA 235 respectively, had developed symptoms. The mean incidence for all cultivars was 46.7% (Fig. 4.5). From 35 to 50 days after inoculation, some plants produced symptoms for the first time but between 50 and 55 days after inoculation no plants produced symptoms for the first time (Fig. 4.5). From 55 to 60 days after inoculation a few more plants began to show initial systemic symptoms. There were, therefore, indications of possibly two phases of symptom production best seen when the data are presented as the number of plants showing symptoms for the first time at various times after inoculation (Fig. 4.6). The first phase of symptom production occurred from 25 to 50 days after inoculation when most plants which were going to produce symptoms had done so and similar trends were evident for all three cultivars. Within this period most plants produced symptoms 25-35 days after inoculation. It is considered likely that symptoms produced during this initial phase resulted from successful systemic infection following the initial sap inoculation with an incubation period of from 30 to 50 days. The second phase of new symptom production started 55-60 days after inoculation involving relatively few plants but seen in all three cultivars. It is considered probable that these new symptoms resulted from infection spread by root-to-root contact as this is known to occur in rice (Bakker, 1974). A two phase incubation period is probably unlikely from initial infection by sap inoculation. There were two plants per pot in this experiment and all late (post-55 days) systemic symptom expression occurred in plants growing in pots containing one plant which had developed systemic symptoms in the first phase. Thus, for the purposes of this experiment, all plants with symptoms up to 50 days after inoculation were considered

Fig. 4.5 The proportion (%) of inoculated plants which developed systemic symptoms for resistant cultivars Lac 23, Moroberekan and ITA 235 inoculated with RYMV-N irrespective of the removal date of inoculates leaves
(50 inoculated plants per cultivar)

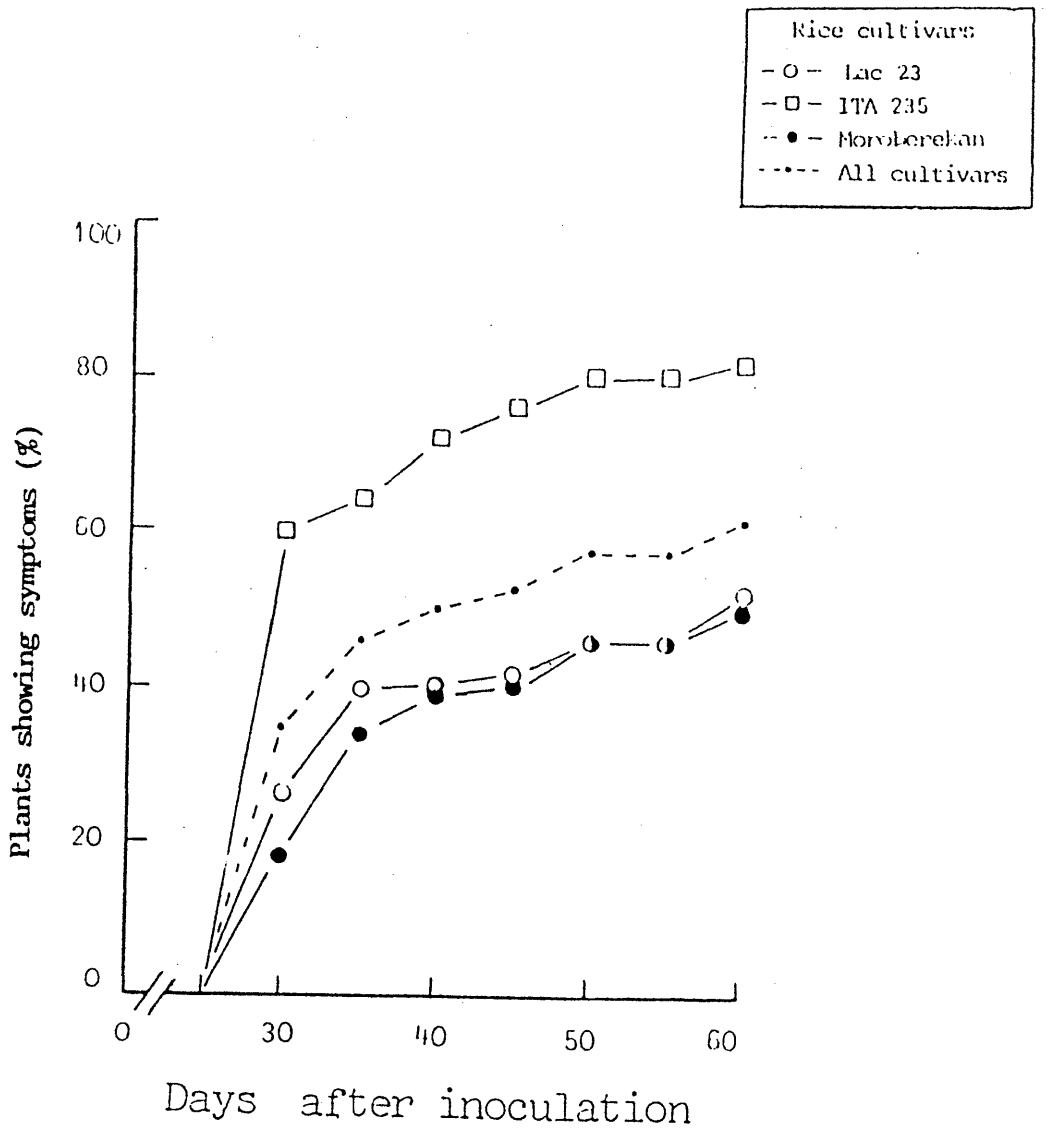
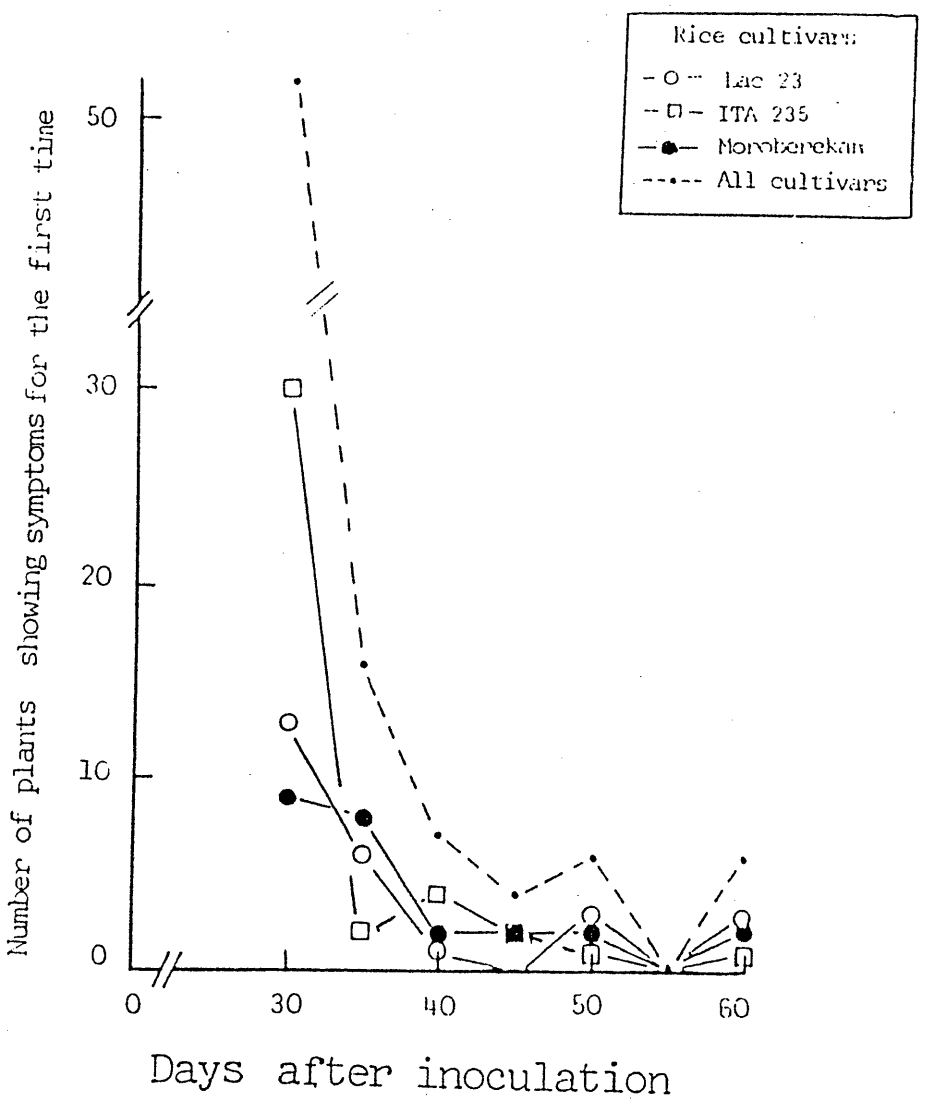


Fig. 4.6 **Numbers of plants showing symptoms for the first time in resistant cultivars inoculated with RYMV-N irrespective of removal date for inoculated leaves (50 inoculated plants per cultivar)**



as successful primary infections resulting directly from initial sap inoculations, and all plants showing initial symptoms after 55 were considered most likely to have arisen by secondary infection from root to root contact. The proportions of successful infections as evidenced by the presence of systemic symptoms at 50 days after inoculation were 46%, 46%, 80% and 57.3% for Lac 23, Moroberekan, ITA 235 and all cultivars combined, respectively. The slight increases in the numbers of plants showing symptoms after 60 days following secondary root to root spread did not markedly affect the relative values for the three cultivars but obviously increased the absolute values.

4.2.2.3 RESULTS (experiment 3)

Data presented for experiment 2 indicated that when inoculated leaves were removed 5 days after inoculation, some plants showed systemic symptoms later (Table 4.8). This was the case in both susceptible and resistant cultivars. Therefore an experiment was instigated to test the effect of inoculated leaf removal on susceptible and resistant cultivars from the day of inoculation to 5 days after inoculation.

Detection of RYMV-N in inoculated and the youngest non-inoculated leaf: When the inoculated leaf (leaf 3) was removed 1 or 2 days after inoculation and tested by ELISA, virus was not detected in the inoculated leaf of either resistant cultivar (Lac 23 and ITA 235) but when removed at 3 and 5 days after inoculation, the number of successfully infected inoculated leaves increased from 10% to 30% (Lac 23) and 20% to 50% (ITA 235) respectively (Table 4.9). By 12 days after inoculation, the majority of inoculated leaves of both resistant cultivars indexed RYMV positive. In the susceptible cultivar ITA 212, RYMV was detected in 80% of the inoculated leaves removed 1 or 2 days after inoculation and, after 3 or more days, all inoculated leaves were sucessfully infected as evidenced by a positive ELISA reaction (Table 4.9).

Results of ELISA tests of extracts from the youngest non-inoculated leaves of resistant and susceptible cultivars showed that virus was not detected from 1 to 5 days after inoculation. After 12 days the youngest leaf of one out of 20 plants (5%) was RYMV positive for Lac 23, 30% for ITA 235 and 100% for susceptible ITA 212 (Table

Table 4.9 ELISA detection of RYMV-N in the inoculated and youngest non-inoculated leaves of resistant and susceptible rice cultivars following inoculation of leaf 3 and subsequent removal of the inoculated leaf at different time after inoculation.

Removal of inoculated leaf (days after inoculation)	Cultivar	Proportion (%) of plants in which RYMV-N antigen detected in extracts of[a]	
		Inoculated leaf (leaf 3)	Youngest leaf
1	Lac 23	0	0
	ITA 235	0	0
	ITA 212	80	0
2	Lac 23	0	0
	ITA 235	0	0
	ITA 212	90	0
3	Lac 23	10	0
	ITA 235	20	0
	ITA 212	100	0
5	Lac 23	30	0
	ITA 235	50	0
	ITA 212	100	0
12	Lac 23	80	5
	ITA 235	90	30
	ITA 212	100	100

[a] Twenty plants of each cultivar on each occasion

4.9).

Relationship between infection of inoculated leaves and subsequent systemic infection: Results of the previous experiment (experiment 2) showed that with some inoculated plants of resistant cultivars, although virus was detected in extracts of inoculated leaves, subsequent systemic infection did not invariably occur. In the present experiment virus was not detected in inoculated leaf extracts 1 or 2 days after inoculation but three plants of cv. ITA 235 eventually showed systemic symptoms (Table 4.10). For plants in which inoculated leaves were removed and tested 3 days after inoculation some plants (3 out of 20) of cv. Lac 23 had detectable RYMV in their inoculated leaf but did not develop any subsequent systemic symptoms, whereas in cv. ITA 235 RYMV was detected in the inoculated leaf of four plants which eventually developed systemic symptoms. When inoculated leaves were removed after 5 days, some plants of both resistant cultivars, considered infected because RYMV was detected in the inoculated leaf, subsequently developed systemic symptoms but others did not, whereas some plants considered not infected (inoculated leaf RYMV negative) developed systemic symptoms (Table 4.10). With the 12 day treatment where most plants of both resistant cultivars appeared to be successfully infected, a few such plants of Lac 23 did not show systemic symptoms and all of the plants in which virus could not be detected in the inoculated leaf failed to produce systemic symptoms. In ITA 235, all infected plants eventually developed systemic symptoms and those in which inoculation to inoculated leaves was considered to have failed remained symptomless.

Table 4.10 Presence or absence of systemic symptoms after 60 days for RYMV inoculated susceptible and resistant rice cultivars in relation to successful infection of inoculated leaf 3

		Inoculated leaves successfully infected[a]			Inoculated leaves not infected		
Removal of inoculated leaf (days after inoculation)	Cultivar	No. of plants with infected inoculated leaf 3	No. of plants with subsequent systemic symptoms	No. of plants with no systemic symptoms	No. of plants with inoculated leaves not infected	No. of plants with subsequent systemic symptoms	No. plants with no systemic symptoms
	Lac 23	0	0	0	20	0	20
1	ITA 235	0	0	0	20	3	17
	ITA 212	16	6	10	4	0	4
	Lac 23	0	0	0	20	0	0
2	ITA 235	0	0	0	20	3	17
	ITA 212	18	9	9	2	0	2
	Lac 23	3	0	3	17	0	17
3	ITA 235	4	4	0	16	0	16
	ITA 212	20	14	6	0	0	0
	Lac 23	8	5	3	12	1	11
5	ITA 235	10	9	1	10	3	7
	ITA 212	20	20	0	0	0	0
	Lac 23	16	12	4	4	0	4
12	ITA 235	18	18	0	2	0	2
	ITA 212	20	20	0	0	0	0

[a] Twenty plants of each cultivar and for each sampling date were inoculated

With susceptible (ITA 212) plants where inoculated leaves were removed 1 to 3 days after inoculation, only a proportion of plants considered infected ultimately showed systemic symptoms, whereas after 5 or 12 days all infected plants eventually showed symptoms (Table 4.10).

Relationship between timing of inoculated leaf removal and subsequent systemic infection: In all cultivars the number of plants showing systemic symptoms progressively increased as the length of time between inoculation and removal of the inoculated leaf was extended (Table 4.11). When the inoculated leaf 3 of Lac 23 was removed 1, 2 or 3 days after inoculation, plants failed to produce systemic symptoms but removal after 5 and 12 days was associated with 30% and 60% systemic infection respectively. In cv. ITA 235 systemic infection was clearly influenced by timing of removal of the inoculated leaf and a higher proportion of plants eventually produced systemic symptoms than in Lac 23. A similar trend was evident for ITA 212 and if the interval between inoculation and leaf removal was 5 days or longer, all plants developed systemic symptoms. Even if the inoculated leaf was left on the plant for only 24 hours, systemic invasion occurred in a quarter of the inoculated plants, in marked contrast to the 3 days required in Lac 23.

Relationship between virus content in the inoculated leaves and systemic infection: Means of ELISA readings for extracts of those inoculated leaves considered infected were obtained. In Table 4.12 shows in resistant cultivars Lac 23 and ITA 235, it was difficult to determine the relationship between virus content in the inoculated leaves and subsequent systemic infection when inoculated leaves were

Table 4.11 Proportion (%) of RYMV-N inoculated resistant and susceptible rice plants which developed systemic symptoms in relation to the time of removal of the inoculated leaf.

Cultivar	Proportion (%) of plants showing systemic symptoms[a]				
	Days between inoculation and leaf removal				
	1	2	3	5	12
Lac 23	0	0	0	30	60
ITA 235	15	15	20	60	90
ITA 212	25	45	70	100	100

[a] Presence or absence of systemic symptoms recorded 60 (Lac 23 and ITA 235) or 30 (ITA 212) days after inoculation

tested up to 5 days after inoculation. This may be because the amounts of RYMV antigen in these leaves were either undetectable or relatively very low (Table 4.12). But, by 12 days after inoculation, the amount of virus in infected inoculated leaves of Lac 23 plants that eventually developed systemic infection was about twice that in infected inoculated leaves of plants which did not show systemic symptoms whereas all RYMV positive inoculated leaves of ITA 235 occurred on plants which eventually produced systemic symptoms (Table 4.12).

In the susceptible cultivar ITA 212, the amounts of virus in inoculated leaves on plants that ultimately produced systemic symptoms were more than in those inoculated leaves of plants that did not show systemic infection when inoculated leaves were removed 1-3 days after inoculation. If inoculated leaves were removed after 5 or more days, all inoculated plants produced systemic symptoms.

Table 4.12 Relationship between mean ELISA absorbance values for extracts of inoculated leaves considered RYMV-N positive in plants eventually producing systemic symptoms or remaining symptomless.

Sampling time (days after inoculation)	Cultivar	A_{410} x 100 for infected inoculated leaves	
		Leading to systemic infection	Not leading to systemic infection
1	Lac 23	a	a
	ITA 235	a	a
	ITA 212	41.0±5.0[b] (6)	20.0±1.0 (10)
2	Lac 23	a	a
	ITA 235	a	a
	ITA 212	63.0±6.0 (9)	20.0±0.3 (9)
3	Lac 23	c	16.0±1.0 (3)
	ITA 235	15.0±0.5 (4)	d
	ITA 212	84.0±8.0 (14)	19.0±1.0 (6)
5	Lac 23	16.0±1.0 (5)	15.0±1.0 (3)
	ITA 235	21.0±2.6 (9)	16.0 (1)
	ITA 212	82.0±8.0 (20)	d
12	Lac 23	53.0±6.0 (12)	29.0±5.5 (4)
	ITA 235	85.0±13.0 (18)	d
	ITA 212	120.0±10.0 (20)	d

a All inoculated leaves were RYMV negative by ELISA

b Average absorbance (A_{410} x 100) measured 30 minutes after the addition of substrate; mean of those inoculated leaves RYMV positive ± standard error of the mean

c None of inoculated leaves RYMV positive led to systemic infection

d All plants systemically infected therefore no absorbance values for this category.

Values in parentheses show number of test leaf extracts (1:100 w/v) from which the means were calculated.

4.2.3 Experiment 4. The effect of leaf position, inoculum dilution and the number of inoculations on infection of resistant and susceptible rice by RYMV-N

Results for the previous experiment (experiment 2) showed that when leaves 2 and 3 of resistant cultivars at the 4-5 leaf stage were inoculated with RYMV-N the number of successful leaf 3 infections was usually greater than those for leaf 2 (Table 4.4). In addition, the amount of virus accumulated, estimated from ELISA data, was also greater in leaf 3 than in leaf 2 (Table 4.6). Furthermore, in the susceptible ITA 212, although more virus was detected in leaf 3 (Table 4.6), successful infection was obtained in all of both inoculated leaves. An experiment was next carried out to look more closely at the effect of leaf position on inoculated leaf infection and on subsequent systemic symptoms, as well as the frequency of successful plant infections. Further objectives aimed to determine if the degree of resistance to virus infection could be affected by inoculum pressure (virus content of inoculum) and multiple inoculations.

4.2.3 MATERIALS AND METHODS

Plant materials: Seeds of cvs. ITA 212, ITA 235 and Lac 23 were sown on 15th July, 1988. After germination, seedlings were transplanted on 22nd July, 1988 into 10 cm diameter plastic pots with one plant per pot. Plants were grown under glasshouse conditions without supplementary lighting and the temperature was maintained between 25 and 33°C with a daily mean of 26-27°C.

Mechanical inoculation: Frozen leaves of cv. ITA 212 infected with RYMV-N, harvested 3 weeks after inoculation, were used as inoculum. Plants were inoculated at the 4-5 leaf stage by stroking three times on both sides of inoculated leaves using cotton wool swabs. Inoculum dilutions and the position of inoculated leaves are detailed below. When plants were inoculated twice, inoculations were separated by 5 days. After each inoculation, and before inoculating the next plant, inoculated leaves were rinsed with distilled water.

Sample harvesting: As indicated by the results of experiment 2 (section 4), 20 days after inoculation appeared to be sufficient to allow the virus to replicate in the inoculated leaves and to be reliably detected by means of ELISA. Therefore, it was decided to harvest in inoculated leaves 20 days after inoculation but, 10 days after inoculation, inoculated leaf 2 began to dehydrate so this leaf was harvested 10 days after inoculation while other inoculated leaves (leaf 3 and leaf 4) were harvested 20 days after inoculation. The youngest leaf of each plant was harvested 20 and 60 days after inoculation. All samples were stored at -20°C prior to ELISA tests. ELISA work was essentially as described for experiments 2 and 3 (section 4). For the purposes of this experiment, the inoculated leaf or leaves of each plant was treated as one sample (e.g. inoculated leaves 2 + 3 + 4 considered as one sample).

Experimental design: A randomized complete block design was applied with 21 treatments (7 treatments/cultivar x 3 cultivars). Treatments applied to each cultivar were:

Treatment	Inoculated leaf position	Inoculum dilution (w/v)	Number of inoculations
1	2	1:10	1
2	3	1:10	1
3	4	1:10	1
4	2 + 3 + 4	1:10	1
5	2 + 3 + 4	1:10	2
6	2 + 3 + 4	1:1000	1
7	2 + 3 + 4	1:1000	2

Each treatment consisted of 10 replicate plants (one per pot). Treatments (plots) were arranged on the glasshouse benches (immediately after virus inoculation) according to a randomized complete block design using five blocks.

4.2.3.2 RESULTS

Detection of RYMV-N in inoculated and non-inoculated leaves: Results for cv. Lac 23 showed that when leaf 2 was inoculated with RYMV-N and removed 10 days after inoculation, the proportion of successful infections as evidenced by the presence of RYMV-N antigen in extracts of the inoculated leaf was 40%, a value which doubled when either leaf 3 or leaf 4 or all three leaf positions were inoculated (Table 4.13) and tested 20 days after inoculation. Systemic invasion was not detected when the youngest leaves were tested 20 days after inoculation except for one plant, in which all three leaf positions had been inoculated. However, when tip leaves were tested after 60 days, the number of systemically infected plants appeared to be related to the position and number of the inoculated leaves as values

Table 4.13 Detection of RYMV-N by ELISA in inoculated and non-inoculated tip leaves of rice cultivars resistant or susceptible to RYMV-N as affected by the number and position of leaves inoculated.

Cultivar	Leaf position(s) inoculated	Proportion (%) of plants (out of 10) in which antigen was detected in an extract[a] of:		
		Inoculated leaf	Youngest tip leaf	
		Days after inoculation	Days after inoculation	
		20	20	60
Lac 23	Leaf 2	40	0	10
	Leaf 3	80	0	50
	Leaf 4	80	0	60
	Leaf 2+3+4	80	10	60
ITA 235	Leaf 2	60	10	60
	Leaf 3	80	40	80
	Leaf 4	100	60	100
	Leaf 2+3+4	100	60	100
ITA 212	Leaf 2[b]	90	90	-[c]
	Leaf 3	100	100	-
	Leaf 4	100	100	-
	Leaf 2+3+4	100	100	-

[a] Extracts of leaves specified diluted 1:100 (w/v) for tests

[b] Leaf 2 was tested 10 days after inoculation

[c] Most infected plants were killed by 60 days after inoculation

progressively increased for plants inoculated at leaf 2, 3 or 4 and all three leaf positions. Far more systemic infections were detected when plants were tested after 60 than afer 20 days (Table 4.13).

With cultivar ITA 235, successful infections occurred in 60% of leaf 2 inoculations increasing to 80% for leaf 3 and all inoculations were successful when leaf 4 or all three leaf positions were inoculated (Table 4.13). When the youngest leaves were tested 20 days after inoculation the proportion of plants in which systemic virus was detected increased with leaf position inoculated (leaf 2 to leaf 4) and tests 60 days after inoculation showed that all plants with successfully infected inoculated leaves had become systemically infected.

In the susceptible cultivar ITA 212, all plants in which inoculated leaves were successfully infected became systemically infected and systemic virus was detectable by ELISA in tip leaves at 20 days after inoculation. All inoculated leaves became infected except for one leaf 2 inoculation tested 10 days after inoculation,.

Relationship between infection of inoculated leaves and systemic infection: Irrespective of the leaf or leaves inoculated, some inoculated leaves of Lac 23 plants although successfully infected (designated infected in Table 4.14) did not show systemic symptoms or the presence of RYMV-N antigen in tip leaves 60 days after inoculation. Failure to detect RYMV antigen in inoculated leaves tested 20 days after inoculation was always associated with the absence of systemic symptoms and systemic RYMV antigen (Table 4.14).

Table 4.14 Effect of inoculated leaf position on the number of successfully infected plants and subsequent systemic inoculation of susceptible and resistant rice cultivars by RYMV-N.

Cultivar	Inoculated[a] leaf	Inoculated leaves infected			Inoculated leaves not infected		
		No. of infected plants (out of 10)	Leading to systemic symptoms	No systemic infection	No. of plants not infected (out of 10)	Leading to systemic infection	No systemic infection
Lac 23	L2	4	1	3	6	0	6
	L3	8	5	3	2	0	2
	L4	8	6	2	2	0	2
	L2+3+4	8	6	2	2	0	2
ITA 235	L2	6	6	0	4	0	4
	L3	8	8	0	2	0	2
	L4	10	10	0	0	0	0
	L2+3+4	10	10	0	0	0	0
ITA 212	L2	9	9	0	1	0	1
	L3	10	10	0	0	0	0
	L4	10	10	0	0	0	0
	L2+3+4	10	10	0	0	0	0

[a] Inoculated with RYMV-N in leaf sap diluted 1:10 (w/v)

With both ITA 235 (resistant) and ITA 212 (susceptible), if virus was detected in inoculated leaves the plant subsequently became systemically infected and if virus was not detected, systemic invasion did not occur. Thus, the presence of virus in inoculated leaves tested 20 days after inoculation was always associated with subsequent systemic invasion in ITA 235 and ITA 212 but not necessarily in Lac 23.

Relationship between leaf position and disease incidence: Results in Table 4.14 show that the position of the leaf inoculated markedly affected the incidence of systemic symptoms in cv. Lac 23. Systemic symptoms were uncommon if leaf 2 only was inoculated but increased when inoculations were made to leaf 3 or leaf 4 or leaves 2+3+4. However, systemic symptoms never exceeded 60%. Similar trends were apparent for cv. ITA 235 but the overall incidence of systemic symptoms was greater than in Lac 23. Systemic infection occurred in nearly all ITA 212 inoculated plants irrespective of the leaf inoculation (Table 4.14).

Relationship between inoculum dilution and number of inoculations on infection of inoculated leaves: When RYMV-N at 1:10 (w/v) dilution was inoculated once to Lac 23 plants the inoculated leaves of eight of the 10 inoculated plants became infected but two of the plants did not develop systemic infection but when plants were inoculated twice, all inoculated leaves were infected and 80% of such plants developed systemic symptoms (Table 4.15). In ITA 235, largely as expected, all inoculated leaves were infected irrespective of inoculum dilution or number of inoculations and the virus invaded all plants systemically (Table 4.15). However, using inoculum at a dilution of 1:1000 (w/v)

Table 4.15 Effect of inoculum dilution and the number of inoculations on infection of inoculated leaves and subsequent systemic infection of susceptible and resistant rice cultivars with RYMV

			Inoculated leaves infected			Inoculated leaves not infected		
Cultivar	Inoculated leaves	Inoculum dilution (w/v)	Number of inoculation	No. of infected plants (out of 10)	No. of plants leading to systemic infection	No systemic infection	No. of plants not infected (out of 10)	No. of plants leading to systemic infection	No systemic infection
Lac 23	L2+3+4	1:10	1	8	6	2	2	0	2
		1:10	2	10	8	2	0	0	0
		1:1000	1	0	0	0	10	0	10
		1:1000	2	0	0	0	10	1	9
ITA 235	L2+3+4	1:10	1	10	10	0	0	0	0
		1:10	2	10	10	0	0	0	0
		1:1000	1	0	0	0	10	0	10
		1:1000	2	4	4	0	6	0	6
ITA 212	L2+3+4	1:10	1	10	10	0	0	0	0
		1:10	2	10	10	0	0	0	0
		1:1000	1	10	10	0	0	0	0
		1:1000	2	10	10	0	0	0	0

inoculated leaves of Lac 23 were not infected even when plants were inoculated twice and with cv. ITA 235 inoculations were only partially successful when inocula were applied twice, when 40% became infected and all eventually showed systemic symptoms (Table 4.15).

When the youngest leaves were tested 20 days after inoculation with ELISA the number of inoculations had little effect on the number of plants with systemic antigen when the inoculum was diluted 1:10. Proportions were for single and double inoculations, 100%, 100% (ITA 212); 60%, 70% (ITA 235) and 10%, 20% (Lac 23). However, for inocula diluted 1:1000 and inoculated once RYMV antigen was not detected in the tip leaves of cultivars ITA 235 or Lac 23 but antigen was found in 60% of the ITA 212 plants. When two inoculations were made (1:1000 dilution), the proportions of tip leaves with RYMV antigen were 90% (ITA 212), 20% (ITA 235) and 0% (Lac 23).

Symptom severity: Results (Table 4.16) showed that in general rice cultivars inoculated with inoculum diluted 1:10 were more severely affected than plants inoculated with inoculum diluted 1:1000 irrespective of the number of inoculations and, for a given dilution, symptoms tended to be slightly more severe following two inoculations than a single inoculation. As expected, symptoms were most severe in ITA 212, mild in Lac 23 and intermediate in ITA 235. In the susceptible ITA 212 plants inoculated once with RYMV-N diluted 1:1000, disease severity value at 30 days after inoculation had not reached the discolouration (rating 5.0) stage (Table 4.16).

Incubation period: Inoculum dilution and the number of inoculations affected the incubation period in both resistant and susceptible

Table 4.16 Disease severity and incubation period for RYMV-N inoculated once or twice and at a dilution of 1:10 or 1:1000 (w/v) to susceptible and resistant rice cultivars.

Cultivar	Inoculum dilution	Number of[a] inoculations	Incubation time (days)		Disease[b] severity
			Mean	Range	
Lac 23	1:10	1	36	28-44	2.1
	1:10	2	28.8	16-44	2.5
	1:1000	1	-[c]	-	-
	1:1000	2	45[d]	45	1.0
ITA 235	1:10	1	25.8	16-40	3.5
	1:10	2	22.4	14-32	4.0
	1:1000	1	-	-	-
	1:1000	2	30	20-40	1.5
ITA 212	1:10	1	12.8	12-16	6.8
	1:10	2	12.4	10-14	7.0
	1:1000	1	19.4	12-24	4.6
	1:1000	2	16.4	12-20	6.2

[a] Inoculated leaves were leaf positions 2+3+4

[b] Thirty and sixty days after inoculation for susceptible ITA 212 and resistant Lac 23 and ITA 235 cultivars respectively.

[c] None of the inoculated plants produced symptoms

[d] Only one plant produced symptoms

cultivars (Table 4.16).

For Lac 23 plants inoculated once with sap diluted 1:10, initial symptoms appeared 28 days after inoculation whereas when inoculated twice, first symptoms appeared after only 16 days (Table 4.16). The single plant which produced symptoms when inoculated twice with sap diluted 1:1000 did so in 45 days; symptoms were not produced in plants inoculated once and twice respectively with a 1:10 sap dilution but symptoms took slightly longer (20 days) when a 1:1000 sap dilution was used twice. As with Lac 23 plants, plants did not produce symptoms when inoculated once (1:1000 dilution). In the susceptible cultivar ITA 212 symptoms appeared rapidly after 12 and 10 days when RYMV-N was inoculated once or twice using inoculum diluted 1:10 or 1:1000 (Table 4.16).

The average length of the incubation period increased when inoculum was further diluted from 1:10 to 1:1000 but when the same dosage was used increasing the number of inoculations tended to decrease the incubation period (Table 4.16). Generally incubation periods were long in Lac 23 (28.8 - 45 days), of intermediate length in ITA 235 (22.4 - 30 days) and short in ITA 212 (12.4 - 19.4 days) (Table 4.16).

Virus content: Results (Table 4.17) showed that the amount of virus (in terms of absorbance at A_{410} x 100) detected in the inoculated leaves tested after 20 days were similar for each cultivar when plants were inoculated once or twice with sap diluted 1:10 and consistently higher than in inoculated leaves of plants inoculated at 1:1000 dilution. Virus content (inoculum 1:10 dilution) was higher

Table 4.17 Mean absorbance values (A_{410} x 100) from ELISA tests on extracts from inoculated and youngest leaves of resistant and susceptible rice cultivars when inoculated once or twice with sap diluted 1:10 or 1:1000 (w/v).

Cultivar	Inoculum dilution	Number inoculations	A_{410} x 100		
			Inoculated leaves[a]	Youngest leaves after:	
			20 days after inoculation	20 days	60 days
Lac 23	1:10	1	30±4[b] (8)	16 (1)	*66±7 (6)
	1:10	2	38±5	51±6 (2)	*99±4 (8)
	1:1000	1	0[c]	0	0
	1:1000	2	0	0	169 (1)
ITA 235	1:10	1	46±9 (10)	101±23 (6)	*95±3 (10)
	1:10	2	53±6 (10)	78±22 (7)	*99±4 (10)
	1:1000	1	0	0	0
	1:1000	2	34±3 (4)	42±6 (2)	*43±2 (4)
ITA 212	1:10	1	117±3 (10)	*119±4 (10)	-[d]
	1:10	2	110±5 (10)	*124±6 (10)	-
	1:1000	1	61±3 (10)	102±22 (6)	*78±14 (10)
	1:1000	2	86±4 (10)	167±15 (19)	

*Extracts diluted 1:500; remaining extracts diluted 1:100. Reaction was stopped 30 minutes after the addition of substrate by adding 50µl 3N NaOH.

[a] Inoculated leaves were leaves 2+3+4

[b] Mean absorbance of ELISA readings of ELISA positive (2 x healthy control or higher) ± standard error of the mean.

[c] None of the inoculated plants showed symptoms

[d] Most of the infected plants were killed

in ITA 212 followed by ITA 235 with Lac 23 extracts giving the lowest absorbance values. When the inoculum was diluted 1:1000 RYMV antigen was not detected in the inoculated leaves of Lac 23 but reached detectable levels in ITA 235 plants inoculated twice, but not when they were inoculated once. High amounts were present in inoculated leaves of ITA 212 irrespective of the number of inoculations.

The virus contents of the tip leaves were always greater in extracts of leaves tested after 60 days than after 20 days irrespective of virus dilution, number of inoculation or rice cultivar (note that absorbances in Table 4.17 for tip leaves were for extracts diluted 1:100 or if the values were off scale, the extract was further diluted to 1:500). Virus accumulation was greatest in the non-inoculated young leaves of ITA 212, least in Lac 23 with ITA 235 extracts yielding intermediate amounts. Amounts were similar in plants of three cultivars when inocula were applied once or twice at a dilution of 1:10. When inocula were diluted 1:1000 virus accumulation was slower than with inocula diluted 1:10 and systemic antigen was only detected in Lac 23 plants after 60 days following a double inoculation, In ITA 235, RYMV was not detected following a single inoculation (dilution 1:1000) but was detected following two inoculations. All ITA 212 plants were systemically infected irrespective of the number of inoculations after 20 days but most plants did not survive for testing after 60 days (Table 4.17).

In general, RYMV accumulation in both inoculated and tip leaves was greatest in ITA 212 followed by ITA 235 then Lac 23. Accumulation was unaffected by the number of inoculations with 1:10 inocula; infections were more frequent and virus accumulated most

following two inoculations rather than a single inoculation with sap diluted 1:1000 and lastly, RYMV reached higher amounts in tip compared with inoculated leaves, particularly when the former were tested after 60 days.

Experiment 5. Variation in infection and symptom production within resistant rice cultivars to RYMV

Rice is self pollinated (Grist, 1986), therefore cultivars are probably homogenous and homozygous for resistance or susceptibility to RYMV infection. Results of the host-virus combination experiments (section 3) revealed the unexpected presence of variability in the occurrence of RYMV infection and symptom production in resistant cultivars when they were mechanically inoculated with high concentration inocula under glasshouse conditions. Plant to plant variation in incubation period length and the production of symptoms in inoculated plants was very marked in cvs. Lac 23 and Moroberekan. ELISA tests on inoculated leaves showed that a few plants usually failed to become infected in that sufficient antigen accumulated in inoculated leaves to be detectable by ELISA. Nevertheless, some of these 'virus-positive' plants failed to produce systemic symptoms and RYMV antigen was not detected in extracts of the youngest non-inoculated leaf.

Plant to plant variability in symptom expression has often been attributed to one or more of the following possibilities (Scott and Rosenkranz, 1987):-

1. Not all plants received the threshold level of inoculum required for symptom expression. This factor is not heritable.
2. Differences in the microclimate of individual plants affected symptom expression. This factor also is not heritable.
3. Plants may differ in the number of sites of infection and rate of virus replication and movement. This factor is heritable and

thus it is likely that progenies from plants showing symptoms are more susceptible than progenies from symptomless plants.

The objective of this experiment was to determine if variability in disease symptom production following RYMV inoculation to plants of resistant cultivars could be attributed to genetic variation within a cultivar or to environmental factors.

4.2.4.1 MATERIALS AND METHODS

Plant materials: Seeds of resistant cultivars collected from RYMV inoculated plants showing symptoms and from symptomless plants (see section 4.2.2.1) were sown in vermiculite on 15th October, 1988 and later transferred into 9 cm plastic pots with one plant per pot. Seeds from each 'mother' plant were collected and planted separately. Seeds of ITA 235, Moroberekan and Lac 23 were used. Seeds of these three cultivars as obtained in packets from IITA (the seed source for all other experiments in this study) were also sown for comparative purposes. Cultivar ITA 212 was included to check the efficiency and infectivity of inoculation and inoculum respectively.

Virus isolate: RYMV-N isolate was used.

Inoculation procedure: Leaves 2 and 3 were inoculated by stroking both sides of the lamina three times using a cotton wool swab then rinsing with distilled water. Inoculum was prepared from infected leaves of cv. ITA 212 in 0.067 M Sorensen's buffer (pH 7.0) as before.

Experimental design: A randomized complete block design was used with 9 treatments (3 cultivars x 3 seed sources). Each treatment consisted of 60 plants from each seed source. There were three blocks each containing 20 plants of each treatment. For seed gathered from RYMV inoculated symptomless and symptom producing plants there were six mother plants and 10 seeds were used from each plant, thus giving the total of 60 plants per treatment. For IITA seed, 60 seeds were sown. In allocating to blocks, each block received seed from two mother plants (20 seeds). In total, 540 plants were used; 180 of each cultivar.

Inoculated leaves of each plant were harvested 20 days after inoculation, weighed, labelled and stored at -20°C prior to ELISA tests. After initial symptom appearance, disease incidence was recorded at 7 day intervals.

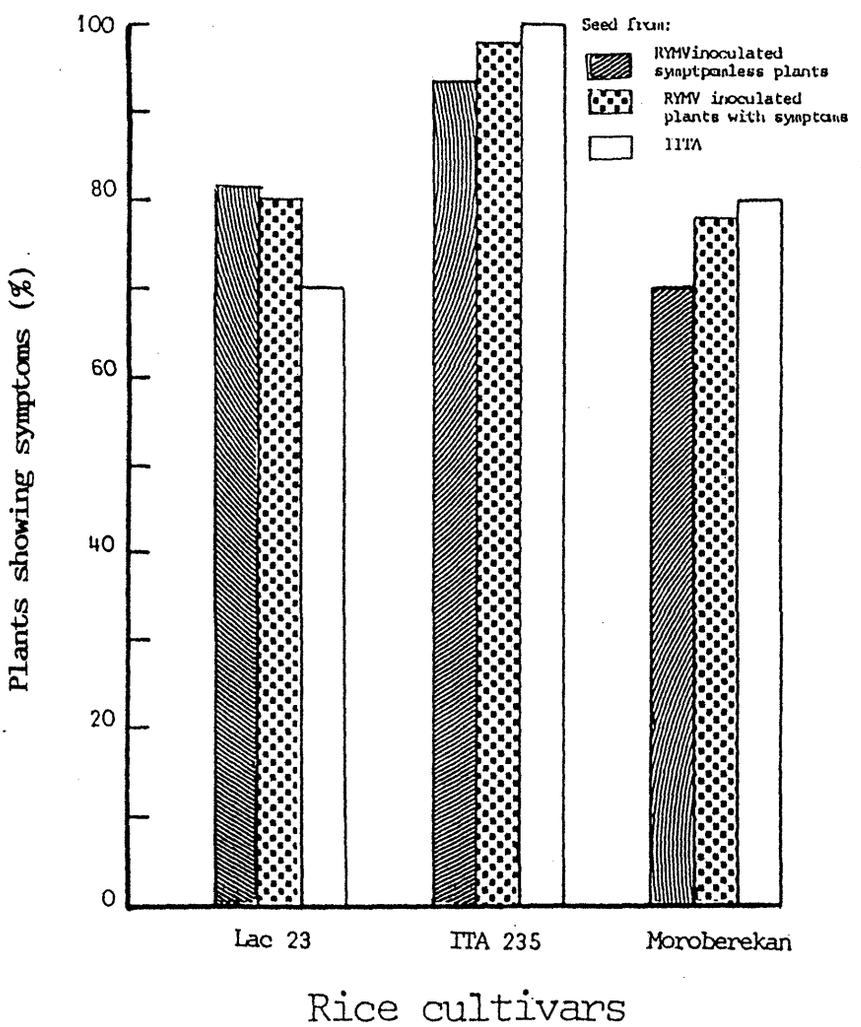
4.2.4.2 RESULTS

Disease incidence: Results showed that irrespective of seed origin cv. ITA 235 had a higher disease incidence (mean 97.2%) than Lac 23 or Moroberekan, in which disease incidence was similar (means 76.1% and 74.4% respectively [Fig. 4.7]).

For all three cultivars disease incidence in plants grown from seed collected from symptomless inoculated plants was similar to the incidence in plants grown from seed collected from plants showing disease or from the usual IITA seed source.

Values in Fig. 4.8 are the mean from 60 inoculated plants per

Fig. 4.7 Mean disease incidence (60 days after inoculation) for plants grown from seed a) collected from RYMV inoculated plants with symptoms, b) as a) but symptomless and c) normal seed source (IITA) and inoculated with RYMV-N



seed source and, from seed collected from RYMV-N inoculated sources, seed from six mother plants was tested separately using a sample of 10 seeds from each mother plant. Results for each of the three cultivars and the individual mother plant sources of seed collected from symptomless and diseased material are shown in Fig. 4.8. For Lac 23 the range of disease incidence was similar for seed from symptomless mother plants (range 60-100%) and diseased plants (50-100%). Variation was similar in Moroberekan ranging from 50 to 100%. For both seed sources variation was less and overall incidence higher in cv. ITA 235 (overall range 80-100%).

Detection of RYMV-N from inoculated leaves: When inoculated leaves were tested by ELISA 20 days after inoculation, only 14 out of 540 inoculated plants were ELISA negative and most of these (9 plants) were of cv. Moroberekan (Table 4.18). Of those plants with successfully infected inoculated leaves, most subsequently produced systemic symptoms, particularly in cv. ITA 235, and the source of seed had no effect on either the proportion of inoculated leaves infected or the development of systemic symptoms in any of the three cultivars. Absorbance readings from inoculated leaf extracts showed, irrespective of seed source, greater (about twice) accumulation of RYMV antigen in ITA 235 than in cvs. Moroberekan and Lac 23 in which virus accumulation was similar. For plants which remained symptomless, absorbance values were low and unaffected by cultivar or seed source.

Fig. 4.8 Disease incidence (%) in batches of plants grown from seed gathered from six RYMV-inoculated mother plants (either diseased or symptomless) of three resistant cultivars 60 days after plants were inoculated with RYMV-N.

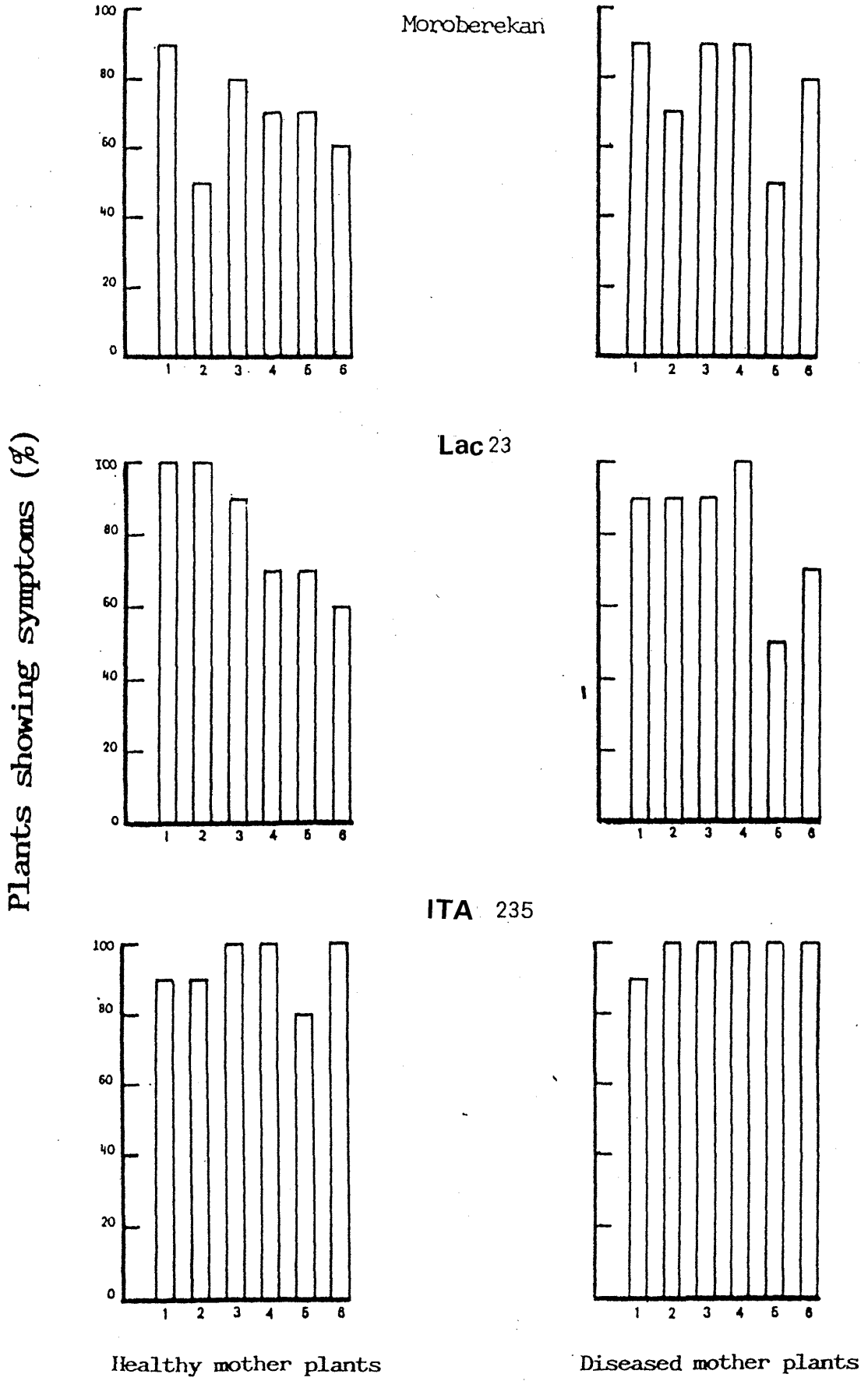


Table 4.18 Infection of inoculated leaves by RYMV-N and ELISA absorbance valaues for extracts of inoculated leaves from plants which did or did not eventually produce systemic symptoms. Plant grown from seed gathered from successfully infected mother plants (disease), from inoculated symptomless plants (healthy) and imported seed from IITA

		Inoculated leaves infected[a]			Inoculated leaves not infected		
Cultivar	Seed source	No. of plants infected (out of 60)	Leading to systemic symptoms	Absorbance A_{410} nm x 100	No.with no systemic symptoms	Absorbance A_{410} nm x 100	No. not infected (out of 60)
Lac 23	Healthy	60	49	93.8±[b]5.5	11	29±[c]1.2	0
	Diseased	58	49	91.2±3.4	9	32±3.2	2
	IITA	59	42	87.9±4.6	17	31±2.4	1
ITA 235	Healthy	58	56	171.2±6.5	2	32±3.2	2
	Diseased	60	59	170±7.2	1	26	0
	IITA	60	60	164.8±5.1	0	-	0
Moroberekan	Healthy	56	42	87.5±3.3	14	29-2.7	4
	Diseased	57	47	94.4±5.2	10	35±4.3	3
	IITA	58	48	90.0±4.6	10	35±4.8	2

[a] Inoculated leaves tested by ELISA 20 days after inoculation using extracts diluted 1:100 (w/v)
[b] Mean absorbance for ELISA readings of extracts from plants which developed systemic symptoms ± standard error or the mean
[c] Mean absorbance for ELISA reading of extracts from plants which remained symptomless ± standard error of the mean

Absorbance was measured 30 minutes after the addition of substrate

4.3 SOME POSSIBLE MECHANISMS OF RESISTANCE TO RYMV

Previous results in this section suggested that when resistant cultivars were inoculated with RYMV-N several types of resistance mechanisms may be involved. As some plants failed to become infected, there may be some resistance to infection *per se*. In some plants, virus was detected in inoculated leaves but did not move systemically, suggesting resistance to virus movement, whereas in others RYMV invaded non-inoculated leaves but with a marked delay compared with susceptible cultivars. Some resistance to virus multiplication and accumulation occurred in plants showing symptoms for the amount of virus in inoculated and non-inoculated leaves was much less than in comparable tissue of the susceptible cultivar ITA 212.

These findings le d to further examination of possible resistance mechanisms. The first possibility considered was the presence of an inhibitory substance(s) in resistant cultivars. Such a substance(s) had been associated with resistance to virus infection (Simon and Moss, 1963; Salehuzzaman and Wilkins, 1983). The second possibility was that morphological differences existed between the leaves of resistant and susceptible cultivars such as the number and size of hairs on the leaf surface which might affect susceptibility to infection *per se* following sap infection.

4.3.1 Inhibitory properties of sap from resistant and susceptible rice cultivars on the infectivity of RYMV

4.3.1.1 MATERIALS AND METHODS

Experiment 1: Effects of healthy saps on RYMV-N

Seeds of susceptible cultivars ITA 212 and BG 90-2 and of resistant cultivars Lac 23, Moroberekan and ITA 235 were sown on 22nd March, 1988 and transplanted (1-2 leaf stage) 7 days later into 10 cm plastic pots with one plant per pot.

Fresh RYMV-N infected leaves (cv. ITA 212, 3 weeks after inoculation) were homogenized in 0.067 M Sorensen's buffer (pH 7.0; 1 ml buffer:1 g infected tissue) and the extract expressed through four layers of muslin. Leaves of healthy plants (all five cultivars) at the same age as the ITA 212 test plants were collected, washed with distilled water, dried to remove excess water and extracts (1 ml buffer:1 g tissue) prepared as above.

Inoculum extract was divided into three aliquots and diluted with buffer to give w/v dilutions 1:5, 1:100 and 1:500. Next, an equal volume of healthy sap was added to each diluted inoculum extract to give final w/v dilutions of 1:10, 1:200 and 1:1000 for inoculum extracts. Thus sap from healthy plants of all five cultivars was separately mixed with dilutions of RYMV-containing sap from cv. ITA 212 which gave a final test healthy sap dilution of 1:2. Aliquots of the three virus dilutions were also mixed with an equal volume of buffer to give final dilutions of 1:10, 1:200 and 1:1000; these served as controls.

After leaving the inoculum mixture to stand for 4 hours at room temperature, test plants of ITA 212 at the 4-5 leaf stage were inoculated by stroking leaves 2 and 3 on both sides of the lamina three times with cotton wool swabs.

Plants were arranged in the glasshouse according to a randomized complete block design with 18 treatments (5 test sap sources + buffer control x 3 virus dilutions). Each treatment consisted of 10 plants (one per pot) with two replicate pots in each of the five blocks.

Experiment 2: Effects of healthy saps on RYMV isolates

Seeds of cultivars ITA 212, Lac 23 and ITA 235 were sown on 23rd May, 1988 and transplanted after one week into 10 cm plastic pots with one plant per pot.

All five isolates of RYMV were tested with inocula prepared and inoculated as described for experiment 1 but using final dilutions of 1:10 and 1:1000 (w/v) for each isolate after mixing with an equal volume of healthy sap from cvs. ITA 212, Lac 23 or ITA 235 or buffer only.

Plants were arranged in the glasshouse according to a randomized complete block design with 40 treatments (3 test sap source + buffer control x 5 isolates x 2 virus dilutions). Each treatment consisted of 10 plants (one per pot) with two replicate pots in each of the five blocks.

EXPERIMENT 3: Effects of healthy sap on RYMV-N distribution in ITA 212

Seeds of ITA 212, Lac 23 and ITA 235 cultivars were sown on 29th May, 1988 and one week later seedlings were transplanted into 10 cm plastic pots with one plant per pot.

The Nigerian isolate of RYMV was used for preparing inoculum which was inoculated to cv. ITA 212 as described for experiment 1 but with two final dilutions of 1:10 and 1:1000 (w/v). Inoculated leaves were immediately rinsed with distilled water.

There were two main treatments. In the first, inoculated leaves and the youngest tip leaf were removed after 5 days and a further removal of the youngest leaf on the same plant was made after 30 days. In the second main treatment inoculated leaves were not removed but the youngest leaf present at 15 and 30 days was removed. All harvested leaves were tested for RYMV antigen by ELISA.

Plants were arranged in the glasshouse according to a randomized complete block design with 16 treatments (3 test sap sources + control buffer x 2 virus dilution x 2 (inoculated leaves removed or retained). Each treatment consisted of 10 plants (one per pot) with two replicate pots in each of the five blocks.

EXPERIMENT 4: Effect of healthy sap contact time on RYMV-N

Seeds of cv. ITA 212, Lac 23 and ITA 235 were sown on 28th July, 1988 and transplanted after 7 days into 10 cm plastic pots with one plant per pot.

As before, healthy saps from the three cultivars were mixed with RYMV-N sap extracted from infected leaves of ITA 212. The final dilution of healthy sap was 1:2 and for virus was 1:1000 (w/v). After leaving the mixtures to stand for 2, 4, 8, 12 or 24 hours at room temperature, test plants (ITA 212 at the 4-5 leaf stage) were inoculated as described for experiment 1.

Plants were arranged according to a randomized complete block design with 20 treatments (3 sap sources + buffer control x 5 contact times). Each treatment consisted of 10 plants with two replicates in each of 5 blocks.

4.3.1.2 RESULTS

Experiment 1

Effect of rice extracts on disease incidence: Diluting RYMV-N with buffer to 1:1000 (w/v) did not decrease the infectivity of the virus when tested on susceptible ITA 212. All test plants treated with virus-buffer mixture showed symptoms (Table 4.19). Dilution of inoculum (to 1:10 or 1:200) with sap from susceptible cultivars ITA 212 and BG 90-2 did not change the infectivity and even at 1:1000 disease incidence was 90%. Using sap from the resistant cultivar ITA 235 as a diluent, the disease incidence was 100% at final inoculum dilutions of 1:10 and 1:200 but only 60% at a dilution of 1:1000 (Table 4.19). With Lac 23 or Moroberekan sap as diluent, incidence declined to 40% with RYMV-N tested at 1:1000 (w/v).

Incubation periods: In control treatments (virus-buffer mixture) inoculated plants displayed initial symptoms 10-12 days after inoculation (Table 4.19). Diluting beyond 1:10 delayed symptom expression and increased the mean incubation period from 11.4 days (1:10) to 15.2 days (1:1000). The mean incubation period for virus mixed with sap extracted from susceptible plants ranged from 12.0 days (1:10 dilution) to 18.6 days (1:1000 dilution) whereas mixtures containing sap from resistant rice cultivars showed extended incubation periods ranging from 13.8-22.5 days after inoculation. In general, incubation periods in all treatments increased with increasing virus dilution but particularly so when the virus was mixed with resistant plant extracts.

Table 4.19 Disease incidence and severity rating after 40 days and incubation period of RYMV-N in rice (cv. ITA 212) inoculated with virus extracts diluted with buffer or with healthy sap from five rice cultivars.

Virus dilution	Source of diluent sap	Disease incidence (%)	Disease severity	Incubation period (days)	
				Mean	Range
1:10	Lac 23	90	6.5	14.2	10-16
	Moroberekan	90	6.1	13.8	10-16
	ITA 235	100	6.6	13.8	10-16
	BG 90-2	100	6.8	12.0	10-14
	ITA 212	100	6.8	12.8	10-14
	Buffer control	100	6.8	11.4	10-14
1:200	Lac 23	90	5.2	17.1	12-28
	Moroberekan	90	4.7	19.1	12-28
	ITA 235	100	5.6	17.2	12-26
	BG 90-2	100	6.4	16.2	12-20
	ITA 212	100	6.4	16.6	12-20
	Buffer control	100	6.8	13.8	12-20
1:1000	Lac 23	40	4.0	22.0	14-28
	Moroberekan	40	3.3	22.5	14-28
	ITA 235	60	4.5	20.3	14-28
	BG 90-2	90	5.0	18.6	12-24
	ITA 212	90	5.4	18.0	12-24
	Buffer control	100	6.2	15.2	12-20

Disease severity scale ranged from 0 (symptomless) to 7.0 (complete leaf necrosis) and means calculated only from plants which produced symptoms.

Symptom severity: diluting the virus with buffer to 1:10, 1:200 and 1:1000 did not affect the disease severity rating 40 days after inoculation; all plants gave a disease severity score of 6.2 or above (Table 4.19). For inocula containing sap from susceptible rice cultivars disease severity scores were high (above 6.4) at dilutions of 1:10 and 1:200 but, when diluted 1:1000, the severity values decreased to 5.0-5.4 (Table 4.19). When inocula contained resistant plant extracts, the disease severity was affected by virus dilutions in that at a dilution of 1:10 values were similar to the buffer controls but at dilutions 1:200 and 1:1000 disease severity was much reduced compared with buffer controls (Table 4.19).

Experiment 2

Results of experiment 1 (section 4.3.2.2) indicated that clear effects on infection and disease progression were only obtained when inocula contained extracts from resistant plants and when tested at a dilution of 1:1000. However, results were essentially similar for inocula containing saps from Lac 23 and Moroberekan and for inocula containing ITA 212 and Bg 90-2. Therefore in the next experiment two dilutions (1:10 and 1:1000 w/v) were tested and sap from Lac 23, ITA 235 and ITA 212 were used. The experiment was also extended to include inocula of all five isolates available.

Disease incidence: Irrespective of virus isolate or source of diluent all test plants produced symptoms. However, for all inocula diluted 1:1000 with rice extracts overall means (all isolates combined) gave disease incidences of 88%, 68% and 52% when sap from ITA 212, ITA 235 and Lac 23 were used as diluents respectively. Differences between isolates were slight (Table 4.20).

Disease severity: Symptom severity scores were similar irrespective of virus isolate or diluent source when tested at a final virus dilution of 1:10 (Table 4.20). When tested at 1:1000 and irrespective of virus isolate severity scores were highest for inocula diluted with buffer only and the values progressively decreased when inocula contained sap from healthy ITA 212, ITA 235 and Lac 23.

Incubation period: Initial symptoms appeared 9-10 days after inoculation with all RYMV isolates when diluted 1:10 with healthy

Table 4.20 Disease incidence and severity rating of cv. ITA 212 plants producing symptoms within 40 days following inoculation with five RYMV isolates diluted with buffer or with sap from three rice cultivars

Virus dilution (w/v)	Virus isolate	Virus diluted with sap from Lac 23 Disease incidence[a]	Lac 23 Disease severity[b]	ITA 235 Disease incidence	ITA 235 Disease severity	ITA 212 Disease incidence	ITA 212 Disease severity	Buffer control Disease incidence	Buffer control Disease severity
1:10	IC	100	6.6	100	6.5	100	6.5	100	6.5
	N	100	6.0	100	6.2	100	6.8	100	6.8
	SL	100	6.2	100	6.3	100	6.5	100	6.5
	Nr	100	6.5	100	6.4	100	6.8	100	6.8
	K	100	6.8	100	6.9	100	6.7	100	6.6
1:1000	IC	60	5.2	70	5.4	90	5.7	100	6.2
	N	50	5.1	70	5.8	90	6.1	100	6.0
	SL	40	4.9	60	5.3	80	6.0	100	5.8
	Nr	50	5.4	70	6.1	90	6.4	100	6.5
	K	60	5.4	70	6.1	90	6.4	100	6.5

[a] There were 10 plants per treatment

[b] Disease severity scale ranged from 0 = symptomless to 7.0 = complete necrosis. Means calculated only from plants which produced symptoms

Table 4.21 Incubation periods of five RYMV isolate inoculated to rice (cv. ITA 212) following dilution with healthy sap from three rice cultivars

Virus dilution (w/v)	Virus isolate	Incubation periods days: Virus diluted with sap from: Lac 23 Mean	Lac 23 Range	ITA 235 Mean	ITA 235 Range	ITA 212 Mean	ITA 212 Range	Buffer control Mean	Buffer control Range
1:10	IC	11.0	9-14	12.6	10-16	11.8	9-14	11.4	10-16
	N	13.8	10-16	10.6	10-16	11.3	9-14	12.2	10-14
	SL	12.1	9-14	12.2	10-16	11.8	9-14	11.8	10-16
	Nr	11.4	9-16	11.3	9-16	11.0	9-12	10.6	9-14
	K	10.8	9-14	10.9	9-14	10.9	9-14	10.9	9-14
1:1000	IC	21.5	14-28	17.3	14-28	18.6	14-24	16.8	12-20
	N	22.4	14-32	20.8	14-28	18.0	14-24	16.8	12-20
	SL	25.5	14-32	21.0	14-28	21.2	14-24	20.8	12-28
	Nr	22.0	12-28	19.7	14-28	18.0	12-24	16.6	12-20
	K	17.6	12-28	18.3	14-24	16.4	12-24	14.2	10-20

rice extracts or buffer; neither diluent source nor virus isolate had a clear effect on the incubation period at this dilution (Table 4.21). For all virus isolates tested at a diluent of 1:1000 the mean length and range of the incubation period increased according to the dilution used. Thus, values were shortest with buffer as diluent and tended to increase when inocula contained sap from ITA 212, ITA 235 and Lac 23 (Table 4.21).

Experiment 3

Results of experiments 1 and 2 indicated the possible presence of an inhibitory compound(s) in extracts of resistant rice cultivars which affected the proportion of inoculated plants successfully infected, lengthened the incubation periods and reduced symptom severity. An experiment was made to examine the distribution of RYMV antigen in ITA 212 plants inoculated with RYMV in the presence or absence of saps from healthy rice cultivars.

Disease incidence: All plants inoculated with RYMV-N at a final dilution of 1:10 (w/v) eventually showed symptoms whether inoculated leaves were removed 5 days after inoculation or not (Table 4.22). With inocula diluted 1:1000 (w/v) disease incidence was influenced by removal of inoculated leaves. When inoculated leaves of ITA 212 were removed 5 days after inoculation, disease incidence was 70% for inocula mixed with buffer, 10% for inocula containing sap from cv. Lac 23, whereas inocula containing sap from ITA 235 and ITA 212 both gave an intermediate value of 50%. When inoculated leaves were not removed, disease incidence reached 100% in plants inoculated with the virus-buffer mixture then progressively decreased when inocula

Table 4.22 Disease incidence 40 days after inoculation of RYMV-N on cv. ITA 212 plants when virus inoculum diluted with rice extracts or buffer and inoculated leaves removed or retained 5 days after inoculation.

Virus dilution	Proportion (%) of plants showing symptoms[a]							
	Source of diluent:							
	Lac 23		ITA 235		ITA 212		Buffer control	
	R[b]	N[c]	R	N	R	N	R	N
1:10	100	100	100	100	100	100	100	100
1:1000	10	50	50	70	50	90	70	100

[a] Ten plants were used per treatment

[b] R = Inoculated leaves removed 5 days after inoculation

[c] N = Inoculated leaves not removed

contained sap from ITA 212, ITA 235 and Lac 23 (TAble 4.22).

Detection of RYMV in inoculated and non-inoculated tip leaves: Table 4.23 shows that RYMV antigen was detected after 5 days in inoculated leaves of all plants inoculated with inoculum diluted 1:10 but antigen was not detected in the tip leaves. When inocula were diluted 1:1000 virus was not detected in either inoculated or non-inoculated tip leaves after 5 days. However, when tip leaves were removed after 30 days, 10% (one plant) of plants inoculated with inocula containing Lac 23 leaf sap contained antigen. Values then increased for inocula containing sap from ITA 235 (50%), ITA 212 (50%) or buffer (70%). When inoculated leaves were not removed and tip leaves were tested after 15 days, all extracts were RYMV-positive with inocula diluted at 1:10 whereas the proportion ranged from 0 (Lac 23) to 70% (buffer control) for inocula diluted 1:1000 (Table 4.23). On testing tip leaves after 30 days for the 1:1000 dilution inoculation, the number of extracts RYMV-positive increased from 50% (diluent sap Lac 23) to 100% (buffer control).

Experiment 4

As there was evidence that healthy saps affected the interaction between RYMV and ITA 212, the next experiment attempted to discover whether the sap had a direct effect on the virus. Therefore the *in vitro* contact period between mixing RYMV inoculum with buffer or healthy sap and inoculation was varied.

Effect of sap contact and time on virus infectivity: RYMV extracted from ITA 212 infected leaves was diluted 1:1000 with healthy sap

Table 4.23 Detection of RYMV antigen by ELISA in the inoculated and tip leaves of ITA 212 plants following inoculation with RYMV-N diluted with healthy sap extracts or buffer.

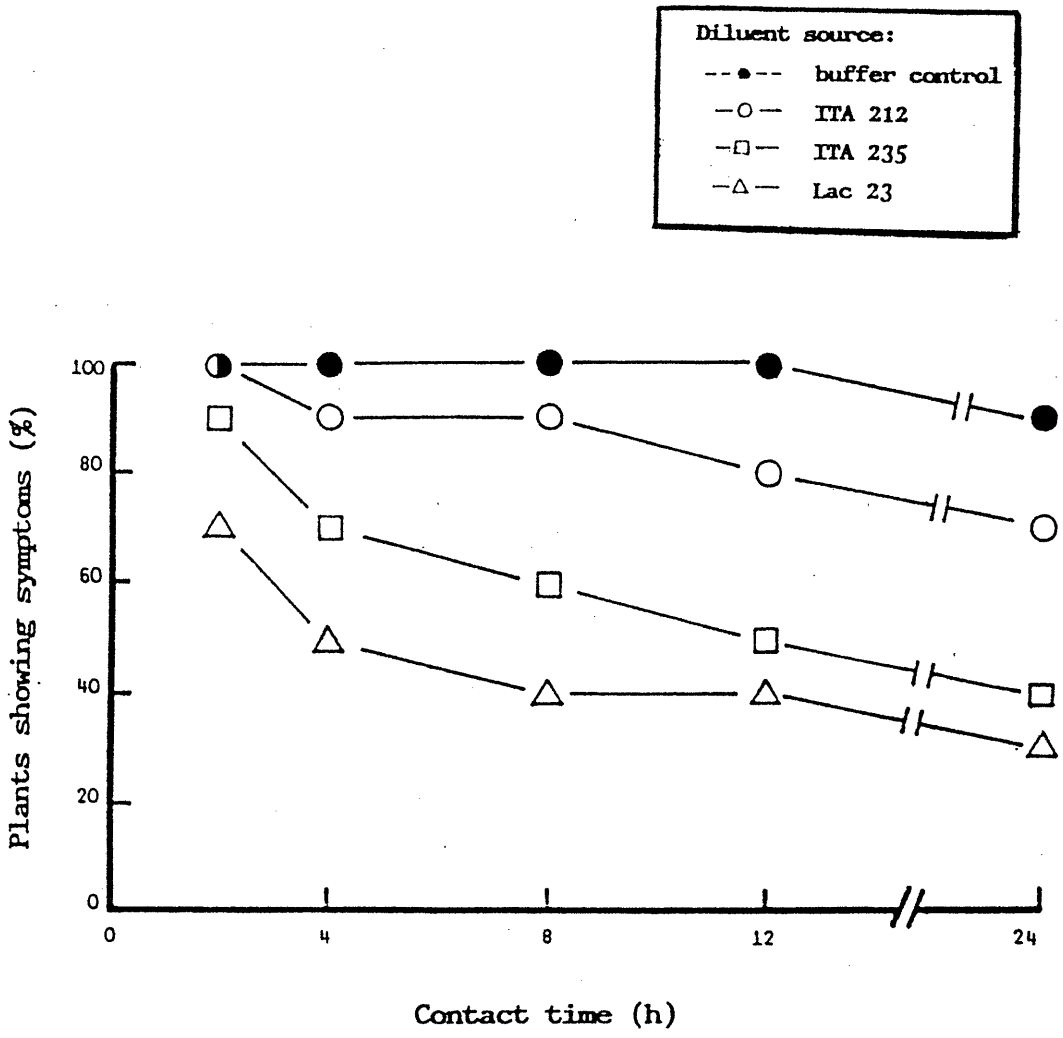
Harvest (days after inoculation)	Virus dilution	Leaves tested	Proportion (%) of test leaf extracts RYMV-positive			
			Source of diluent:			
			Lac 23 sap	ITA 235 sap	ITA 212 sap	Buffer control
5	1:10	Inoculated	100	100	100	100
		Youngest	0	0	0	0
	1:1000	Inoculated	0	0	0	0
		Youngest	0	0	0	0
30[a]	1:1000	Youngest	10	50	50	70
15[b]	1:10	Youngest	100	100	100	100
	1:1000	Youngest	0	40	50	70
30[c]	1:1000	Youngest	50	70	90	100

[a] Leaves taken from the same batch of plants from which inoculated leaves were removed after 5 days.

[b] Inoculated leaves not removed.

[c] Same batch of plants from which tip leaves removed after 15 days.

Fig. 4.9 Effect of time of inoculation on the infectivity of RYMV mixed with sap from cvs. ITA 212, ITA 235 or Lac 23 or with buffer control.



extracts from cvs. Lac 23, ITA 235 and ITA 212 and buffer as before, but mixtures were allowed to stand at room temperature for varying periods before inoculation.

Fig. 4.9 shows that increasing the contact time from 2 to 24 hours had little effect on the infectivity of RYMV preparations diluted with buffer but caused a slight decrease for inocula containing ITA 212 sap. The proportion of ITA 212 plants showing symptoms was, however, much reduced with inocula containing ITA 235 and Lac 23 following only 2 hours contact time. Increasing the contact time to 4 hours sharply increased the effect and there was a steady further decline in the number of plants producing symptoms as the contact time was extended to 24 hours.

4.3.3 Scanning electron microscopy

The objective of this study was to examine the leaf surface of resistant and susceptible cultivars to see if any differences existed which might explain the variation in the proportion of plants successfully infected by sap inoculation noted in earlier experiments.

4.3.3.1 MATERIALS AND METHODS

Leaf 3 of each cultivar tested was cut into 5 mm segments and immediately immersed in vials containing 2.5% cold glutaraldehyde in 0.05 M phosphate buffer (pH 7.0). Capped vials were stored at -4°C for fixation for 24 hours, then most of the fixative was removed and the tissue pieces washed with two changes of buffer, 20 minutes each.

Specimens were quickly transferred to critical point dryer specimen containers and dehydrated by passage through a series of increasing concentrations of ethanol (50, 70, 80, 90% ethanol; 20 minutes each) then exposed to three changes of 100% ethanol for 20 minutes each. The next stage was critical point drying where the ethanol is exchanged for liquid CO_2, temperature and pressure are increased until the 'critical point' is reached when liquid CO_2 becomes gaseous. The material was critical point dried in Emscope model CPD 750 for 4 hours after which it was kept dust-free and dry before fixing to an aluminium stub using araldite or colloidal silver as adhesive. Specimens were left to dry under dust-free conditions. The specimen, adhesive and stub were coated with a very thin film of gold in a 'sputter coater' (Nanotech Semprep 2). Specimens were finally viewed with an Hitachi S430 SEM operating at an accelerating voltage of 15 kv.

4.3.2.2 RESULTS

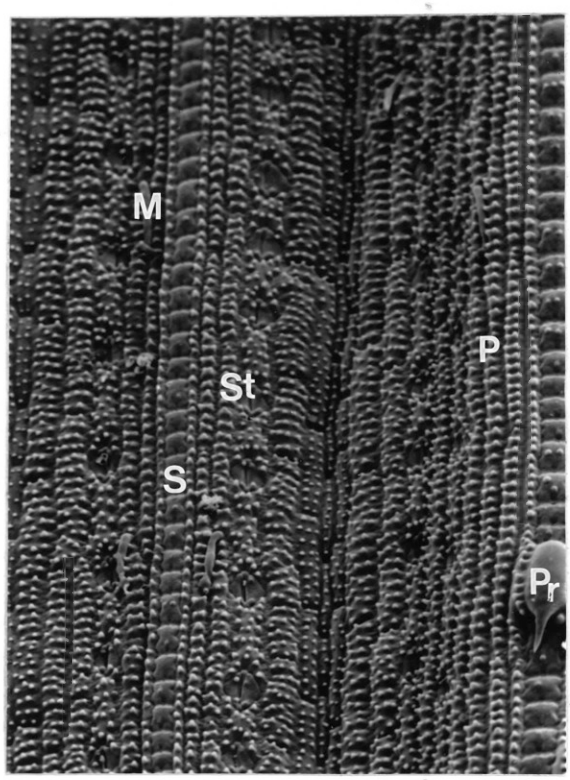
Comparisons were made between the susceptible rice ITA 212 and the resistant rice cultivars ITA 235 and Lac 23.

Like most cereal leaves, the anatomy of the leaf surface of rice leaves was quite complex. Over the veins there were single or double rows of dumb-bell shaped silica cells (Fig. 4.11.s) and in between each silica cell was a cork cell (Fig. 4.11 s) with a distinct papilla over each cork cell (Fig. 4.11 p). Sometimes the silica cells were replaced by prickle hairs but such hairs were also seen elsewhere (Fig. 4.10pr). In the intercostal region there were one or two parallel rows of stomata (Fig. 4.10 st) and also many rows

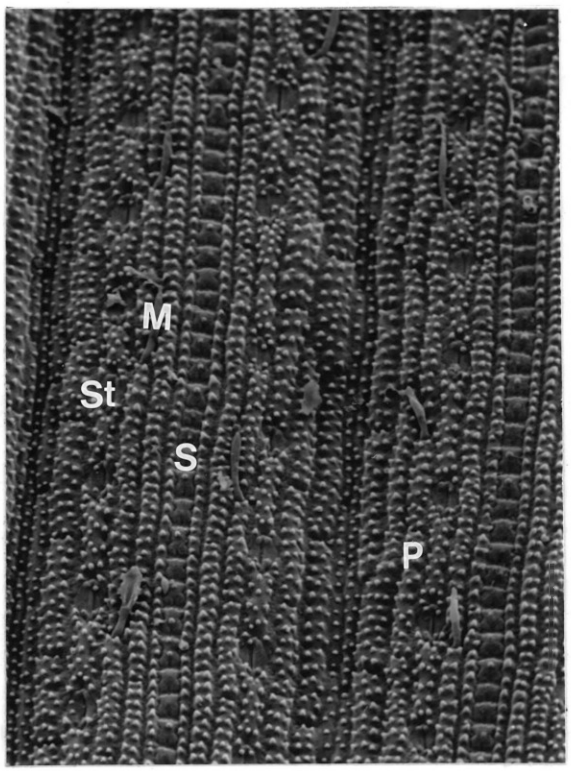
Fig. 4.10 Scanning electron micrographs of the leaf surface of rice cultivars. A) Lac 23, B) Moroberekan, C) ITA 235, D) ITA 212.

Scale bar = 50 um

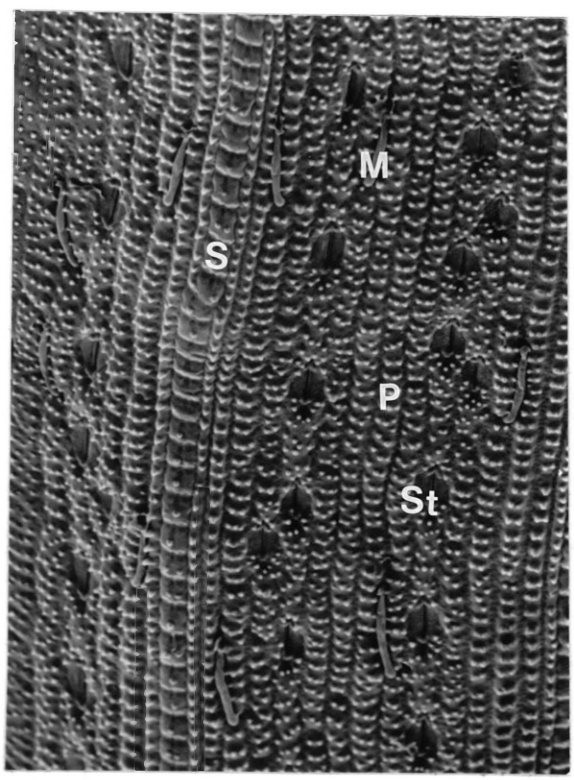
S = rows of silica cells and cork cells
Pr = Prickle hair
St = stoma
M = microhair
P = papilla
C = celite particles
Ma = macrohair



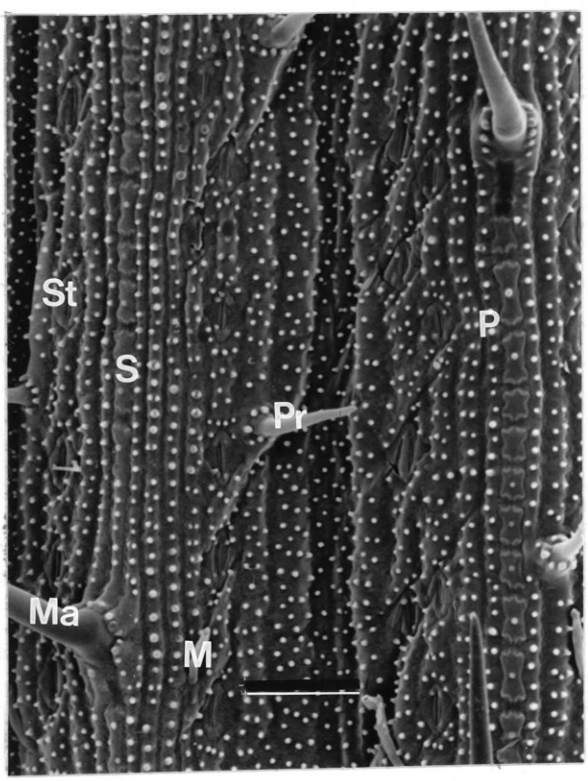
A



B



C

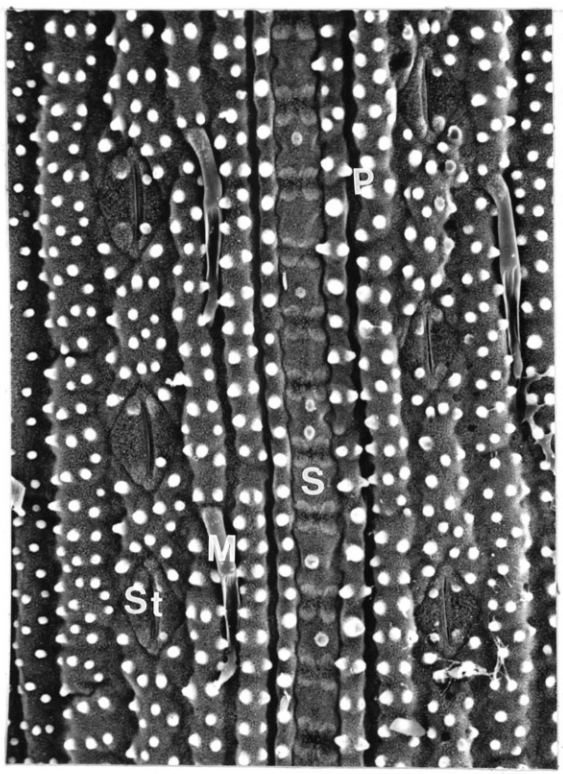


D

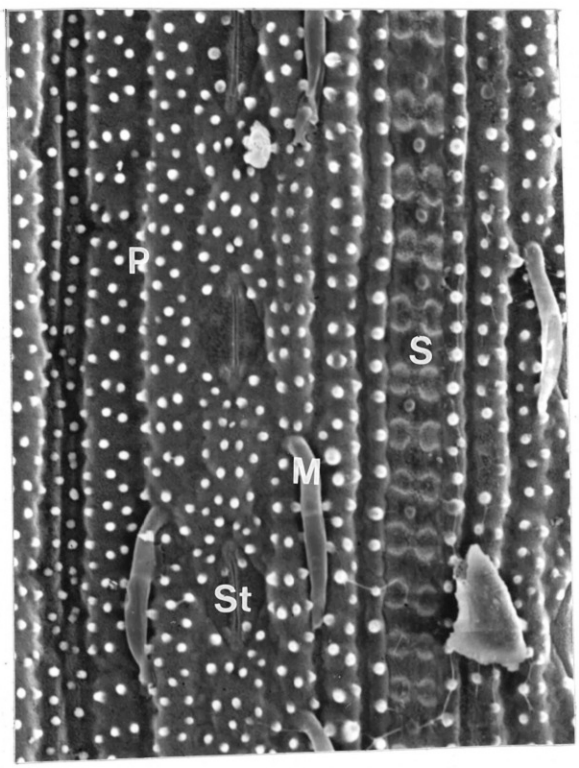
Fig. 4.11 Scanning electron micrographs of the leaf surface of rice cultivars after abrasion with buffer.

Scale bar = 50 um

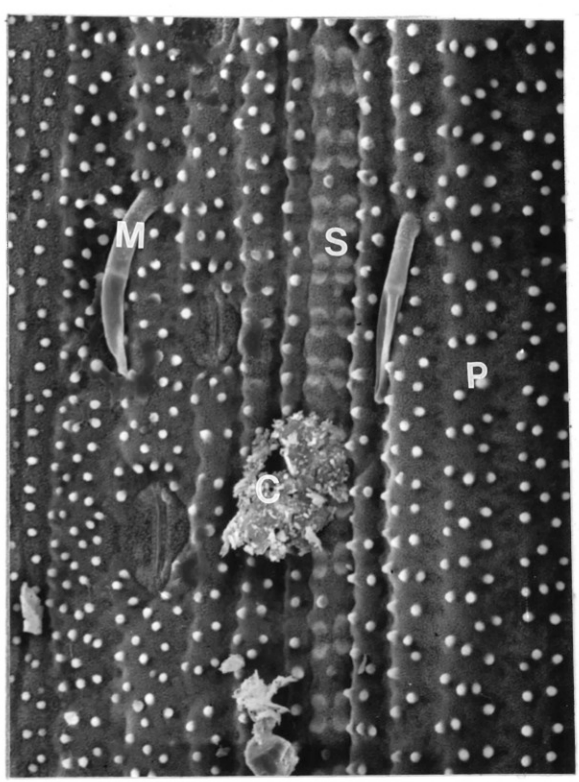
S = rows of silica cells and cork cells
Pr = prickle hairs
St = stoma
M = microhair
P = papilla
C = celite particles
Ma = macrohair



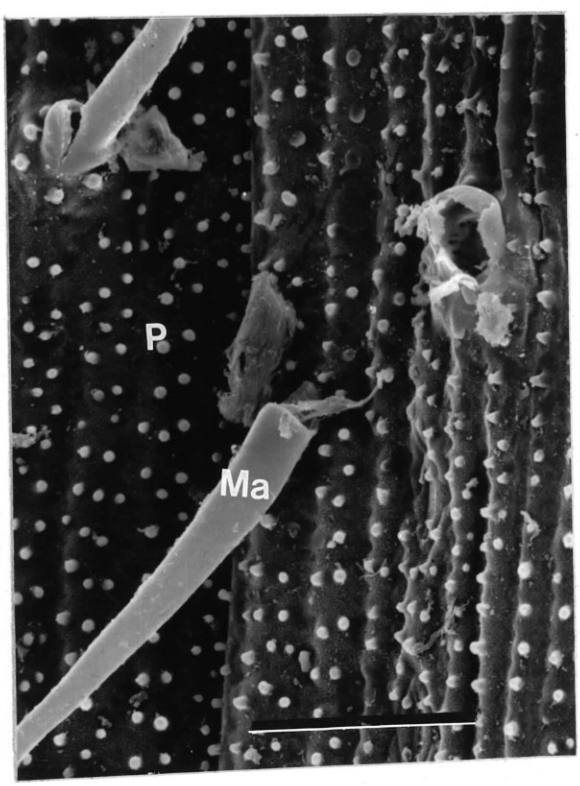
A



B



C



D

of long cells. Above the surface of some long cells were macrohairs (Fig. 4.10 Ma) which were unicellular, sharply pointed and with a ring of small cuticular papillae at the base. Another type of hair present on long cells was the microhair, delicate, two celled and without a ring of papillae at the base (Fig. 4.11 M). In the middle of the intercostal region were 2-3 parallel rows of long cells with papillae but without hairs.

Observation of the surface of leaf 3 showed that the upper leaf surface of resistant cultivars was in general similar to susceptible ITA 212 but differed in type, size and number of epidermal trichomes (Fig. 4.10). Trichomes were generally more numerous in cv. ITA 212 and this cultivar also possessed numerous large macrohairs not found in any of the resistant cultivars.

When leaf 3 was abraded with buffer containing celite (Fig. 4.11) celite particles could be seen on the micrographs. The only clear evidence of abrasion damage was complete breakage of the macrohairs present in cv. ITA 212 creating clear potential virus entry sites (Fig. 4.11, ITA 212). In the resistant cultivars microhairs were frequently damaged as evidenced by the collapsed distal cell of many microhairs (Fig. 4.11) although clear entry ports for virus particles was not seen in the micrographs. Visible damage to epidermal cells was not seen other than the removal of the distal portion of papillae (not shown in Fig. 4.11) but this damage did not create obvious virus entry ports. Microhairs and prickle hairs and papillae were mostly damaged on the ribs, there being little damage in the intercostal regions.

4.3.4 DISCUSSION

In this section an attempt was made to examine some of the features of resistance to RYMV shown by some rice cultivars. The cultivars used (Lac 23, Moroberekan and ITA 235) were known to be resistant to Nigerian isolates of RYMV (John *et al.*, 1985) and experiments in section 3 had shown that these cultivars were also resistant to isolates RYMV-SL, -IC, -Nr and -K. The experiments in this section compared various aspects of RYMV pathogenesis in the three resistant cultivars with pathogenesis in susceptible cv. ITA 212 and sometimes BG 90-2.

A convenient starting point is infection of inoculated leaves. Infection of these leaves was usually determined by ELISA serology as infected inoculated leaves never displayed symptoms in either resistant or susceptible cultivars inoculated with any of the five RYMV isolates. Infection of inoculated leaves was particularly important in resistant cultivars because initial interaction studies (section 3) had established that not all inoculated resistant plants became systemically infected which may have been a result of failure to successfully infect the inoculated leaf or inability of the virus to be translocated to non-inoculated young leaves.

ELISA tests with extracts of inoculated leaves of susceptible ITA 212 showed that RYMV antigen could be detected in the majority (80%) of inoculated leaves as early as one day after inoculation and, by 3 days, all inoculated leaf extracts were RYMV positive. Thus, inoculated leaves of this cultivar were extremely susceptible to infection and the early detection of RYMV by ELISA suggested that

virus replication in these leaves was rapid.

Plants were routinely inoculated at the 4-5 leaf stage with virus sap diluted 1:10 and inoculations made to leaves 2 and 3. When these conditions were varied by inoculating leaf 2 or 3 or 4 or leaves 2+3+4 once or twice with inoculum diluted 1:10 or 1:1000 all inoculated ITA 212 leaves became infected, so susceptibility to infection was unaffected by inoculated leaf position or the number of inoculations made.

In the resistant cultivars, RYMV antigen was first detected after about 3 days in inoculated leaves of a few plants of Lac 23 (10%) and ITA 235 (20%) (Table 4.9) and, after 5 days, in Moroberekan (40%) (Table 4.4). The proportion of inoculated leaves containing detectable levels of RYMV-N then progressively increased until after 15 days total infection (100%) had occurred in ITA 235. However, total infection never occurred in Lac 23 or Moroberekan even when inoculated leaves were tested 30 days after inoculation, maximum infection being 80-90%. Thus, antigen accumulation appeared to be slower in resistant than susceptible cultivars and, for Lac 23 and Moroberekan, virus was not detected at all in some inoculated leaves. The slow accumulation of antigen in inoculated leaves of resistant cultivars compared with ITA 212 meant that ELISA values were low and often only just above the threshold value when inoculated leaves were tested soon after inoculation. This could lead to some uncertainty regarding the success or failure of inoculation. However, when leaves were tested about 20 days after inoculation, antigen amounts were sufficient to yield A_{410} values well above the threshold value thus leaving no doubt which inoculated leaves had been successfully

infected. Inoculated leaves of Lac 23 and Moroberekan ELISA tested 20 days after inoculation still gave 10% of extracts which were RYMV negative. These plants never developed symptoms and virus was not found in young leaves tested 60 days after inoculation. This tendency to escape virus infection was rarely seen in ITA 235 and was never encountered in ITA 212.

In contrast to the situation in susceptible ITA 212, infection of inoculated leaves in resistant cultivars was influenced by the position of the inoculated leaves, inoculum concentration and the number of inoculations. Thus, when leaf 2 of Lac 23 was inoculated less than half (40%) of the inoculated leaves were successfully infected, whereas 80% of inoculated leaves were ELISA positive when leaf 3 or leaf 4 or leaves 2+3+4 were inoculated (Table 4.13). Furthermore, total infection occurred when combined leaves (2+3+4) of Lac 23 were inoculated twice with RYMV-N diluted 1:10 (Table 4.15) whereas 80% infection was obtained when leaves were inoculated once but no infection occurred in leaves inoculated either once or twice with RYMV-N diluted 1:1000 (w/v) (Table 4.15).

Such results showed that resistance to infection *per se* occurred in cultivars Lac 23 and Moroberekan and that the extent of this resistance could be influenced by inoculum dilution, inoculated leaf position and the number of inoculations made. The tendency of some plants to escape infection when a population of a given cultivar, inbred line or clone from the same mother plant is exposed to equal chances of virus infection following mechanical or vector inoculation has been reported in other virus/host systems. For example, the phenomenon has been observed in rice with rice tungro

virus (Ling, 1969; Srinivasan, 1981), maize and maize dwarf mosaic virus (Anzola *et al*., 1982) and in ryegrass and ryegrass mosaic virus (Gibson *et al*., 1980; Salehuzzaman and Wilkins, 1984). Claims for resistance to virus infection should be based on experiments which include tests on the inoculated leaves. In some studies such resistance was based mostly on symptom expression (e.g. Hibino *et al*., 1988, rice and rice tungro spherical virus) and/or the absence of the virus in extracts of non-inoculated tip leaves of inoculated plants tested by ELISA (Allen *et al*., 1982; cowpea and cowpea mottle virus, Cho and Goodman, 1982; soybean and soybean mosaic virus). In such cases it is possible that latent infection may have occurred in inoculated symptomless plants and unless inoculated and young leaves were tested for virus, claims for resistance to infection based on symptoms only must be treated with caution. Moreover, unless tests are made on inoculated leaves the possibility of virus restriction to inoculated leaves cannot be discounted.

In the present study identification of resistance to infection was based not only on the absence of visual symptoms 60 days after inoculation, but also the absence of RYMV-N following ELISA tests on extracts of both inoculated and young non-inoculated leaves. Although the virus was not detected in some inoculated plants, they may not be immune (Walkey, 1985). The possibility exists that the inoculated leaves of such plants were subliminally infected and the ELISA tests made were insufficiently sensitive to detect the very low amounts of virus involved. It is possible that the reduced infection of resistant compared with susceptible plants following sap inoculation might be a reflection of difficulties in sap inoculating resistant plants due to mechanical barriers on the leaf surface.

However, although scanning electron microscopy examination of the surface of leaf 3 of resistant and susceptible rice cultivars revealed some differences in the leaf surface anatomy, no obvious features were seen which might explain the comparative ease whereby susceptible plants could be infected. Celite-induced damage was seen on all leaves evident mainly as the breakage of macrohairs in susceptible ITA 212 and collapse of the distal cells of microhairs in resistant cultivars.

It is noteworthy that resistance to infection has not been reported in studies made in Africa with resistant and susceptible rice genotypes although the general experiments made there were not designed specifically for this purpose (e.g. John *et al.*, 1985; Fomba, 1988). However, if such resistance had been frequent within a given genotype population the likelihood is that it would have been noticed. The possibility exists that environmental factors may influence the frequency of plant resistance to infection *per se* but this has yet to be examined.

Consideration will now be given to the relationship between infection of inoculated leaves and the subsequent distribution of RYMV in rice. A potentially very significant finding was that in resistant but not in susceptible cultivars, successful infection of inoculated leaves was not an inevitable prelude to systemic invasion. When susceptible ITA 212 plants were inoculated with RYMV-N all inoculated leaves were successfully infected and the virus always subsequently invaded the plants systemically and induced symptoms. By contrast, in Lac 23 and Moroberekan although the majority (about 80%) of inoculated plants contained RYMV in inoculated leaves tested

30 days after inoculation, some of these plants (20-40%) did not develop systemic symptoms and the virus was not detected in young symptomless leaves tested up to 60 days after inoculation. This phenomenon was observed in Lac 23 and Moroberekan plants whether plants were inoculated once or twice with inoculum diluted 1:10 and irrespective of leaf position inoculated. When the inoculum dilution, leaf inoculated and number of inoculations made resulted in infection of the inoculated leaf, ITA 235 plants were subsequently systemically invaded by RYMV-N. These results indicated the operation of a resistance mechanism involving restriction of virus to symptomless inoculated leaves in cultivars Lac 23 and Moroberekan but not in cvs. ITA 212 and ITA 235. Restriction of virus to the inoculated leaves is usually associated with the hypersensitive reaction and the production of conspicuous necrotic or chlorotic lesions. There are, however, instances where, as in the present case, restriction is not accompanied by symptom production in inoculated leaves. For example, Wyatt and Kuhn (1979) found that in a resistant cowpea introduction (PI 186465) no symptoms were produced in either primary or trifoliate leaves following inoculation with cowpea chlorotic mottle virus (CCMV). The amount of CCMV in the inoculated leaves of PI 186465 was much less than in susceptible lines. They suggested that restriction of virus movement was more effective than virus replication. In a very interesting paper, Lei and Agrios (1986) examined maize dwarf mosaic virus strain B (MDMV-B) movement within the inoculated leaves of susceptible and resistant maize genotypes using fluorescent antibody staining. They found that resistant and susceptible genotypes produced similar amounts of MDMV-B in the inoculated leaves but virus remained localized in the resistant inbred lines whereas susceptible plants became systemically

infected. They also found that the number of infection sites in the symptomless inoculated leaves of resistant inbred lines were either higher or equal to those in susceptible lines and therefore suggested that the mechanism of resistance was not resistance to virus infection. In addition Lei and Agrios (1986) discovered that the rate of cell to cell movement was higher or equal in resistant compared with susceptible inbred lines. Thus, the evidence suggested that virus restriction was not mediated by effects on cell to cell spread but against systemic movement.

In the present study, no information is available on the relative number of infection loci or the rate of RYMV cell to cell spread in inoculated leaves of resistant and susceptible rice cultivars, so an explanation of the basis of virus restriction to inoculated leaves in just some plants of Lac 23 or Moroberekan awaits further studies along the lines of those used by Lei and Agrios (1986).

It was considered possible that there might be a correlation between the rate and extent of virus accumulation in inoculated leaves and subsequent systemic infection in resistant compared with susceptible cultivars in Lac 23 plants showing either virus restriction or subsequent systemic invasion. Generally the results supported this notion. The ELISA absorbance values for extracts of inoculated leaves of those Lac 23 (Table 4.12) plants that eventually displayed systemic symptoms were about twice those of infected leaves on plants in which the virus did not invade systemically when inoculated leaves were tested 12 days after inoculation. The conclusion was further supported when a larger number of resistant

plants were inoculated (Table 4.18). Here the A_{410} ELISA absorbance readings for extracts of inoculated leaves of Lac 23 plants that eventually showed systemic infection were at least three times higher than in inoculated leaves of plants which did not show systemic infection. A similar trend was also evident for cv. Moroberekan. The absorbance values for inoculated leaf extracts of ITA 212 were always substantially higher than for Lac 23 (e.g. Table 4.12) and, as noted previously, the inoculated leaves always became infected and all plants eventually produced systemic symptoms. Similarly for cv. ITA 235, virus amounts, as evidenced by A_{410} values, were higher than in cvs. Lac 23 and Moroberekan (although lower in cv. ITA 212) and here successful infection of inoculated leaves was always associated with systemic invasion. Overall, the greater the amount of virus in the inoculated leaves, the greater was the probability of systemic invasion.

It is becoming increasingly likely that systemic virus movement is not a passive process (e.g. Atabekov and Dorokhov, 1984) and that a distinct virus-specific transport function (TF) may be associated with a virus coded protein (Atabekov and Dorokhov, 1984). This function has been shown to be associated with a 30K protein for TMV and similar proteins exist for several other plant viruses. Thus hypotheses attempting to explain virus (e.g. RYMV) restriction to inoculated leaves or variable rates of egress from inoculated to non-inoculated leaves will have to take into account the TF. The TF is likely produced at the initial infection sites and is responsible for systemic movement, but to induce systemic movement it may be that the TF has to accumulate to a certain threshold level. This hypothetical level is very likely associated with the rate of replication and/or

the number of infection loci in the inoculated leaves.

In the present work, studies were not made on the number of infection loci in inoculated leaves but assessments were made of virus content at various times after inoculation. Determination of virus content in inoculated leaves by ELISA showed that there was a gradual increase in virus content with time for all rice cultivars inoculated with RYMV-N. Virus accumulated more readily in leaf 3 than leaf 2 (Table 4.6) but in both leaves RYMV-N reached substantial amounts (*c.* 40-270 µg/g tissue) within 15 days of inoculation in susceptible ITA 212. This situation was in marked contrast to the very slow and low virus content of resistant cultivars Lac 23 and Moroberekan where amounts of only 5-25 µg/g tissue were found 20 to 30 days after inoculation. This difference may be either due to an intrinsicly lower rate of increase in the resistant cultivars and/or the creation of fewer infection loci and reduced cell to cell spread in the two resistant cultivars. As with other aspects of pathogenesis noted earlier, ITA 235 was intermediate between the two extremes. Here, RYMV-N reached 12-175 µg/g tissue after 20 to 30 days.

The possible relationship between virus accumulation rate in inoculated leaves and the likelihood of later systemic movement in resistant and susceptible cultivars was further examined in experiments where the virus source (inoculated leaves) was removed and tested for RYMV antigen at varying intervals after inoculation. This approach gave an indication of the time of initial egress from inoculated leaves leading to the successful establishment of systemic infection and leaves could be removed at a time when virus content

was too low to detect (up to *c.* 10 days) in the inoculated leaves of resistant cultivars. The failure of virus recovery from non-inoculated leaves and the absence of systemic symptoms 60 days after inoculation was interpreted to mean that the virus had not moved systemically from inoculated leaves by the time of leaf removal. When inoculated leaves were removed one day after inoculation, some inoculated plants of both ITA 212 (25%) and ITA 235 (15%) eventually showed systemic symptoms (Table 4.11) whereas in cultivars Lac 23 and Moroberekan systemic symptoms failed to appear and RYMV antigen was not detected in young leaves when inoculated leaves were removed after 5 days. As the time between inoculation and removal of the inoculated leaf increased, the number of plants showing systemic symptoms progressively increased.

When extracts of young non-inoculated leaves were tested by ELISA, RYMV-N was detected in ITA 212 leaves after only 5 days, in ITA 235 leaves after 10 days and then in Lac 23 and Moroberekan 15 days after inoculation.

Results relating to the amount of virus in the inoculated leaves, detection of RYMV-N in inoculated and young leaves and the relationship between removal of virus source and subsequent systemic symptom production were, overall, suggestive of some type of resistance to virus movement in resistant compared with susceptible rice cultivars. However, whether this is a consequence of the slow rate of replication in the inoculated leaves of resistant cultivars and possibly the creation of fewer infective sites together with a reduction in cell to cell movement, is not known. To show that the largely unknown factors which might affect systemic virus movement

per se probably required experiments to be made along the lines of those of Lei and Agrios (1986). If systemic invasion is slower or occurs in a lower proportion of resistant compared with susceptible plants (as occurs with RYMV) and if virus content and the number of infection loci are similar in resistant compared with susceptible plants, then it would seem likely that movement *per se* is less efficient. The reduced content of RYMV-N in resistant rice cultivars suggests that reduced movement may be a consequence of factors affecting replication in the inoculated leaves.

The movement of RYMV-N in rice has not previously been studied but resistance to virus movement has been claimed for several cereal host/virus combinations. Earlier Tu and Ford (1970) had reported that MDMV-B reached similar amounts in the inoculated leaves of resistant (Illinois A) and susceptible (Seneca Chief) maize varieties, but systemic mosaic symptoms rarely developed in the resistant varieties. They suggested that resistance to MDMV-B involved resistance to virus movement in the vascular system. Jones and Tolen (1972) reported that MDMV concentration in a resistant (T8 x 07B) maize hybrid was equivalent to that in susceptible maize even though symptom expression was delayed by 5 to 6 days in the resistant hybrid. They suggested that the resistance mechanisms did not involve resistance to virus infection or multipliction but did involve movement of the virus in the host. In wheat, oat and barley, Jensen (1973) found that cultivars which differed in their susceptibility to barley yellow dwarf virus (BYDV) also showed differences in the rate of systemic BYDV movement.

In many systems studied, with the exception of MDMV-B in

maize, it is difficult to distinguish between a direct effect on movement *per se* and related factors such as differential rates of replication in inoculated leaves which could have a more indirect effect on movement.

Following discussion of the results obtained relating to infection of inoculated leaves, virus accumulation in inoculated leaves and movement of RYMV-N into non-inoculated leaves, attention will now be focussed on RYMV in successfully systemically invaded leaves.

As mentioned earlier, RYMV was not detected in young leaf extracts of ITA 212 tested 5 days after inoculation but after 10 days the virus was found in young leaves of all inoculated plants. In resistant cultivars the systemic presence of RYMV was delayed. The virus was detectable 10 days after inoculation in ITA 235 (20% of plants inoculated) and after 15 days in Lac 23 and Moroberekan (both 10%). Thereafter there was a progressive increase in the number of resistant plants containing RYMV in young leaves.

Concerning the amount of virus in non-inoculated leaves, amounts estimated for non-inoculated leaves of susceptible ITA 212 were consistently greater than in the three resistant cultivars irrespective of leaf position (Table 4.3) or time after inoculation tested (Table 4.6). It should be noted that estimates of RYMV content in young leaves of ITA 212 were made on leaves of the main shoot up to about 20 days after inoculation (e.g. Table 4.6) but, after 20 days, many (sometimes all) plants were killed by infection. Estimates for ITA 212 later than 20 days were from tillers produced

by the few plants which survived. Young leaves on the main shoot of resistant cultivars were tested up to 90 days after inoculation.

Initial experiments (Table 4.3) showed that at 20 days after inoculation RYMV was present in all non-inoculated leaf positions in cv. ITA 212. Amounts of RYMV were least in leaf 4 in which symptoms were not produced, and progressively increased to about 4.6 mg/g tissue in the youngest leaf (leaf 7) in which symptoms were conspicuous. However, RYMV-N was only just detectable in Lac 23 at all leaf positions after 20 days. Determinations for Lac 23 after 30 and 40 days showed that RYMV amounts increased at each leaf position with time and that amounts progressively increased in each newly produced leaf. Trends for changes in virus content were related to symptom severity; as symptoms increased so did virus content. However, virus content was always very much lower in Lac 23 compared with values obtained for ITA 212 after 20 days. The maximum virus content obtained for Lac 23 was in leaf 8 after 40 days and was only 168 µg/g tissue; far lower than the 1-4 mg/g leaf tissue for ITA 212 leaves.

A more extensive experiment with all three resistant cultivars showed essentially similar trends (Table 4.6) when the youngest leaf was sampled at various times after inoculation. Here RYMV could not be detected in resistant cultivars until 20 days after inoculation. Thereafter, amounts increased progressively in tests made after 30 and 40 days, reaching values of 328, 645 and 1128 µg/g tissue for cvs. Lac 23, Moroberekan and ITA 235 respectively. When tested after 90 days, amounts decreased to about half the above values. Clearly, by about 45 days substantial RYMV-N accumulation had occurred in all

three resistant cultivars but leaves still showed mild symptoms. Moreover, amounts were always much less than in susceptible ITA 212. In ITA 212, RYMV was first found in tip leaves after 10 days and the amounts increased rapidly to about 6.4 mg/g tissue by 30 days. As with resistant cultivars, there was a decrease in RYMV content in the later samples (45 and 90 days). In this experiment the virus content of susceptible ITA 212 was 50-100 times greater than in resistant cultivars after 15 days but thereafter, although RYMV-N reached consistently greater amounts in the susceptible than in the resistant cultivars, the magnitude of the difference declined until after 90 days there was two to three times more virus in the tillers of the surviving ITA 212 plants than in the three resistant cultivars.

The high amounts of RYMV-N detected in susceptible ITA 212 warrant further comment. Amounts of up to 6 mg/g tissue regularly obtained from ELISA absorbance values and the dose response curves with partially purified preparations were generally greater than recovery amounts of about 1 mg/g tissue calculated from spectrophotometric values obtained for purified preparations (detailed later in section 5). The 1 mg/g tissue obtained for purified preparations was in general agreement with the similar values also obtained spectrophotometrically by Bakker (1974) and Hull (1977). The latter worker also reported recoveries of *c.* 1 mg/g tissue for southern bean mosaic virus (SBMV) and several other sobemoviruses and Kahn and Dawson (1973) found up to 3 mg/g tissue of SBMV in leaves of *Phaseolus vulgaris* harvested 15 days after inoculation. Clearly, estimates using partially purified virus will likely underestimate the *in vivo* amounts due to almost inevitable losses during purification. As far as the writer is aware, there

are no previous reports of amounts of sobemoviruses calculated from ELISA data. Estimates of viral antigen levels by ELISA may overestimate the amounts of intact or infective particles as antibodies do not discriminate between infective or non-infective particles or between intact and partially degraded virions. It is possible that the several and usually high dilutions of sap required to obtain ELISA values 'on scale' may have introduced some error to the determination. Nevertheless, RYMV-N clearly reaches exceedingly high amounts in susceptible rice leaf tissue; amounts probably approaching or even exceeding the 5-10 mg/g tissue frequently found for TMV (Zaitlin, 1979).

The results obtained for virus content of non-inoculated leaves clearly show that RYMV invasion of these leaves is slower in resistant than susceptible cultivars and that once invaded, virus accumulation in resistant cultivars never reached the amounts found in susceptible ITA 212 after a relatively short time. This strongly suggests that some form of resistance to virus replication operates in resistant cultivars. Whether this is in the form of an inherently low replication rate in each invaded cell, reduced cell to cell movement in non-inoculated leaves or that fewer cells are actually supporting RYMV replication in resistant non-inoculated leaves is not known. This type of resistance expressed as reduced virus accumulation in systemically infected leaves has been reported for other cereal host/virus combinations including oats and barley yellow dwarf virus (Jedlinski *et al.*, 1977), wheat and wheat streak mosaic virus (Hunger and Sherwood, 1985), rice and rice tungro virus (Hibino *et al.*, 1987) and oat and oat mosaic virus (Catherall and Valentine, 1987).

The reduced virus titre of resistant cultivars may play a role in the epidemiology of the virus in that it could be one factor which could lead to a decrease in disease incidence. Certainly a low virus inoculum dose, for example as obtained from resistant compared with susceptible rice, could reduce the probability of natural infection. As discussed earlier (see also Table 4.15), a 1:1000 w/v dilution of sap inoculum was insufficient to infect resistant but not susceptible cultivars. It is possible that plant to plant spread of RYMV by the beetle vector may well be more rapid and efficient in crops of susceptible than resistant rice because of the higher virus inoculum dose obtained by the vector from the leaves of susceptible plants. This possibility has yet to be explored in the natural spread of RYMV in Africa. Such a situation has been shown to exist for viruses with aphid vectors in that reduced virus content of resistant cultivars has been associated with a reduced likelihood of transmission by natural aphid vectors. For example, Zitter (1975) showed that resistant pepper varieties contained reduced amounts of pepper mottle virus (PMV) and were less likely to serve as a source of PMV for aphids than susceptible pepper varieties.

Virus content studies also showed there was an association between virus content and symptom severity. Thus, for example, the virus content of ITA 235 (moderately resistant; disease severity moderate but higher than Lac 23) was higher than in Lac 23 (highly resistant, mild symptoms) but less than in ITA 212 (highly susceptible; severe symptoms). Such associations have been found in other experimental systems; for example, Jedlinski *et al*. (1977) found a strong association between disease severity in oats infected

with barley yellow dwarf virus and virus titre. However, such associations are not inevitable.

The above has shown that resistance in rice to RYMV may involve at least four components. First, resistance to the establishment of infection which was shown best in some plants of cultivars Lac 23 and Moroberekan and particularly obvious when plants were inoculated with RYMV-N diluted 1:1000. Secondly, restriction of the virus to the inoculated leaves found particularly in some plants of cvs. Lac 23 and Moroberekan but much less common in ITA 235. Thirdly, the possibility of resistance to virus movement and lastly, resistance to virus accumulation in both inoculated and systemically infected non-inoculated leaves. The last two components may be directly related to the delay in symptom appearance and hence the lengthy incubation period and to the mild symptoms produced in resistant cultivars. No single factor alone can explain the contrasting features exhibited by resistant compared with susceptible rice genotypes. Resistance based upon multiple factors is not uncommon. Resistance in some maize lines and hybrids was characterized by lower disease incidence (resistance to the establishment of infection) together with a delay in symptom appearance when infected and also a reduced rate of virus accumulation (Kuhn and Smith, 1977; Anzola *et al*., 1982). In perennial ryegrass resistance to virus infection (most effective) and post-infection (resistance to virus multiplication and movement) resistance were found together in some ryegrass clones tested against ryegrass mosaic virus (Salehuzzaman and Wilkins, 1984).

An intriguing feature of RYMV infection of resistant cultivars

(particularly Lac 23 and Moroberekan) was that for every experiment with these cultivars there were three possible interactions with RYMV. Some plants were not infected, in others virus was restricted to inoculated leaves and in yet others the plants became systemically infected and symptom expression or the lack of could be related to virus distribution. It was possible that the seed source was not homogeneous and that such variability was a reflection of seed variation. However, the results of experiment 5, in which plants were grown from seeds collected from symptomless inoculated plants, from inoculated plants which developed symptoms and from the usual IITA seed source suggested seed variation was not the cause. The proportion of plants with infected inoculated leaves and the proportion of these which did or did not lead to subsequent systemic invasion and concomitant systemic symptoms was unaffected by the seed source. If such variation was inheritable, then it is reasonable to suppose that seed from plants which had produced symptoms would give rise, in their progeny, to a higher proportion of symptom-producing plants than plants from seed gathered from inoculated but symptomless plants. This did not happen. It is possible that not all plants received the necessary threshold of virus for infection of inoculated leaves to occur - this would seem unlikely in view of the high amounts of RYMV shown to be present in ITA 212 which provided donor leaves for inoculum preparation and the leaves were only diluted 1:10 (w/v). Where inoculated leaves were infected perhaps the required virus threshold was not always reached to ensure systemic invasion when non-inoculated 'sink' leaves were showing optimal 'sink strength'. Overall, the results suggested that factors governing systemic invasion were not inherited and that they existed in all the seed sources tested to a similar extent and must, for the time being,

be accommodated under "plant to plant variation". It is possible that environmental factors were involved such as the microclimate around individual plants, as suggested by Scott and Rosenkranz (1987).

In section 4.3.2 the possibility was examined that inhibitory substances might be present in greater amounts or with greater potency in sap from resistant than susceptible rice and that such a compound(s) might, in some way, be associated with resistance.

Results showed that materials were present in leaf sap from healthy plants which, when mixed with virus inoculum obtained as a crude sap from ITA 212 and inoculated as a mixture to susceptible ITA 212, reduced the proportion of plants successfully infected, extended the incubation period and reduced symptom severity. Such effects were apparent for all five RYMV isolates but a clear effect was dependent on the source cultivars from which sap was obtained and also on the final concentration of the virus in the inoculum applied to the ITA 212 test plants. Changes in the numbers of plants infected, incubation period and symptom severity were most pronounced when the healthy sap source was Lac 23 or Moroberekan, less pronounced but usually evident when ITA 235 sap was used and minimal or absent when ITA 212 sap was included in the inoculum. However, if the final virus dilution was 1:10 (w/v) then the preceding effects were minimized or overcome. Clearly sap effects required that the virus be diluted beyond 1:10 and ideally to about 1:1000 (w/v). ELISA determination of RYMV distribution in treated plants confirmed the slower systemic invasion of ITA 212 plants when virus was mixed with sap from resistant rice cultivars compared with the small effect

caused by ITA 375 and ITA 212 saps.

When the various mixtures were allowed to stand for varying times before inoculation, increasing this 'contact time' was accompanied by a progressive reduction in inoculum infectivity indicating a direct effect on the virus *in vitro*. It would appear that all the rice saps tested could affect infectivity this way but that the greater effects were obtained with saps from Lac 23 and Moroberekan, ITA 235 was intermediate and the effect of ITA 212 sap was negligible. These direct effects on the virus probably explain the subsequent changes in incubation periods and symptom severity when inocula were used at 1:1000; a dilution which was probably approaching the threshold for infection. A more pronounced *in vitro* inactivation or inhibition would probably be needed for effects to be evident with the high virus concentration present at a dilution of 1:10 (w/v). No evidence was obtained of an *in vivo* effect of the healthy sap treatments. That the saps of resistant plants had the greater effects may be fortuitous. It cannot, however, be ruled out that further study might reveal a more profound reason for the fact that effects were greatest with Lac 23 and Moroberekan saps, intermediate with ITA 235 sap and least with sap from ITA 212, a sequence identical to the degrees of resistance exhibited by these cultivars to RYMV. It is not known if sap constituents affect the probability of infecting resistant rice but the *in vitro* action suggests that they should.

It is noteworthy that the clear progressive *in vitro* reduction in the infectivity of RYMV in the presence of Lac 23 and Moroberekan sap might be of some consequence in the natural spread of the virus

by the beetle vectors. Clearly, the acquisition of leaf sap by such chewing insects will expose RYMV particles to possible inhibitors. Might RYMV be rapidly inactivated on the mouthparts of vectors which have fed on RYMV resistant rice but inactivated much more slowly after feedings on susceptible cultivars? Moreover, such a possibility is, perhaps, even more likely in view of the fact that results in this study have shown that RYMV concentrations are much reduced in resistant compared with susceptible cultivars and, as stated above, the effects of leaf sap were greatest with dilute virus preparations.

S E C T I O N F I V E

RELATIONSHIPS AMONG RICE YELLOW MOTTLE VIRUS ISOLATES

5.0 RELATIONSHIPS AMONG RYMV ISOLATES

5.1 INTRODUCTION

Although much effort has been made at I.I.T.A. in Nigeria to develop high-yielding rice varieties with resistance to RYMV (Rossel, 1986), little attention has so far been given to the relationships between isolates of RYMV from different geographical regions in Africa. The only serological study, by Fauquet and Thouvenel (1977) in the Ivory Coast, showed that with gel-double diffusion tests spur formation occurred between the Kenyan (type isolate) and an isolate from the Ivory Coast in reciprocal tests using antisera produced against both isolates. No other serological comparisons between RYMV isolates appear to have been made.

In a comparative study of Kenyan and Nigerian isolates at Wageningen (IITA, 1986), a set of *Oryza glaberrima*, *O. barthii* and *O. sativa* accessions were inoculated with both isolates. Symptoms in susceptible rice accessions were slightly more severe with the Kenyan than the Nigerian isolate. One *O. barthii* accession (TOb 5701) and one *O. glaberrima* accession (TOg 7235) differentiated between the two isolates in that symptoms failed to develop in both accessions with the Nigerian isolate whereas the Kenyan isolate induced the production of distinct symptoms (IITA, 1986). It was later shown (IITA, 1987) that *O. glaberrima* accessions TOg 5674 and TOg 5681 did not develop symptoms when inoculated with both Kenyan and Nigerian RYMV isolates and the virus was not detected from the two accessions using ELISA. Such studies suggested that accessions of African *Oryza* species may be used to differentiate between RYMV isolates.

Knowledge of the serological relationships between RYMV isolates is valuable in determining the effective use of ELISA in diagnostic work and may prove to be important in epidemiological studies and disease control.

In this study, serological comparisons were made among the five isolates of RYMV by means of agar-double diffusion, DAS-ELISA (direct ELISA) and immunosorbent electron microscopy (ISEM) techniques. The relative infectivity of the five isolates of RYMV in extracts from susceptible and resistant cultivars and interactions of the virus isolates with *O. glaberrima* accessions were also briefly studied.

5.2 MATERIALS AND METHODS

5.2.1 Purification of RYMV

Two purification methods were used. The first method tried was used for experiments with RYMV-N for which homologous antiserum was available and was based on the method of Bakker (1974).

Seedlings (cv. ITA 212) were mechanically inoculated with RYMV-N at the 4-5 leaf stage as before with inocula prepared from systemically infected leaves of rice cv. ITA 212 inoculated 3 weeks previously. Plants were maintained in the glasshouse and systemically infected leaves harvested one week after symptom appearance but before the leaves turned necrotic. Infected leaves were stored at -20°C as 5 g samples until used.

Five grams of frozen leaves were cut into *c.* 0.5 cm lengths and homogenized for 2 minutes in a Sorval Omni-mixer with 50 ml of 0.1 M phosphate buffer (Na_2HPO_4-KH_2PO_4) pH 5.0, containing 0.1 M ascorbic acid. The homogenate was filtered through two layers of muslin and the pulp re-extracted with a further 50 ml of grinding medium and strained. Chloroform (50 ml) was slowly added to the combined homogenate (100 ml) and the mixture stirred for 30 minutes at 4°C. The emulsion was separated by low speed centrifugation (1500 g; 15 minutes; 4°C) using a Denley 13 R 401 centrifuge and the aqueous phase collected.

Solid ammonium sulphate was added to the aqueous phase (1 g:50 ml) with stirring until the ammonium sulphate had completely

dissolved. The preparation was allowed to stand for 30 minutes at laboratory temperature. The precipitate was removed by centrifugation (2600 g; 15 minutes). The supernatant was collected and solid ammonium sulphate (1 g:5 ml) added with stirring at room temperature. After standing for 30 minutes, the suspension was centrifuged at 4200 g for 15 minutes and the precipitate resuspended in 10 ml of 0.1 M phosphate buffer (pH 5.0) containing 0.1 M ascorbic acid and kept for 4 hours at 4°C.

The suspension was dialysed for 18 hours at 4°C against 400-500 ml of 0.1 M phosphate buffer (pH 5.0) containing 0.1 M ascorbic acid. The buffer was renewed once. The dialysis tubing was boiled prior to use in 10^{-4} M EDTA for 15 minutes. After cooling, the tubing was rinsed with double-distilled water for 30 minutes and then kept in 0.01 M phosphate buffer (pH 7.0) at 4°C.

After low speed centrifugation (4200 g; 15 minutes; 4°C) the supernatant was subjected to high speed centrifugation (78,000 g; 100 minutes; Centrikon Ultracentrifuge; rotor TFT 70.38; 4°C). The clear supernatant was discarded and the pellet resuspended overnight in 2 ml 0.01 M phosphate buffer (pH 7.0) then centrifuged for 15 minutes at 4200 g at 4°C. The supernatant was retained for further use.

The same procedure as above was applied to 5 g frozen uninoculated cv. ITA 212 leaves for control purposes.

Partially purified virus and the control preparation at 100 µg/ml was inoculated to rice cv. ITA 212 to test for infectivity. The ultraviolet absorption spectrum was determined with a Pye Unicam

SP8-100 spectrophotometer. Virus concentration was estimated assuming $E^{0.1\%}_{1\ cm,\ 260nm}$ 6.5 (Bakker, 1974).

The partially purified preparation was stored at -20°C with 50% glycerol (Rowhani and Mircetich, 1988).

For quantitative and comparative purposes, a more rapid purification method was used to purify the five RYMV isolates. The purification procedure used was based on the methods of Hull (1977); Denlyo *et al*., (1978) and Brisco *et al*. (1985) for the purification of various sobemoviruses.

Seedlings of rice (cv. ITA 212) were inoculated with each RYMV isolate at the 4-5 leaf stage as before. Systemically infected leaves were harvested one week after initial symptom appearance but before leaves turned necrotic and stored at -20°C.

Five grams of frozen leaves were cut into 0.5 cm lengths and homogenized in a Sorval Omni-mixer for 2 minutes with 50 ml of 0.1 M sodium acetate buffer (pH 4.8). The homogenate was strained through two layers of muslin and the pulp re-extracted in a further 50 ml of buffer and strained. After stirring for 30 minutes at 4°C and centrifugation at 10,000 g for 15 minutes at 4°C (RC5C Sorvall centrifuge, Du Pont), the supernatant was collected. Solid polyethylene glycol (PEG 6000) and NaCl were slowly added to the supernatant while stirring to give a final concentration of 10% (w/v) and 1% (w/v) respectively. Stirring was continued for 1 hour at 4°C. The precipitate was collected by low speed centrifugation (10,000 g; 15 minutes; 4°C) and the pellet collected and resuspended in 10 ml of

0.1 M sodium acetate (pH 5.0). Following low speed centrifugation, the supernatant was subjected to a high speed step (101,00 g; 2 hours; Centrikon Ultracentrifuge; rotor TFT 70.38; 4°C). The clear supernatant was discarded and the pellet re-suspended in 2 ml 0.1 M sodium acetate (pH 5.0) overnight and clarified by low speed centrifugation. The supernatant was retained for further use. This protocol was used for all five RYMV isolates. Infectivity was tested by inoculating cv. ITA 212 plants with the partially purified preparations at a concentration of 100 µg/ml.

Absorbance and virus concentration were determined as before. The partially purified virus was stored at 4°C with 0.02% (w/v) chlorobutanol (Hull, 1985).

5.2.2 Electron microscopy

To determine particle dimensions, suspensions of the partially purified virus with an A_{260} value of 0.65 (100 µg/ml) were stained with 1% aqueous uranyl acetate (pH 4.5) on 300 mesh copper grids coated with formvar and examined with a Hitachi H7000 electron microscope at an instrument magnification of X 60000. At least 100 particles were measured for each isolate and TMV particles were included on each grid as an internal standard (Bos, 1975).

Measurements of virus particle size were done using Micro Measure, a computer programme for the Commodore Amiga computer, developed at Wye College by Jon Varley. The system consisted of a microcomputer (Amiga 500) connected to a video camera via a Genlock device which allows the computer graphics to be overlaid onto the

video picture. Using the computer mouse and pointer, measurements of objectives on the video picture can be made, and using a simple calibration technique, measurements can be converted into appropriate real units.

5.2.3 Agar gel double-diffusion tests

Agar gel-diffusion tests (Ouchterlony, 1948) were performed in plastic petri dishes (9 cm diameter) containing 15 ml of agar medium. For all tests 1.0 g agar (Ionagar No. 2, Oxoid Ltd., London) was dissolved in 100 ml distilled water containing 0.85 g NaCl and 0.02% (w/v) sodium azide. Antiserum and antigen wells were 5 mm in diameter and 5 mm apart; six peripheral wells surrounded a central well which usually contained the antiserum. Antisera to RYMV-N, -K and -IC were kindly provided by Drs. G. Thottappilly (IITA, Nigeria), D.A. Peters (Agricultural University, Wageningen) and C. Fauquet (ORSTROM, Ivory Coast), respectively.

Antisera were diluted 1:32 (v/v) with 0.85% NaCl (Bakker, 1974; Fauquet and Thouvenel, 1977). RYMV antigens were prepared from infected leaves or rice, cv. ITA 212 showing typical symptoms of RYMV. Leaves were ground (tissue:medium ratio 1:16 w/v) in 0.85% NaCl and the sap expressed through two layers of muslin. Healthy rice (cv. ITA 212) sap was prepared similarly for control purposes. The plates were incubated at room temperature for 4 days. Precipitation lines were first visible after 24 hours when virus isolates were tested against homologous or heterologous antisera, but no reaction was observed with healthy sap controls with any antiserum.

5.2.4 Immunosorbent electron microscopy (ISEM)

The method used was based upon the methods of Derrick (1973) and Milne (1986). Fresh formvar (1.5%)-coated electron microscope grids were floated, film side down, on a 15 µl droplet of the γ-globulin fraction (1 mg/ml) of the antiserum prepared for ELISA (section 2.6) diluted 1:100 (v/v) with 0.1 M Sorensen's buffer (pH 7.0). After 5 minutes (Milne, 1986) at room temperature, grids were then washed with 30 drops of Sorensen's buffer. After draining, grids were placed, coated side down, on 20 µl droplets of test leaf extract (diluted 1:100 (w/v) with 0.1 M Sorensen's buffer, pH 7.0) for 15 minutes at room temperature, then washed with 20 drops of buffer and 30 drops of distilled water. Without drying, grids were stained with 10 drops of 2% (w/v) uranyl acetate in distilled water. Excess stain was removed with filter paper, grids dried and examined in the electron microscope.

The 'decoration' extension of the ISEM technique involved an additional step. After the second wash with buffer, grids were drained and floated on a 15 µl droplet of γ-globulin, coated side down, for 5 minutes at room temperature. Grids were then washed with 20 drops of buffer and 30 drops of distilled water, stained with 10 drops of 2% uranyl acetate and examined in the electron microscope.

Leaf extracts were prepared by grinding infected tissue (1:100 w/v) in 0.1 M Sorensen's buffer (pH 7.0), squeezing through muslin and subjection to low speed centrifugation (4200 g for 5 minutes).

5.2.5 ELISA

Direct double antibody sandwich, enzyme linked immunosorbent assay (DAS-ELISA) methods were essentially as described in the general material and methods (section 2.6). For each experiment at least two replicate wells per treatment were used and each experiment was repeated twice. Absorbance values presented in the results were the average of two experiments. ELISA tests were made with serial half-$\log_{10}$ dilutions of partially purified preparations of all five RYMV isolates stored at 4°C with 0.02% chlorobutanol and with serial 10-fold dilutions of sap extracts from RYMV-infected rice cv. ITA 212 leaves. Antisera to RYMV-N, -K and -IC were used. Extracts of healthy leaves were always included. Gamma globulins were used at 1 mg/ml and alkaline phosphatase conjugates at a dilution of 1:1500 (v/v).

5.2.6 Infectivity

The infectivity and infectivity dilution end points of RYMV preparations from resistant and susceptible cultivars were, in the absence of a local lesion host (Bakker, 1974), tested on rice cv. ITA 212 using serial extract dilutions.

Plants of cvs. ITA 212, ITA 235 and Lac 23 were inoculated with RYMV isolates at the 4-5 leaf stage and kept in the glasshouse where temperatures ranged from 21-30°C with a daily average of 24-25°C. The youngest leaves of plants showing symptoms were harvested 20, 40 and 60 days after inoculation. Frozen harvested leaves were thawed and ground with a mortar and pestle to give a 1:1 (w/v)

dilution with Sorensen's buffer. A ten-fold dilution series (10^{-1}-10^{-7} w/v) was made and each dilution tested on five plants of cv. ITA 212. Symptoms were recorded on the 1575 plants inoculated 40 days after inoculation.

5.2.7 Reaction of RYMV isolates in *Oryza glaberrima*

Two accessions, TOg 7235 and TOg 5674 of African rice (*O. glaberrima*) were inoculated with all five RYMV isolates at the 4-5 leaf stage. Inoculum was prepared by grinding infected leaves of cv. ITA 212 in 0.067 M Sorensen's buffer (pH 7.0) (1:10 w/v). Using five plants of *O. glaberrima* per isolate, leaves 3 and 4 were inoculated. Thirty days after inoculation the inoculated leaves were harvested, then 60 days after inoculation, the youngest leaf was harvested. Leaves 3 and 4 of each plant (inoculated leaves) were combined as one sample. Each sample of inoculated or non-inoculated leaves was divided into two, one sub- sample for ELISA indexing and the other for back inoculation tests to cv. ITA 212 (four plants/subsample).

5.3 RESULTS

5.3.1 Purification and spectrophotometry

Isolate RYMV-N was initially purified using the method based on Bakker (1974). Spectrophotometric determinations on the partially purified preparation gave an absorption maximum at 260 nm and a minimum at 242 nm. The $A_{260:280}$ ratio (not corrected for light scattering) was 1.51±0.02 indicative of a nucleic acid content of *c.* 20% (Paul, 1959). The method gave yields (estimated spectrophotometrically) of 300-500 µg RYMV/g tissue from infected ITA 212 leaves.

The second purification method, used for comparative purposes, was satisfactory. All isolates could be purified within 2-3 days whereas Bakker's method required 3 or more days per isolate. Furthermore, the second procedure yielded more virus than the first.

Purified preparations of the five isolates gave UV absorption spectra typical of nucleoproteins. Figure 5.1 shows the UV spectrum of RYMV-N which was typical of all five RYMV isolates examined.

Ultraviolet absorption spectra results (Table 5.1) showed that maximum absorption was at 260 nm for all RYMV isolates and the minimum at 242 nm (IC, N, Nr, K) and 243 nm for isolate SL. Mean values for the $A_{260:280}$ ratio (not corrected for light scattering) ranged from 1.43-1.49 (Table 5.1). The A_{max}/A_{min} ratio was 1.32±0.02 for all isolates.

Fig. 5.1 UV absorption spectrum of RYMV-N (not corrected for light scattering)

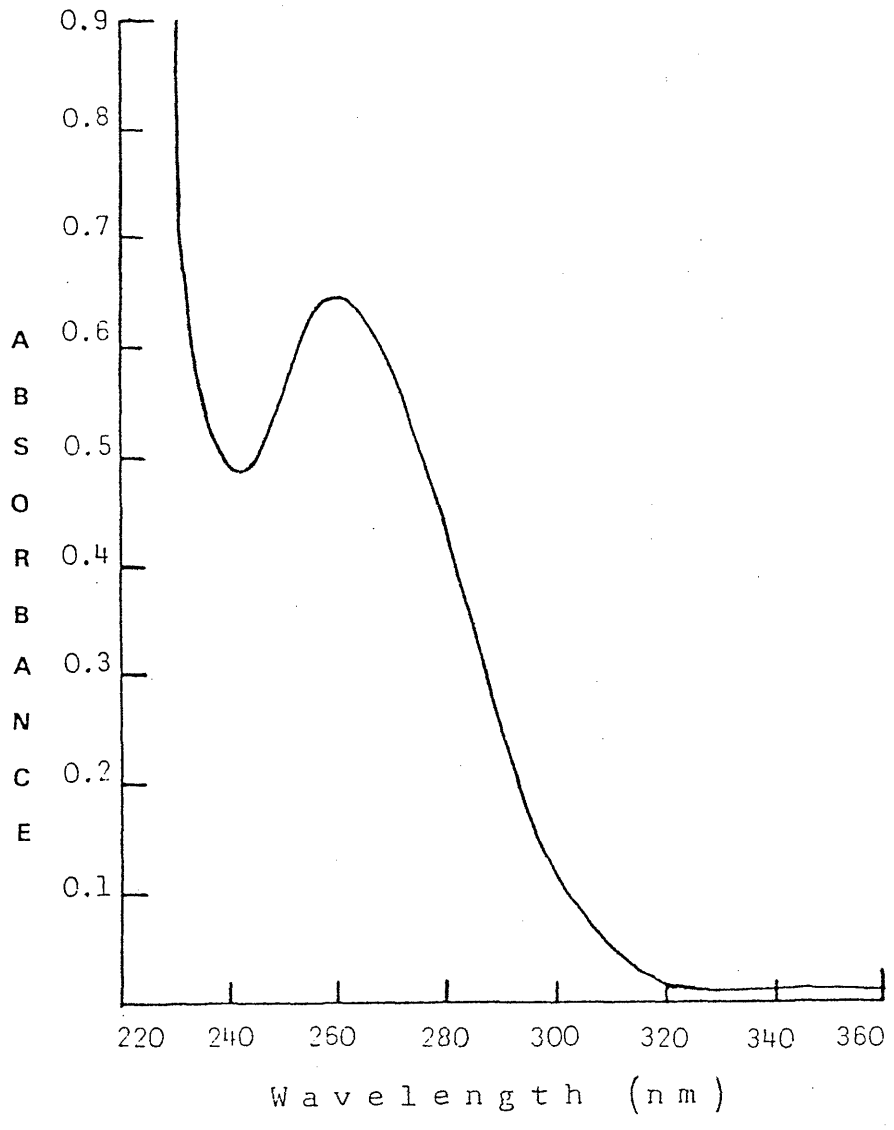


Table 5.1. Ultraviolet absorption spectra data (not corrected for light scattering) for partially purified preparations of five isolates of RYMV.

RYMV isolate	A_{max} (nm)[a]	A_{min} (nm)[a]	Ratio[a] $A_{260:280}$	Estimated[b] recovery (mg/g tissue)
IC	260	242	1.49±0.02	0.50
N	260	242	1.48±0.01	0.75
SL	260	243	1.43±0.02	0.50
Nr	260	242	1.46±0.02	1.00
K	260	242	1.49±0.01	1.00

[a] All preparations were diluted to an A_{260} value of c. 0.65

[b] From systemically infected leaves of rice cv. ITA 212 one week after initial symptom appearance.

The highest virus concentration (about 1 mg/g tissue) occurred in leaves infected with RYMV-Nr and K whereas the lowest (about 0.5 mg/g tissue) was found with RYMV-IC and SL.

The infectivity test results showed that ITA 212 (10 plants/isolate) plants inoculated with each purified isolate at 100 µg/ml exhibited RYMV symptoms 8-10 days after inoculation and all 10 plants developed symptoms; all preparations were therefore infective.

5.3.2 Electron microscopy

Preparations were stained with 1% uranyl acetate and 1% phosphotungstic acid (PTA). The best preparations were obtained with uranyl acetate (Fig. 5.2). Time did not permit examination using other stains such as ammonium molybdate and methylamine tungstate.

Electron microscope examination of partially purified preparations of RYMV isolates at a concentration of 100 µg/ml revealed numerous isometric prticles that often had an angular outline (usually hexagonal) as a result of a clear tendency for particles to aggregate into semi-crystalline arrays (Fig. 5.2). Particles stained clearly and there was no evidence of 'empty' particles or excessive stain penetration into particles. Protein subunit architecture was not clearly seen. There was little evidence of particle disruption or the presence of extraneous material indicative of poorly purified preparations.

Particle size from purified preparations was measured and calculated using TMV as the internal standard. A hundred particles

Fig. 5.2 Electron micrographs of particles of RYMV isolates stained with uranyl acetate. (Scale bar = 100 nm)

a) RYMV-N b) RYMV-SL c) RYMV-IC
d) RYMV-K e) RYMV-Nr

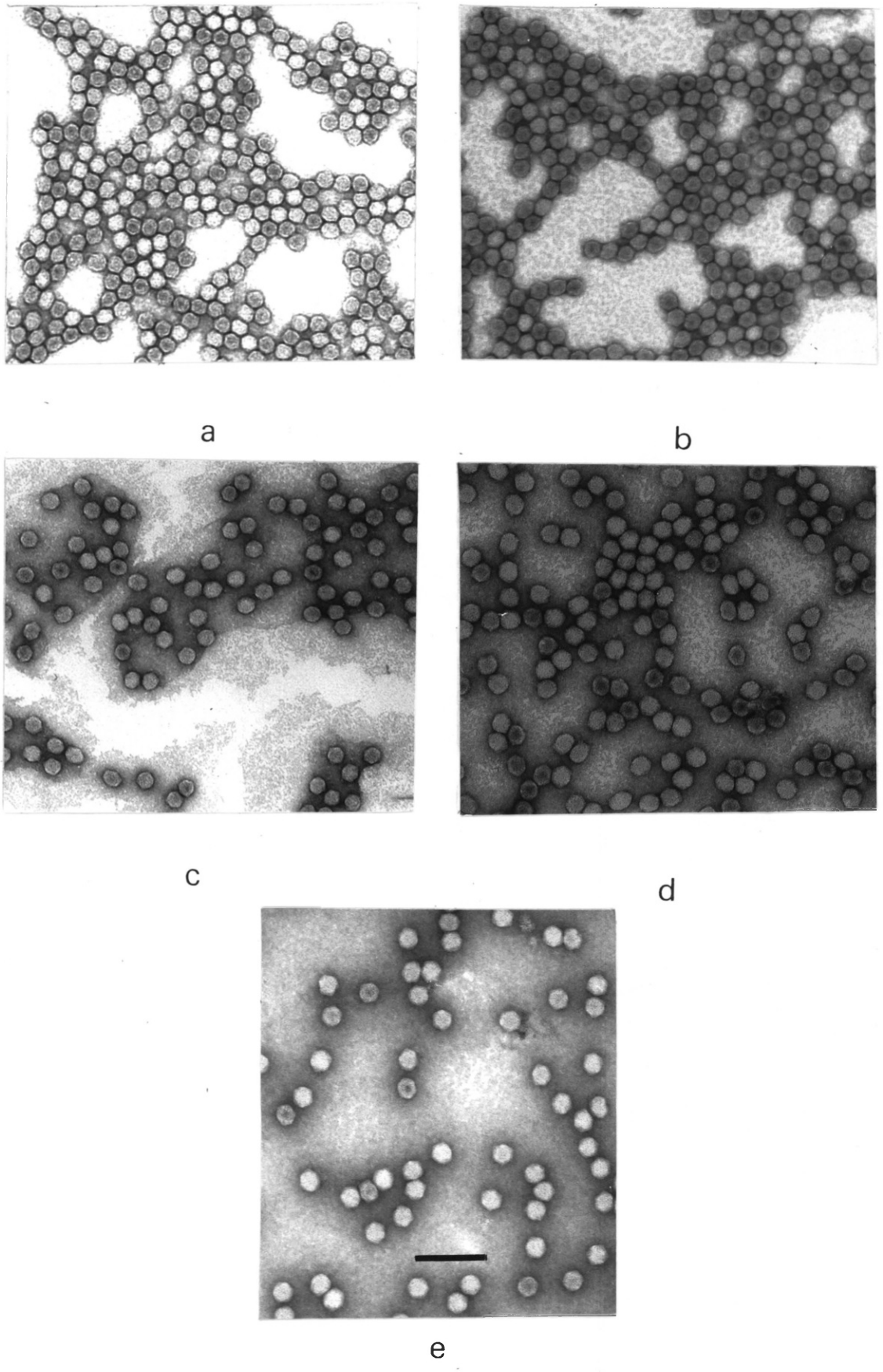


Table 5.2. Particle dimensions of purified preparations of RYMV isolates

RYMV isolate	Particle size (nm) measured: from corner to corner	from edge to edge
IC	[a]29.0±1.4	24.7±0.8
N	28.0±1.0	25.0±1.0
SL	28.0±1.4	24.6±0.7
Nr	28.8±1.0	24.8±0.8
K	28.9±1.0	25.0±0.8

[a]Average of 100 measurements ± standard error of the mean

Table 5.3. Dimensions of crude sap RYMV particles trapped with RYMV antiserum in ISEM tests

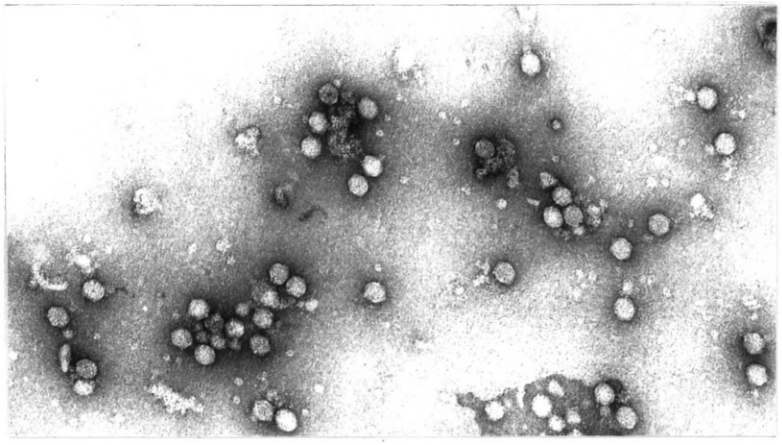
RYMV isolate	Particle size (nm) measured: from corner to corner	from edge to edge
IC	[a]28.5±0.1	24.5±0.08
N	28.6±0.1	24.8±0.08
SL	28.0±0.1	23.9±0.10
Nr	28.0±0.1	24.0±0.10
K	28.7±0.1	24.5±0.10

[a]Average of 50 particles ± standard error of the mean

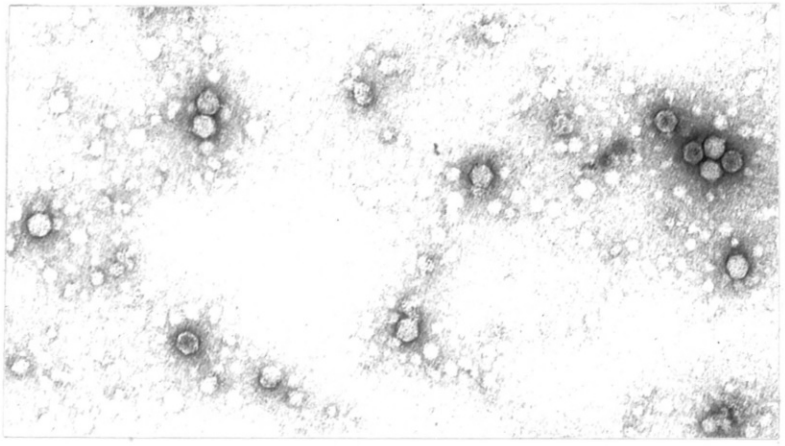
Fig. 5.3 Electron micrographs of RYMV particles trapped from crude sap extracts in ISEM tests using RYMV-N antiserum.

Scale bar = 100 nm

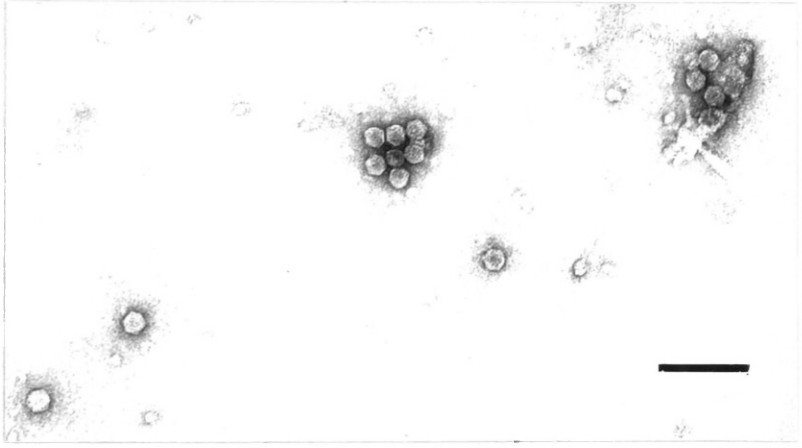
a) isolate N
b) isolate SL
c) isolate IC



a



b



c

of each isolate were measured. In the distinctly angular particles the average diameters from corner to corner and from edge to edge were measured from negatives of photographs taken at X 60000 magnification. There were no obvious differences in size between particles of the five RYMV isolates examined; all had dimensions (Table 5.2) within the range 27-30 nm (measured corner to corner) and 23-26 nm (measured from edge to edge). Trapped particles (Milne, 1984) (50 particles/isolate) of the five isolates were also measured when antisera to RYMV-N was used to coat grids for ISEM tests using crude sap preparations. Results (Table 5.3) showed that particle sizes were again similar for all isolates and, as with the partially purified preparation, the majority of particles had an angular outline (Fig. 5.3).

5.3.3 Serological relationships between RYMV isolates

5.3.3.1 Agar gel double-diffusion tests

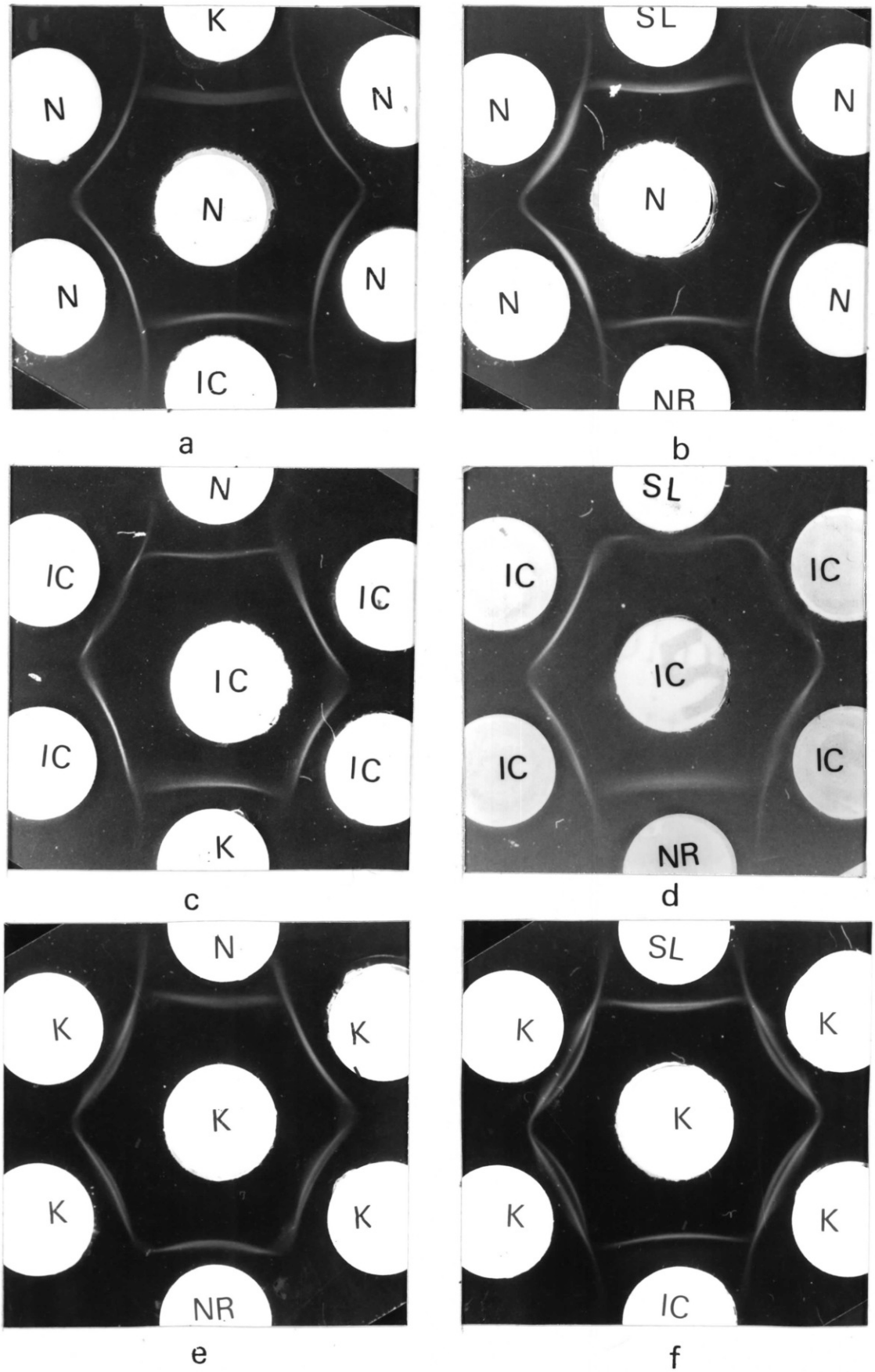
In initial tests, four of the six peripheral wells contained two sets of two adjacent wells containing the homologous antigen arranged so that in each plate heterologous antigen was sandwiched between two wells of homologous antigen; this arrangement was adopted for tests with each of the three antisera (Fig. 5.4). In all tests, as expected, there was a continuous precipitin line between the two adjacent wells containing homologous antigen, i.e. there was no spur formation.

In the tests using RYMV-N antiserum (Fig. 5.4a, b), two heterologous antigens were tested on each plate and placed opposite each other separated by the two sets of wells containing RYMV-N

Fig. 5.4 Gel diffusion tests with five isolates of RYMV using antisera to RYMV-N, RYMV-K and RYMV-IC.

Central wells : N RYMV-N antiserum
K RYMV-K antiserum
IC RYMV-IC antiserum

Peripheral wells : Extracts from rice (cv. ITA 212) infected with isolates N (Nigeria), K (Kenya), SL (Sierra Leone) Nr (Niger) and IC (Ivory Coast)



antigen. Spur formation occurred in all instances where homologous RYMV-N antigen was in a well adjacent to heterologous RYMV-IC, -SL, -K or -Nr and the homologous antigen spurred over the heterologous antigen.

With similar tests and RYMV-K antiserum (Fig. 5.4 c,d), results were the same as those using RYMV-N antiserum in that the homologous antigen K spurred over heterologous antigens SL, IC and N. However, spur formation did not occur when wells containing homologous antigen K were adjacent to RYMV-Nr antigen; here the two precipitin lines fused (Fig. 5.4d). These results suggested that serological differences existed between K and SL, N and IC but that isolates K and Nr were closely related.

Using RYMV-IC antiserum, spur formation indicated serological differences between homologous antigen IC and heterologous antigens N, Nr and K but fusion of precipitin lines where IC and SL antigens were in adjacent wells indicated a close relationship between these two antigens.

Tests were next made with antisera N (Fig. 5.5), K (Fig. 5.6) and IC (Fig. 5.7). With each antiserum, four plates were used and healthy sap was placed in the bottom peripheral well of every plate. The remaining five peripheral wells accommodated one sample of each of the five RYMV isolates under test. These five antigens were arranged so that each antigen was sandwiched between two of the other four antigens. This allowed all 10 combinations of antigen pairs to be tested against each antiserum (Figs. 5.5 to 5.7, Table 5.4).

Fig. 5.5 Gel diffusion tests with five RYMV isolates tested against antiserum RYMV-N (central wells)

For explanation of peripheral well antigens see Fig. 5.4

H = healthy rice saps (cv. ITA 212)

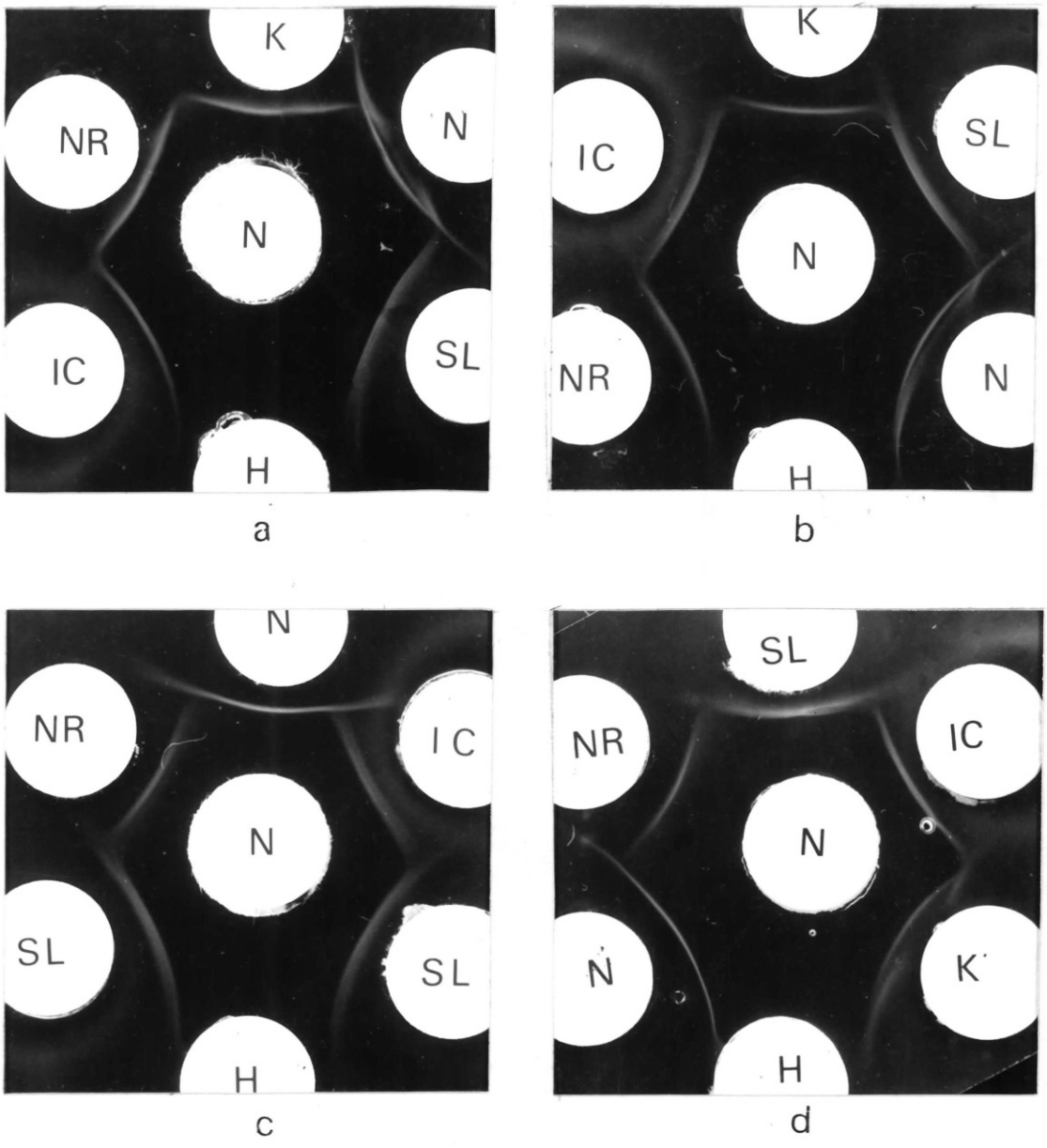


Fig. 5.6 Gel diffusion tests with five RYMV isolates tested against antiserum RYMV-K (central wells).

For explanation of peripheral well antigens, see Figs. 5.4 and 5.5

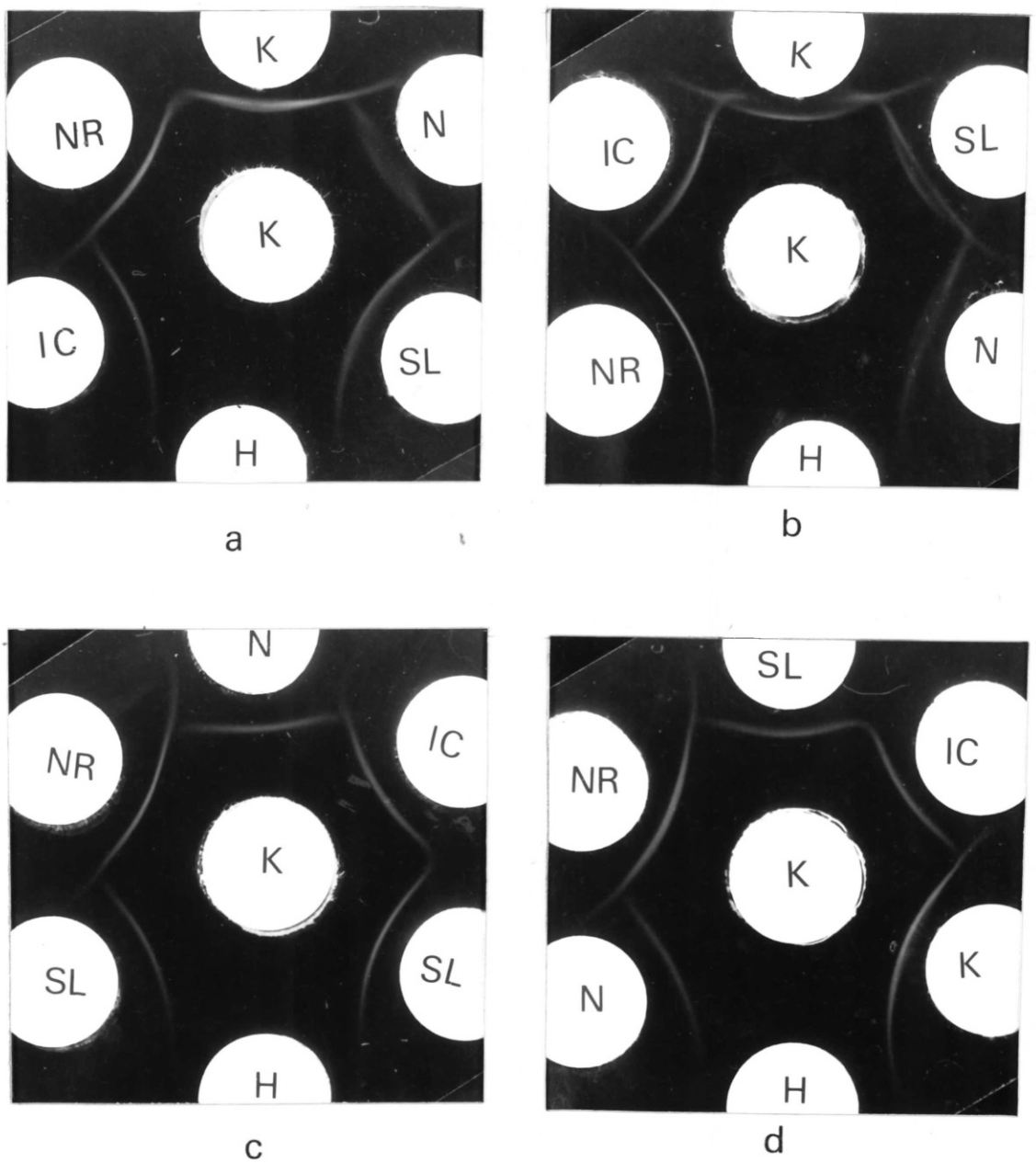


Fig. 5.7 Gel diffusion tests with five RYMV isolates tested against antiserum RYMV-IC (central wells)

For explanation of peripheral well antigens see Figs. 5.4 and 5.5

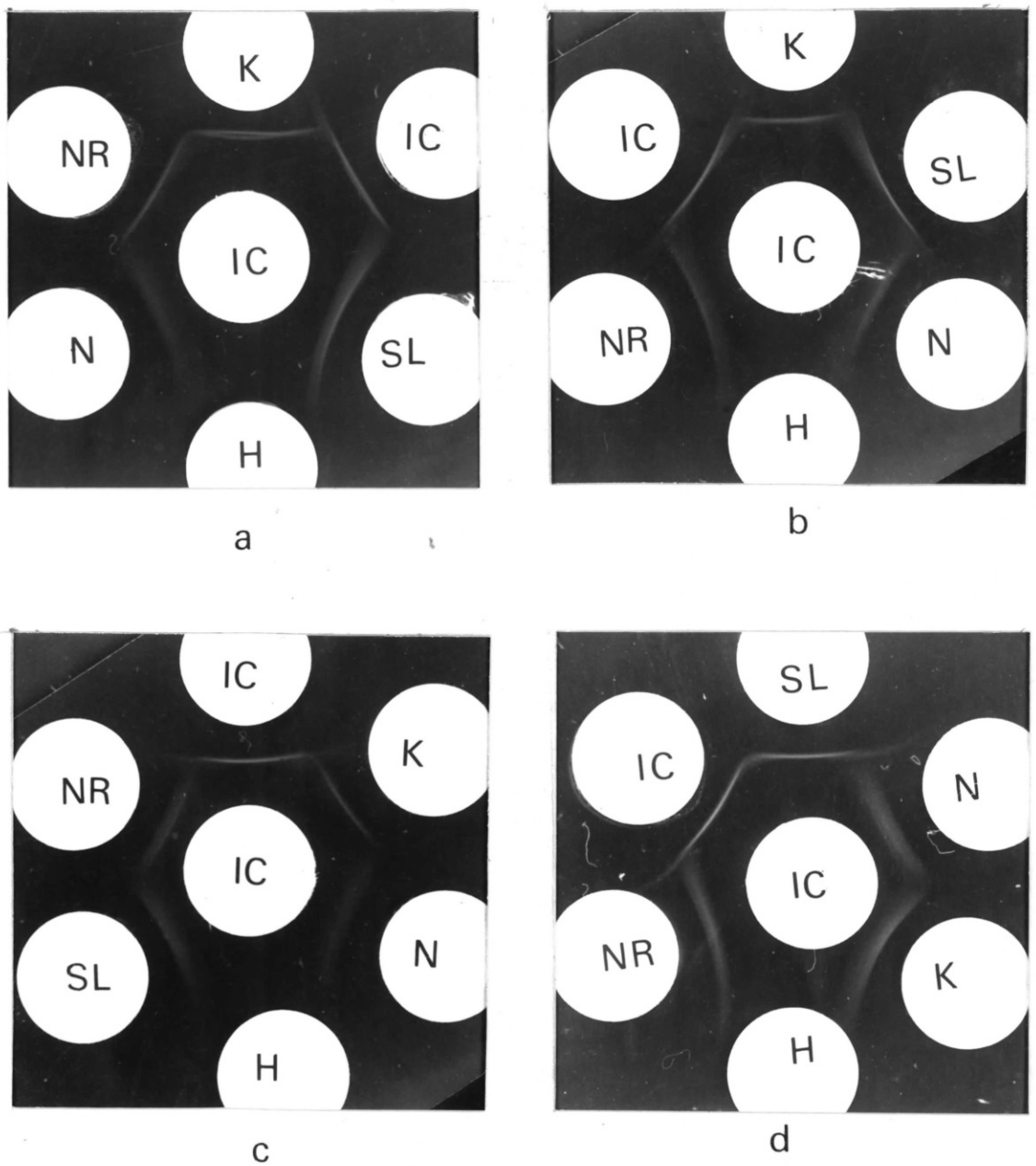


Table 5.4. A summary of the reactions illustrated in Figures 5.5, 5.6 and 5.7 between antigen pairs for all combinations of five RYMV isolates tested against antisera to RYMV-N, -IC and -K in agar double-diffusion tests.

Adjacent antigen pairs	Antiserum to:		
	RYMV-N	RYMV-IC	RYMV-K
N - IC	N[a]	IC	IC
N - SL	N	SL	SL
N - K	N	-[b]	K
IC - SL	SL	-	-
IC - K	-	IC	K
IC - Nr	-	IC	Nr
Nr - K	-	-	-
Nr - SL	SL	SL	Nr
K - SL	SL	SL	K

[a]Letters indicate which antigen of each adjacent pair caused precipitin line extension to form a spur

[b]Precipitin lines fused

No precipitin lines were formed when the antisera were reacted against healthy sap controls (Figs. 5.5 to 5.7).

With all three antisera, when antigens K and Nr were in adjacent wells spur formation did not occur and precipitin lines fused (Figs. 5.5 to 5.7). Thus, antibodies present in the three polyclonal antisera were unable to discriminate between isolates K and Nr, suggesting that these two isolates were serologically closely related or possibly identical.

When antigens IC and SL were placed in adjacent wells, precipitin lines fused when tested with antisera to K and IC (Figs. 5.6 and 5.7) but, with RYMV-N antiserum, antigen SL spurred over antigen IC (Fig. 5.5). Thus there were antibodies in RYMV-N antiserum reacting with antigen SL but not antigen IC and reacting antibodies in RYMV-IC antiserum were common to both antigens. These results suggested that isolates IC and SL were closely related. However, an antibody or antibodies is present in antiserum to RYMV-N which reacts with antigen SL, and this antibody appears not to be present in antisera to RYMV-IC and -K.

When IC, SL, Nr and K antigens were tested against RYMV-N antiserum, spurs always occurred between wells containing antigen SL and the other three isolates with SL spurring over antigens K, Nr or IC (Fig. 5.5). This suggests that within N antiserum there are antibodies which react with antigen SL but not IC, K or Nr, i.e. that a determinant(s) is present on SL and N but not on IC, K or Nr. This may also indicate that antigen SL is more closely related to N than the other three isolates.

When antigens IC and N, or SL and N, were tested with RYMV-K antiserum (Fig. 5.6), antigens IC and SL spurred over antigen N, indicating that there were some K antibodies common to both isolates (IC and SL) and another reacted with antigens IC or SL to produce the spurs. Thus, it appeared that isolates IC and SL were more related to K than N. However, it was not possible to test the relationships of antigen SL with antigen K as RYMV-SL antiserum was not available.

The above results suggested the following: antigens K and Nr were serologically indistinguishable and could be identical; antigens SL and IC were closely related to each other but distinguishable using N antiserum; and isolate N was readily distinguishable from the other four isolates.

5.3.3.2 Immunosorbent electron microscopy (ISEM)

Grids coated with diluted (1:100 v/v) γ-globulin (used for ELISA) of RYMV-N and treated with infected sap of the five RYMV isolates were examined under the electron microscope. The mean number of RYMV particles trapped per field at X 30000 magnification (area = 3,550 nm^2) (Table 5.5) showed that the highest number of particles trapped occurred with RYMV-N (homologous), fewer were trapped with isolates of SL and IC and the lowest numbers trapped were for RYMV-Nr and K. Five grids were examined for each isolate and two fields per grid counted. Grids coated with antiserum to the unrelated cucumovirus, cucumber mosaic virus (crude sap diluted 1:100) or left uncoated but exposed to infected sap of RYMV were included as controls. The control grids (5 grids each) trapped very few virus particles.

Table 5.5. Number of RYMV particles trapped on electron microscope grids coated with RYMV-N antiserum

RYMV isolate	Source of antigen:	
isolate	Crude sap 1:100 (w/v)	Purified virus (100 µg/ml)
N	260±31.6	296±32.1
SL	163±27.2	217±37.3
IC	156±14.2	191±21.5
K	99±12.9	129±24.7
Nr	76±18.3	115±18.7
Control[a]	5±0.95	

[a] Uncoated grids or coated with CMV antiserum

[b] Average of 10 fields (3,550 nm^2) at X 30000 magnification ± standard error

Fig. 5.8 Electron micrographs of RYMV particles decorated with gamma-globulin of RYMV-N diluted 1:100

Scale bar = 100 nm

a) Homologous isolated RYMV-N
b) Heterologous RYMV-SL
c) Heterologous RYMV-IC
d) Heterologous RYMV-K
e) Heterologous RYMV-Nr

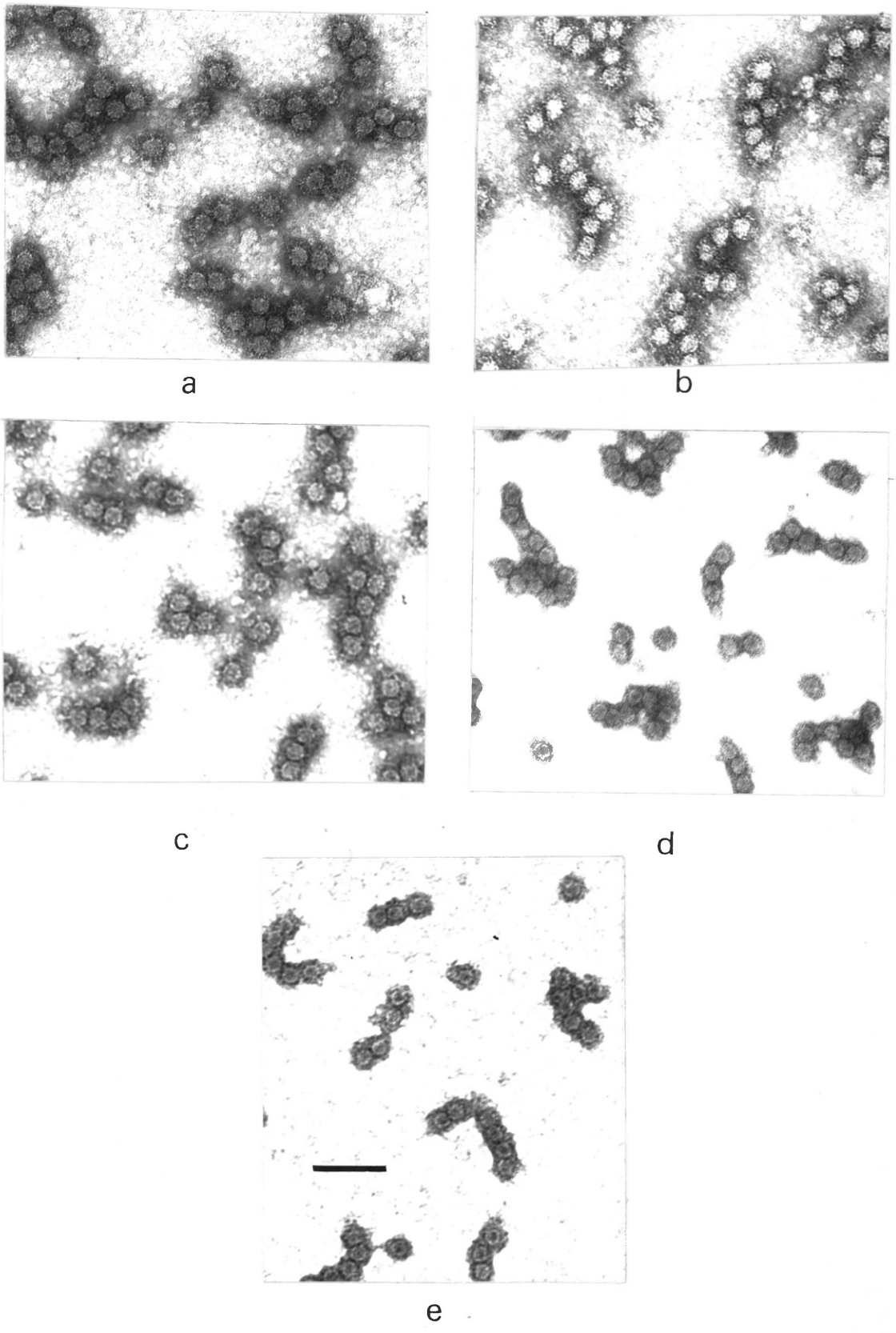
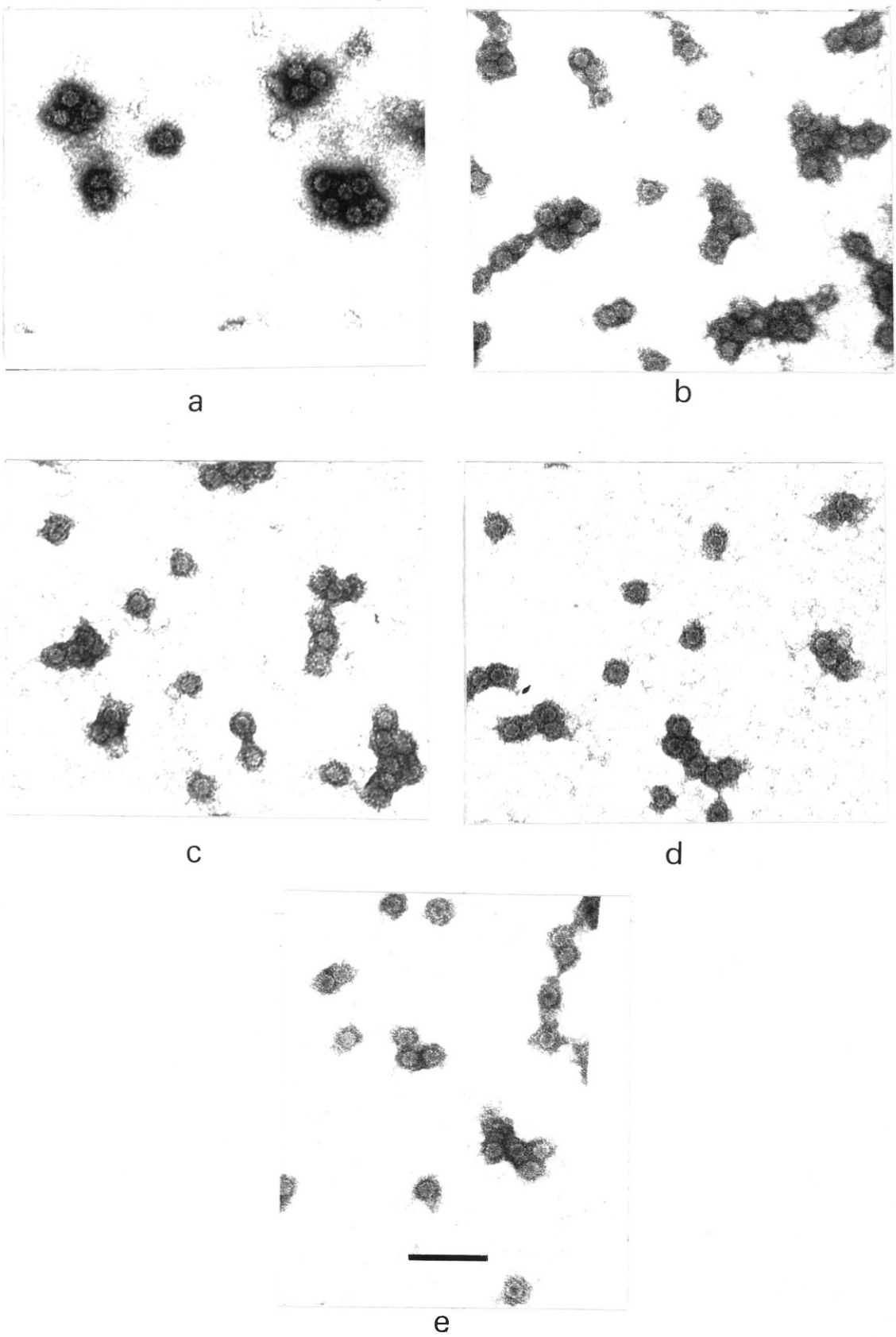


Fig. 5.9 Electron micrographs of RYMV particles decorated with gamma-globulin of RYMV-IC diluted 1:100. Scale bar = 100 nm

a) Homologous RYMV-IC b) Heterologous RYMV-SL

c) Heterologous RYMV-N d) Heterologous RYMV-K

e) Heterologous RYMV-Nr



To avoid the possibility that the number of particles trapped could be a reflection of virus concentration in the host tissue, purified virus preparations adjusted to A_{260} = 0.65 (100 µg/ml) were applied to grids previously coated with 1:100 (v/v) diluted γ-globulin (RYMV-N). The mean numbers of RYMV particles trapped showed the same trend as for crude sap and suggested that the RYMV isolates tested were serologically related.

Results of decoration tests showed that particles of all RYMV isolates whether treated with homologous or heterologous antibody became decorated to some extent (Fig. 5.8, 5.9). This again suggested that the five isolates of RYMV were serologically related. It was difficult to discern consistent and clear differences in the intensity and extent of the antibody halo around virus particles (Fig. 5.8, 5.9) which might give some measure of the degree of relatedness but there was more extensive decoration around the homologous compared with heterologous virus for both N and IC antiserum (Fig. 5.8, 5.9).

5.3.3.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA test results showed that all RYMV isolates reacted with both homologous and heterologous antisera and all five isolates were detected by ELISA in both purified preparations and crude sap extracts of leaf tissue when tested against antisera to RYMV-N, -K and -IC (Figs. 5.10 and 5.11).

Most dose-response curves showed a typical sigmoid shape and sometimes (particularly with crude sap extracts) the experimental

Fig. 5.10 Comparison of the five isolates of RYMV by double antibody sandwich ELISA. Crude sap extracts of the five isolates were serially diluted and tested against antisera to a) RYMV-N, b) RYMV-IC, c) RYMV-K, γ globulin was 1 μg/ml and the enzyme conjugate was diluted 1:1500 (v/v). Absorbance was measured 30 minutes after the addition of substrate

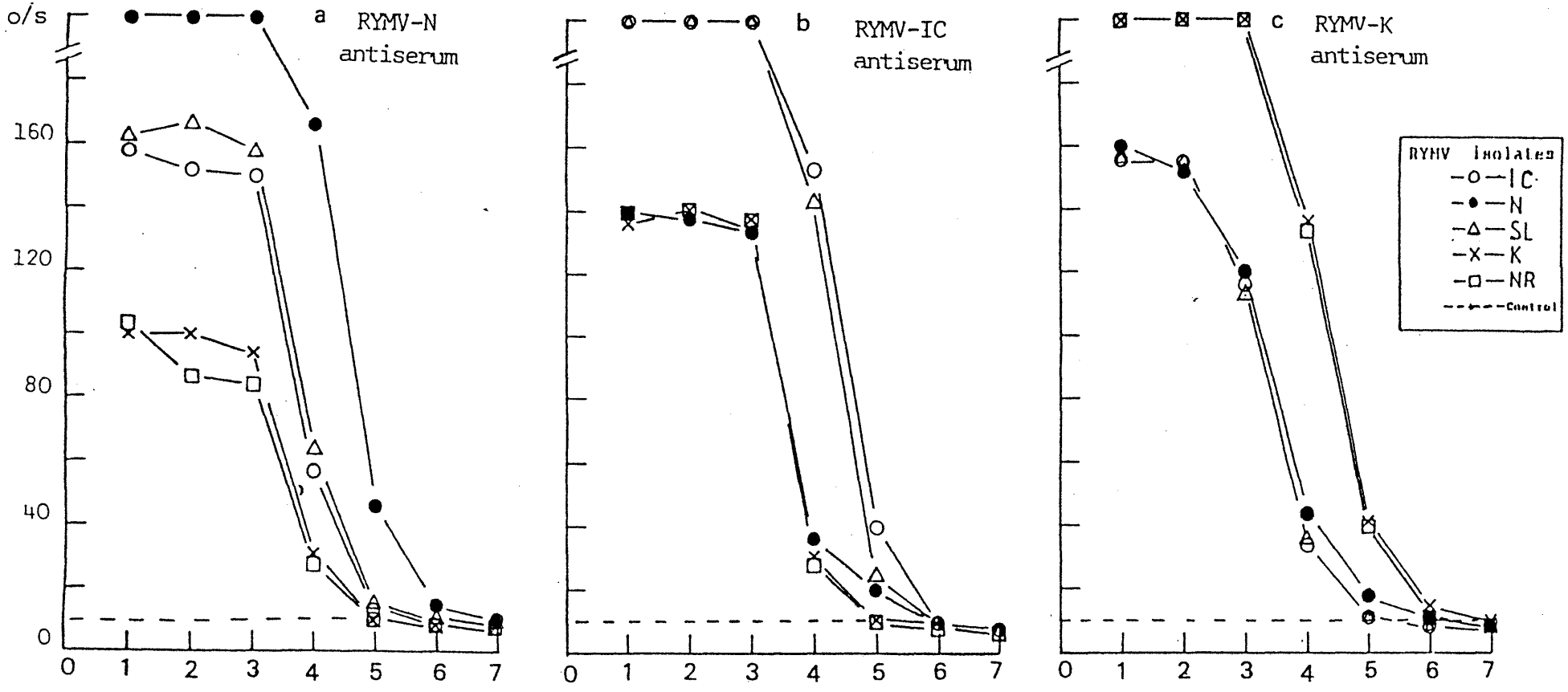
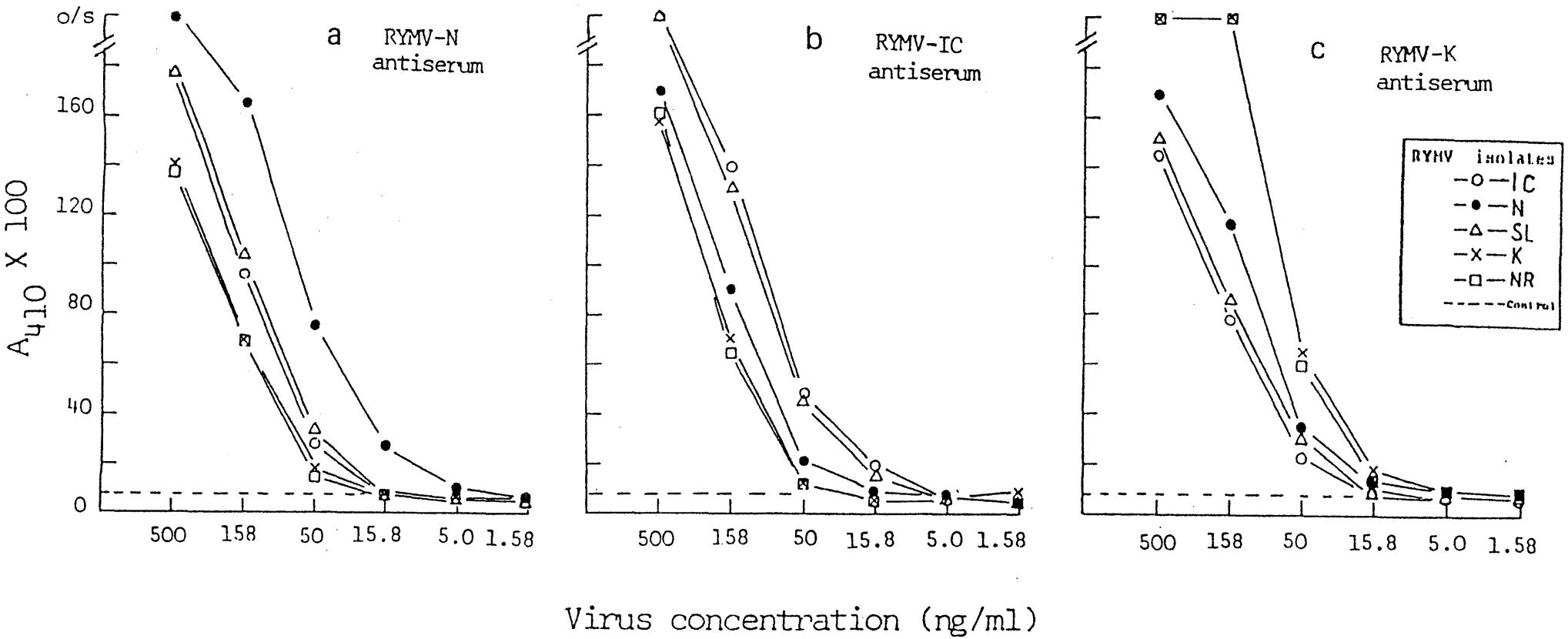


Fig. 5.11 Comparison of the five isolates of RYMV by double antibody sandwich ELISA. Purified virus antigens of the five isolates were serially diluted from 500-1.58 (half-$\log_{10}$ dilution steps) and tested against antisera to a) RYMV-N, b) RYMV-IC, c) RYMV-K. γ-globulin concentration was 1 μg/ml and the enzyme conjugate diluted 1:1500 (v/v). Absorbance was measured 30 minutes after the addition of substrate.



system became 'saturated' such that at low dilutions absorbance no longer increased with increased antigen concentration when absorbance was still on scale.

When extracts from infected ITA 212 plants were used, RYMV-N antibodies reacted most strongly with the homologous antigen giving high A_{410} values; absorbance was off scale for sap diluted 1:10 to 1:1000 (Fig. 5.10a). RYMV-N antibodies reacted least with the heterologous Nr and K antigens and absorbance values were very similar for these two antigens. RYMV-N antibodies produced intermediate A_{410} values with antigens IC and SL and absorbance values were very similar for antigens IC and SL. Differences in absorbance values elicited by N, Nr and K, and SL and IC antibodies were apparent at saturating antibody concentrations (sap dilution 1:10 to 1:1000) and at dilution 1:10,000 when absorbance values were related to antibody concentration. However, clearest discrimination between absorbance and antigen was seen at saturating antigen concentration.

Reaction with RYMV-IC antiserum separated the isolate antigens into two groups (Fig. 5.10b). High absorbance values were obtained with the homologous RYMV-IC and the heterologous RYMV-SL antigens whereas isolates N, Nr and K reacted less strongly but were very similar to each other. Two groupings were also evident with RYMV-K antiserum (Fig. 5.10c) where isolates RYMV-K and Nr induced high A_{410} values, whereas values for antigens N, IC and SL, although very similar to each other, gave values lower than the K and Nr group. In all tests, the differences between the isolates were clear up to a sap dilution of about 1:10,000 but thereafter as absorbance decreased

with antigen dilution differences became less clear.

Trends were essentially similar when serial half-$\log_{10}$ dilutions of partially purified antigen preparations were used (Fig. 5.11) except that antigen saturation was only apparent for antigens K and Nr tested against RYMV-K antigen (Fig. 5.11c). In all instances homologous combinations induced the highest absorbance values together with heterologous antigens SL and Nr when tested against RYMV-IC and RYMV-K antisera, respectively. Isolates could be grouped as in the crude sap tests but with partially purified antigen preparations, antigen RYMV-N was more clearly separable from antigens of the other four isolates.

All isolates were detectable, irrespective of the antiserum used, at sap dilutions up to $1:10^{-5}$ and at virus concentration of 15.8 to 50 ng/ml or above. Differences in antigen-induced absorbance for each antiserum at saturation or high antigen concentrations were also reflected in the lowest antigen concentration detectable.

These results suggested that the RYMV isolates were serologically related, that isolates K and Nr were closely related to each other, that isolates IC and SL were also closely related to each other but distinguishable from isolates K and Nr and that isolate N was sometimes distinguishable from the other four isolates.

5.3.3.4 Infectivity

To obtain an estimate of the relative amount of infective RYMV (all isolates) in resistant and susceptible cultivars, infectivity assays were made using serially diluted extracts 20, 40 and 60 days

after inoculation on the same susceptible systemic host cv. ITA 212. Results (Table 5.6) showed, irrespective of harvesting date, the dilution end points for extracts of all five isolates from the youngest leaves of Lac 23 were less than in cv. ITA 235 which in turn were less than in cv. ITA 212, suggesting that more infective virus accumulated in cv. ITA 212 than Lac 23 with intermediate levels in ITA 235. In cvs. ITA 212 and ITA 235, dilution end points for all isolates increased from 20 to 40 days but then decreased at 60 days whereas in Lac 23 dilution end points progressively increased from 20 to 60 days after inoculation. The tests did not reveal clear differences in infective virus titre between the isolates, although isolates SL and IC appeared to accumulate slightly less in cv. ITA 235 20 and 40 days after inoculation (Table 5.5) and in cv. ITA 212 after 20 and 40 days. Extracts of most isolates were infectious at 10^{-7}, the highest dilution tested, therefore their dilution end points were unknown.

5.3.3.5 Reaction of *O. glaberrima* to RYMV isolates

Further studies on the relationships between RYMV isolates were made using *O. glaberrima* accessions TOg 7235 and TOg 5674. Accession TOg 5674 did not produce symptoms after inoculation with the five RYMV isolates and no reactions were obtained when inoculated and non-inoculated tip leaf extracts were indexed on cv. ITA 212 or tested by ELISA (Table 5.7). Accession TOg 7235 produced mottle symptoms when inoculated with RYMV-N but remained symptomless following inoculation with the remaining four isolates. Extracts from symptomless plants inoculated with isolates IC, SL, K and Nr gave no reaction in ELISA tests or when tested on cv. ITA 212 plants (Table 5.7). Inoculation of *O. glaberrima* accession TOg 7235 with

Table 5.6. The infectivity dilution end points of RYMV isolates in serially diluted extracts from the youngest leaves of susceptible and resistant rice cultivars assayed at 20, 40 and 60 days after inoculation.

RYMV isolate	Rice cultivars								
	ITA 212			ITA 235			Lac 23		
	Days after inoculation			Days after inoculation			Days after inoculation		
	20	40[b]	60[b]	20	40	60	20	40	60
IC	10^{-7}[a]	10^{-7}	10^{-6}	10^{-3}	10^{-5}	10^{-5}	-[c]	10^{-2}	10^{-3}
N	10^{-7}	10^{-7}	10^{-6}	10^{-4}	10^{-6}	10^{-5}	10^{-2}	10^{-3}	10^{-4}
SL	10^{-6}	10^{-7}	10^{-6}	10^{-3}	10^{-5}	10^{-5}	10^{-2}	10^{-3}	10^{-4}
Nr	10^{-7}	10^{-7}	10^{-6}	10^{-4}	10^{-6}	10^{-5}	10^{-2}	10^{-3}	10^{-4}
K	10^{-7}	10^{-7}	10^{-6}	10^{-4}	10^{-6}	10^{-5}	10^{-2}	10^{-3}	10^{-4}

[a] Results presented as the reciprocal of the dilution end point or highest extract dilution tested (10^{-7}) giving rise to symptoms 40 days after indexing extracts on cv. ITA 212. Five plants/dilution were used and an extract was regarded as infective if one or more inoculated plants produced symptoms.

[b] Because most inoculated plants were killed by infection, infectivity determined from the 2-3 plants which survived.

[c] All assay plants symptomless

RYMV-N gave two out of five plants with mottle symptoms in the non-inoculated tip leaves 25 days after inoculation. Infectivity tests with extracts of inoculated leaves on ITA 212 plants and ELISA indexing showed that infective RYMV-N and RYMV antigen were both present in the inoculated leaves of all five TOg 7235 plants inoculated, whereas similar tests with extracts from young tip leaves showed RYMV-N was present only in those leaves of the two plants showing symptoms (Table 5.7).

Table 5.7. Reaction of Oryza glaberrima accessions to five isolates of RYMV and infectivity and ELISA tests on inoculated and tip leaf extracts.

Accesion	RYMV isolate	No. of plants showing symptoms (out of 5)	Infectivity and ELISA indexing			
			Inoculated leaves[a]		Tip leaves[b]	
			ELISA[c]	ITA 212	ELISA	ITA 212
TOg 7235	IC	0	0[d]	0[d]	0[d]	0[d]
	N	2	5	5	2	2
	SL	0	0	0	0	0
	Nr	0	0	0	0	0
	K	0	0	0	0	0
TOg 5674	IC	0	0	0	0	0
	N	0	0	0	0	0
	SL	0	0	0	0	0
	Nr	0	0	0	0	0
	K	0	0	0	0	0

[a]Harvested 30 days after inoculation

[b]Harvested 60 days after inoculation

[c]RYMV-N was used

[d]Number of leaf extracts out of five tested by infectivity test (ITA 212) or ELISA.

5.4 Discussion

The purification method used which was based on the descriptions of Hull (1977), Denlyo *et al*. (1978) and Brisco *et al*. (1985) proved satisfactory in terms of rapidly and ease, as well as in yielding more virus than other methods tried. It was possible to process more than one preparation simultaneously and therefore proved to be of value in the rapid purification of the five RYMV isolates used in this study. All preparations were infective although the dilution end point for infectivity was not determined.

The partially purified preparations of RYMV isolates appeared to be relatively free from host contaminant material as evidenced by ultraviolet absorption spectra (Fig. 5.1) and examination under the electron microscope. The spectrophotometric properties of all five isolates were very similar with an A_{max} of 260 nm, A_{min} of 242 nm, and A_{max}/A_{min} of 1.32 ± 0.02 and an A_{260}/A_{280} ratio in the range of 1.49 ± 0.02 to 1.43 ± 0.02. The amount of virus in leaves of susceptible cv. ITA 212 was very high (0.5 mg-1.0 mg/g tissue) and therefore only 5 g of material were needed to obtain good preparations yielding 2.5 to 5.0 mg virus. Highest amounts of virus were recovered from leaves infected with Nr and K isolates (*c*. 1 mg/g fresh tissue) followed by leaves infected with RYMV-N (0.75 mg/g); amounts were lowest in leaves infected with isolates SL and IC (0.5 mg/g).

Suspensions of purified preparations of the virus isolates consisted of isometric virus particles with no consistent and clear difference discernible in particle appearance and size between the isolates. Spectrophotometric data and electron microscope

observations were in general accord with the previous findings of Bakker (1974) who studied the Kenyan isolate (the same Kenyan isolate as used here). He obtained an A_{max}/A_{min} value of 1.33±0.01 for RYMV-K and estimated that the virus concentration of RYMV-K in infected leaves of rice cv. Sindano (susceptible) was about 1 mg virus/g tissue, similar to the amounts calculated in the present study for isolates Nr and K in rice cv. ITA 212. However, as noted above, isolates N, IC and SL were recovered in lower amounts. RYMV clearly accumulates to very high concentration in rice leaves and this now appears to be the case for several isolates in two susceptible rice cultivars. The present results are in line with the results for an Ivory Coast isolate detailed by Fauquet and Thouvenel (1977). They observed an A_{max}/A_{min} of 1.29±0.03 and calculated the virus content of leaves of an unspecified variety to be 0.4-0.6 mg/g tissue. These values are similar to those obtained for the Ivory Coast isolate used in the present study. Electron microscope examination of partially purified preparations of RYMV isolates revealed numerous isometric particles. Uranyl acetate was a satisfactory negative stain and there was no sign of stain penetration into virus particles. Virus particles often had an angular, usually hexagonal outline and no detailed surface structure was seen. Because most virus particles seen showed an angular outline diameters were measured from corner to corner and from edge to edge. Milne (1984) stated that where isometric particles showed such an angularity these two dimensions ought to be measured. The above findings were in general agreement with results obtained by Bakker (1974). He found that purified virus preparations of a Kenyan isolate (now regarded as the type isolate) contained many isometric particles often with an angular outline and without any clear capsid subunit architecture. PTA partially or

fully penetrated the virus particles but most were not penetrated by the stain. When uranyl acetate was used, particles were seen with an electron-dense central area (Bakker, 1974).

Sehgal (1981) and Hull (1988) stated that when particles of members of sombemovirus group are closely packed, the particles are distinctly hexagonal; most excluded the negative stain from penetrating the capsid and did not show any protein subunit features on the particle surface. The present study showed that the virus particles measured 24-26 nm from edge to edge and 28-30 nm from corner to corner when particles from both purified or crude sap preparations were examined. These results agree with Bakker's (1974) findings with the Kenyan isolate. He found the virus particles measured 25 nm from edge to edge and 26-30 nm from corner to corner. Fauquet and Thouvenel (1977) reported the mean diameter of an Ivory Coast isolate to be 30 ± 3.0 nm.

Although homologous antisera were available for only three of the five isolates studied, interesting results were obtained with gel-diffusion tests which permitted some conclusions to be drawn on the serological relationships among the five isolates. The presence of a strong precipitin line between the three antisera tested and the five isolates of RYMV indicated that all isolates were serologically related. However, spur formation between antigen N and the other four RYMV isolates when RYMV-N antiserum was used suggested isolate N was antigenically distinct from the other four isolates tested. Using RYMV-N antiserum, antigen SL always formed a spur when placed in a peripheral well adjacent to antigens IC, Nr and K (Table 5.4). This suggested that isolate SL was serologically more related to

isolate N than isolates IC, Nr and K. In other words, isolate SL has more determinants in common with isolate N than the remaining three isolates.

When isolates were tested against RYMV-IC antiserum, a spur formed between antigen IC and N, Nr or K antigens suggesting that isolate IC was serologically distinct from isolates N, Nr and K. Fauquet and Thouvenel (1977) also reported spur formation when isolates from the Ivory Coast and Bakker's original Kenyan isolate were tested in adjacent wells using either RYMV-IC or -K antiserum. This was confirmed in the present work. The presence of a fused precipitin band between antigens IC and SL when RYMV-IC and -K antisera were used, suggested that the two isolates were very closely related. However, the presence of spur between antigens IC and SL when RYMV-N antiserum was used indicated a slight serological difference between them.

When RYMV isolates were tested against K antiserum, spurs formed between antigen K and antigens IC, SL and N but not between antigens K and Nr, indicating that isolate K was serologically distinct from isolates IC, SL and N but serologically very close, if not identical with isolate Nr. The absence of spur production between antigens K and Nr, irrespective of the antiserum used, was strong evidence that the two isolates were likely serologically indistinguishable (Table 5.4).

When the antigen pairs N and IC, or N and SL were placed in adjacent wells and tested against RYMK-K antiserum (Table 5.4), antigens IC or SL formed spurs, suggesting that they have more

determinants in common with isolate K then isolate N. However, it was not clear which isolate was more related to isolate K as RYMV-SL antiserum was not available. Nevertheless, it might be anticipated that as isolate SL was more related to isolate N than isolate IC, and both isolates IC and SL were more related to isolate K than isolate N, that isolate IC could be shown to be more related to isolate K than isolate SL if RYMV-SL antiserum were available. Based on the present gel diffusion results, it is also likely that RYMV-Nr antiserum would reveal the presence of a fused line between antigens K and Nr.

In summary, the five isolates of RYMV can be arranged into three serological groups. Firstly, the RYMV-N group, secondly, an SL-IC group, and thirdly a K-Nr group.

The above relationships between the five RYMV isolates proposed on the basis of the gel-diffusion test results were generally confirmed by ELISA and ISEM results.

ISEM studies showed that the number of RYMV particles from partially purified or crude sap preparations (Table 5.5) trapped on grids coated with RYMV-N antiserum was variable suggesting that there were serological differences between the isolates. The results agreed with the three major serological classes revealed by gel-diffusion in that most particles of the homologous antigen N were trapped, fewer particles of isolates SL and IC were trapped than for RYMV-N but numbers trapped were similar for each isolate from either purified or crude sap preparations. Fewest particles were trapped of isolates K and Nr and numbers were similar for these two isolates.

All isolates became decorated when treated with antisera to RYMV-N and -IC. Decoration was most intense (Figs. 5.8 and 5.9) with the homologous combinations and, although heterologous decoration occurred, it did not reveal any clear grouping of the heterologous isolates tested.

Direct ELISA is known to be often highly strain specific (e.g. Uyemoto, 1980; Van Regenmortel and Burckard, 1980), and it was possible to differentiate between some of the RYMV isolates using this method in the present study. Bitterlin and Gonsalves (1988) found that direct ELISA was able to differentiate between isolates of tomato ringspot virus using crude sap from infected tissue as antigen source, whereas indirect ELISA (less strain specific) failed to differentiate the isolates tested. Similarly, when three isolates of potato dwarf virus were serologically compared using ELISA by Falk and Weathers (1983), serotype differences were readily observed by direct DAS-ELISA but were less apparent by indirect ELISA. Koenig (1981) has shown that direct ELISA is a useful method for detecting slight serological differences that are not evident by other serological methods.

In the present study when crude saps of infected ITA 212 leaves were used as antigen source and tested against RYMV-IC antiserum, the isolates could be divided into two groups (Fig. 5.10b). The antigens IC and SL as one group and the heterologous antigens N, Nr and K as another group. Similar results were obtained when RYMV-K antiserum was used, with antigens Nr and K evident as one group and antigens N, IC and SL as a second group. However, when RYMV-N antisera was used, RYMV isolates could be categorized into

three different groups as suggested by the gel double-diffusion results. The same three groups were evident when purified preparations were used as antigen source in ELISA tests using any of the three antisera available (Fig. 5.11). However, in double diffusion tests, it was not possible to categorize the isolates of RYMV using one antiserum whereas in ELISA tests with purified preparations it was possible to group the isolates into 3 different groups. This could be explained by the fact that ELISA is more sensitive than double diffusion tests (Thomas, 1980).

When *O. glaberrima* accessions TOg 7235 and TOg 5674 were inoculated with RYMV isolates, symptoms were not produced in TOg 5674 but TOg 7235 showed symptoms following inoculation with RYMV-N, whereas the other isolates did not induce symptoms. These findings agreed with earlier results (IITA, 1986) where TOg 5674 was inoculated with Kenyan and Nigerian isolates and no symptoms were induced by either isolate. However, when accession TOg 7235 was inoculated with a Kenyan or Nigerian isolate, symptoms developed in plants inoculated with the Kenyan isolate (IITA, 1987). These results conflict with the findings of the present study where TOg 7235 produced symptoms with isolate N but not with isolate K.

There is no ready explanation for this contradiction but it may be due to variations in environmental conditions and/or to the use of different variants of the isolates from the various locations.

In the present study tests were extended by back indexing on ITA 212 rice and ELISA tests using extracts from inoculated and young leaves. Both ELISA and inoculation tests showed that RYMV-N was

present in the inoculated leaves of RYMV-N inoculated plants. Extracts from symptomless plants of both accessions were RYMV-negative when tested by ELISA or by back inoculation to ITA 212 rice.

There was no obvious relationship between serological properties and the geographical origin of RYMV isolates. For example, it was found that the Kenyan (East Africa) and the Niger (West Africa) isolates were serologically indistinguishable whereas Nigerian and Niger isolate were serologically distinct yet the two countries have an extensive common boundary.

S E C T I O N S I X

GENERAL CONCLUSIONS

6.0 GENERAL CONCLUSIONS

Work described here has attempted to investigate the reaction of five rice cultivars to infection with five RYMV isolates; the components and some possible mechanisms of resistance and finally the serological relationships between five RYMV isolates collected from West and East Africa.

Classification of rice cultivars into susceptible and resistant (ITA 212 and BG 90-2 as highly susceptible, ITA 235 as moderately resistant and Lac 23 and Moroberekan as highly resistant) cultivars was based on symptom severity, disease incidence, incubation period, plant height, leaf length and fresh and dry weights of shoots and roots. The results obtained from inoculated rice plants suggested that these parameters were effective indices of resistance to RYMV. For example, in susceptible cultivars severe symptoms and total infection was always associated with short incubation periods and reductions in plant height and fresh and dry weights of shoots and roots, whereas mild symptoms and variable infection coupled with lengthy incubation periods and slight or no effects on shoot and root growth were found in resistant cultivars. Differences in the preceding parameters were also associated with virus content in that amounts of RYMV antigen calculated from ELISA absorbance values were much greater in susceptible than in resistant cultivars. Virus content could therefore also be used as a basis for designating a cultivar as resistant or susceptible, particularly in tests were made 20-30 days after inoculation. Features such as foliar symptom expression (Qualset, 1983), symptom severity and disease incidence (Scott and Rosenkranz, 1981), foliar symptoms and

plant height (Fomba, 1988), symptom expression and virus content (Kuhn *et al.*, 1986), and symptom severity, incubation period and virus content (Kuhn *et al.*, 1989) have often been used previously as a basis on which to express the degree of resistance to virus infection.

Despite the relatively small differences between the five RYMV isolates obtained on the resistant and susceptible cultivars examined, there were indications that the Niger isolate was more virulent than the remaining isolates, whereas the Sierra Leone isolate tended to have the least effect on the rice cultivars tested. It may therefore not be simple coincidence that the severest natural outbreaks of RYMV have been recorded in Niger (Reckhaus and Adamou, 1986).

Inoculation of some *Oryza glaberrima* accessions with the five RYMV isolates revealed some biological differences between the Nigerian isolate and the other four isolates. These and the serological results suggested there may be suitable diagnostic hosts which could discriminate between isolates and thus possibly prove useful for epidemiological and diagnostic purposes. Such hosts may include other *O. sativa* varieties, lines and accessions; wild rice species such as *O. barthii*, *O. punctata* and *O. longistaminata*; *O. glaberrima* accessions and some plant species outside the Oryzeae which were found to be systemically or locally infected when inoculated with the Kenyan isolate (Bakker, 1974). Thus the limited tests made here on rice and *O. glaberrima* ought probably to be extended to search for better differential hosts.

Although the reaction of RYMV isolates on rice cultivars revealed relatively minor biological differences, the various serological tests made (agar double diffusion, ISEM and ELISA) showed clearly that although the five RYMV isolates were serologically related, not all of them were serologically indistinguishable. Detailed comparisons, particularly using agar double-diffusion tests, indicated that the Kenyan and the Niger isolates were indistinguishable, Ivory Coast and Sierra Leone isolates were serologically closely related but could be distinguished by using the Nigerian antiserum and the Nigerian isolate was fairly distinct from the other isolates. Such serological diversity was not evident in the reactions induced in resistant and susceptible rice cultivars.

The direct ELISA used in this study also revealed serological differences between RYMV isolates. Direct ELISA is well known to be highly strain specific and heterologous closely related strains or isolates may not be detected (e.g. Barbara *et al*., 1978; Koenig, 1978; Uyemoto, 1980; Koenig and Paul, 1982), whereas indirect ELISA is less strain specific and can detect slight serological relationships (e.g. Koenig, 1981; Koenig and Paul, 1982; Barbara and Clark, 1982) but cannot be used quantitatively to estimate degrees of serological relatedness (e.g. Koenig, 1981). However, some reports have indicated that serological differences were more pronounced by direct ELISA and less apparent by indirect ELISA (Falk and Weathers, 1983) and effective only with purified preparations (Koenig and Paul, 1982; Bitterlin and Gonsalves, 1988).

The detection of all isolates with all three antisera available suggested that ELISA could be used in survey and

epidemiological work on RYMV, especially when crude sap preparations are to be used. However, the use of both direct and indirect ELISA may reveal further relationships between apparently closely related isolates such as SL and IC or K and Nr, and additional isolates from other countries might usefully be included in an extensive study embracing host range to study further both biological and serological relationships among RYMV isolates.

As stated before, the rice cultivars used in this study were placed into two groups, susceptible cultivars (ITA 212 and BG 90-2) and resistant cultivars (ITA 235, Lac 23 and Moroberekan). Clear features evident in resistant cultivars were low disease incidence, lengthy incubation periods and mild symptoms. These features were found to be associated with low virus content compared with susceptible cultivars. The resistant rice cultivars displayed several features associated with resistance. In inoculated but symptomless plants two conditions existed; resistance to virus infection and virus restriction to the inoculated leaves. Resistance to virus infection (usually about 10-20% of inoculated plants of cultivars Lac 23 and Moroberekan) followed failure of plants to become infected following sap inoculation with RYMV-N such that the virus was not detected in extracts of inoculated or non-inoculated leaves. Virus restriction to inoculated leaves (evident in some plants of Lac 23 and Moroberekan) led to virus detection in the inoculated leaves but not in young non-inoculated leaves. Further investigation is needed to establish whether such restriction is associated with reduced numbers of infection loci and reduced or restricted cell to cell spread in the inoculated leaves of resistant cultivars such as Lac 23 and Moroberekan compared with susceptible

ITA 212. Results in section 5 clearly showed that overall virus content was reduced in the inoculated leaves of resistant cultivars and further work is needed to determine why this is so.

In resistant plants which produced systemic symptoms, reduced virus content and virus systemic movement was found. Again, further work is needed to clarify the underlying mechanisms.

In the present study the Nigerian isolate was mainly used to evaluate the nature and components of resistance. As stated above, RYMV-Nr appeared to be more virulent than the other isolates and thus further work with this isolate may prove fruitful.

In relation to rice production in Africa, the present work has confirmed that RYMV is a devastating disease on susceptible lowland cultivars (ITA 212 and BG 90-2) and three of the many resistant rice lines in use and/or produced by IITA were resistant to RYMV-N and that they were also resistant to other isolates obtained from geographically distant areas. Thus a resistant-breaking isolate was not found but clearly such RYMV strains may still exist. There is still a need to search for better resistance in that current resistant cultivars, although they show mild symptoms and their general growth is little affected, still suffer appreciable yield losses of up to 18.6% (IITA, 1982) although this yield loss is substantially less than the 80-90% decreases often found in susceptible cultivars (IITA, 1982). A further need is the production of high-yielding lowland-type rices with effective RYMV resistance to maximise production in those regions where lowland rice can be grown. It is interesting that cv. ITA 235, a hybrid between lowland and

upland rice parents, although displaying an upland type habit was very often intermediate between the resistant and the susceptible extremes of Lac 23 and ITA 212 in the various aspects explored in the present work.

That resistance to infection and restriction of virus to inoculated leaves can occur, should be borne in mind when various rice genotypes are screened for resistance. Do genotypes exist in which these features are more frequent within a population than found here with cvs. Lac 23 and Moroberekan and are these conditions markedly affected by environmental conditions?

Potentially very interesting studies ought to be made on the pattern and rate of disease spread in resistant and susceptible populations. There may be a double benefit in introducing RYMV resistant cultivars. Not only do resistant plants show increased yield on a per plant basis, but the low virus content, increased resistance to infection or virus restriction and possible *in vitro* effects of sap inhibitors could lead to reduced efficiency in transmission by beetle vectors with consequent reductions in the rate of natural spread.

Overall, resistant plants have much to offer in reducing, in a cost-effective way, the impact of RYMV on rice production in Africa and although there is much current effort directed towards the identification and production of such plants, the underlying resistance mechanisms offer the virologist interesting challenges in the way in which resistance may be brought about.

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7.0 REFERENCES

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