European Journal of Plant Pathology **106:** 167–178, 2000.

Evidence of the presence of two serotypes of rice yellow mottle sobemovirus in Côte d'Ivoire

P. N'Guessan[1], A. Pinel[2], M.L. Caruana[2], R. Frutos[3], A. Sy[4], A. Ghesquière[5] and D. Fargette[2,*]
[1]*CNRA, BP 633, Bouaké 01, Côte d'Ivoire;* [2]*LPRC, CIRAD/IRD BP 5035, 34032 Montpellier cedex 1, France;* [3]*IGEPAM, CIRAD, BP 5035, 34032 Montpellier cedex 1, France;* [4]*ADRAO, BP 2551, Bouaké 01, Côte d'Ivoire;* [5]*Genetrop, IRD, BP 5045, Montpellier cedex 1, France;* [*]*Author for correspondence (Phone: 467 61 7102; Fax: 467 54 7800; E-mail: Denis.Fargette@mpl.ird.fr)*

Accepted 17 November 1999

Key words: Africa, serotype, virus diversity and distribution, strain competition

Abstract

Serological variability of isolates of rice yellow mottle virus (RYMV) collected in Côte d'Ivoire was assessed by immunological tests with polyclonal and monoclonal antibodies (MAbs). Two serotypes (named S1 and S2) were distinguished. The S1 isolates had common epitopes which were absent in S2 isolates, whereas they lacked epitopes shared by S2 isolates. There was no evidence of S1 and S2 mixtures, although S1 and S2 isolates were sometimes found in nearby sites. Serotype S2 was more prevalent in Côte d'Ivoire than S1, and was in a large majority in the centre and the south of the country. By contrast, S1 occurred more widely in the north. S1 isolates were also found in neighbouring countries at the north of Côte d'Ivoire. In tests with monoclonal antibodies, three additional serotypes were found, one in West-Africa and two in East-Africa. Using the primers developed against an S2 isolate from Côte d'Ivoire, all S2 but not the S1 isolates were transcribed and amplified by RT-PCR, and another set of primers was developed to amplify S1 isolates. S1 and S2 have different biological properties, and competition between isolates of the two strains was apparent resulting in S2 dominance over S1. This was assessed using S1 and S2 strain specific MAbs, and it occurred whatever the pattern of inoculation or the rice variety tested. Differences in pathogenicity and virus titre did not account for strain competition, as there was no relation between symptom severity, virus content and serotype of the isolates in *Oryza sativa indica* cultivars.

Introduction

Rice (*Oryza sativa*) is widely grown in Africa and it is the most important cereal in Côte d'Ivoire where annual production exceeds 700 000 metric tonnes (Rochebrun and Sablayrolles, 1993). Rice cultivation throughout the African continent is severely affected by rice yellow mottle virus (RYMV). First reported in Kenya (Bakker, 1974), RYMV is a member of the genus *Sobemovirus* (John and Thottapilly, 1987; Hull, 1988; Matthews, 1991) and the only species so far known to infect rice plants in Africa. RYMV has not been found outside Africa. Under natural conditions, RYMV is transmitted by several species of beetles (*Coleoptera*) mostly belonging to the *Chrysomelidae* (Bakker, 1971), but under artificial conditions it is also sap transmissible. Infected plants show yellow discolouration and mottling of leaves, stunting, reduced tillering, poor panicle exertion and sterility. Early infection of susceptible varieties can cause the death of the plant. Severe yield losses ranging from 20% to 100% after RYMV infection have been reported in many countries (Awoderu, 1991; Awoderu et al., 1987; Taylor et al., 1990).

Although RYMV is now known in most rice growing African countries (Awoderu, 1991; Fauquet and

Thouvenel, 1977; John et al., 1984; Raymundo and Buddenhagen, 1976; Raymundo and Konteh, 1980), there is little information on the variability of the virus. Serological differences were first reported between an isolate from Côte d'Ivoire (West-Africa) and one from Kenya (East-Africa) (Fauquet and Thouvenel, 1977). Later, serological diversity of five isolates was studied, three serogroups were defined, but there was no apparent geographical basis to their distribution (Mansour and Baillis, 1994). By contrast, in Burkina-Faso (West-Africa), the three serogroups found were tentatively linked to their ecological origin and pathogenicity (Konaté et al., 1997).

In this study, the serological variability of RYMV isolates from all rice growing regions of Côte d'Ivoire was assessed using polyclonal and monoclonal antibodies. The existence of serotypes was proved and their distribution in Côte d'Ivoire was assessed. The serological patterns found in Côte d'Ivoire were compared with those of isolates from other countries in Africa. Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the coat protein (CP) gene of isolates of the serotypes was established. Biological properties of the isolates was assessed and interactions between isolates of different serogroups in co-inoculated plants was investigated and tentatively linked to pathogenicity. The epidemiological consequences of these findings are discussed.

Materials and methods

Virus sources, propagation and purification

Nineteen RYMV isolates were collected in all rice growing regions of Côte d'Ivoire from diseased plants showing characteristic mottling symptoms in surveys conducted before 1996. Biological properties of these isolates have also been studied (N'Guessan et al., 1995). First, the serological properties of these 19 isolates were tested with polyclonal antibodies in double-diffusion and absorption tests, and double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). Second, their serological profiles were assessed with monoclonal antibodies (MAbs) in triple-antibody sandwich ELISA (TAS-ELISA), together with those of 24 additional isolates collected since 1996 in Côte d'Ivoire. Third, the serological patterns of the Ivorian isolates were compared to those of 23 isolates collected in Burkina-Faso (2), Ghana (1), Kenya (1), Madagascar (5), Mali (4), Nigeria (5), Sierra-Leone (1) and Tanzania (4). Fourth, RT-PCR amplification of the CP gene of the isolates was tested and appropriate pairs of primers developed. Fifth, biological competition between isolates from different serogroups was tested, in relation to pathogenicity.

All isolates were collected from infected field plants on the basis of symptom expression. They were recovered by mechanical inoculation, which showed that they induced the characteristic yellow discoloration and mottling of leaves after mechanical inoculation to healthy plants of the susceptible *O. sativa indica* cultivar Bouaké 189. They were later determined to be positive for RYMV in ELISA using either polyclonal or monoclonal antibodies raised against the RYMV-Ma isolate from Mali. For biological tests, partially resistant (expressing less severe symptoms) *O. sativa indica* cultivars ITA 212 and Moroberekan were inoculated similarly. Inoculum was prepared by grinding infected frozen leaves in 0.1 M phosphate buffer pH 7.2 (1 g/10 ml). Extracts were mixed with 600-mesh carborundum and rubbed on to leaves of 14-day-old rice seedlings. Test plants were kept in a growth chamber under 13 h illumination at 120 $\mu E\, m^{-2}\, s^{-1}$ of PAR, at 30 °C and 90% humidity to facilitate homogenous symptom development. Infected leaves showing characteristic RYMV symptoms were harvested two weeks after inoculation and stored at −80 °C.

Purified viral preparations were obtained following the method of Bakker (1974) modified by Fauquet and Thouvenel (1977). Concentrations of the purified extracts were adjusted through absorbance at 260 nm, assuming an extinction coefficient of 6.5 for RYMV (Bakker, 1974). Infected leaves (1 g/10 ml) were ground in phosphate buffered saline (PBS) pH 7.2 containing 0.05% Tween 20 for ELISA, and in PBS buffer followed by a centrifugation at $7000 \times g$ for 5 min at 4 °C for double-diffusion tests. Purified virus had an absorption spectrum typical of RYMV, with an A260/A280 ratio of 1.4 to 1.6.

Antibody production

Three polyclonal antisera were used. Two were previously available, one raised against an isolate from Côte d'Ivoire (RYMV-CI) (Fauquet and Thouvenel, 1977), the other one from Mali (RYMV-Ma) (Ngon A Yassi, 1993). An antiserum against an isolate from Madagascar (RYMV-Mg) was also prepared. All antisera were obtained after three to five

intramuscular injections of three month old rabbits, each with 5 mg of freshly purified virus emulsified with Freund's complete adjuvant for the first injection and incomplete adjuvant for the subsequent ones. The three antisera had titres of 1024–2048 in double-diffusion tests.

Two panels of MAbs were prepared against isolates RYMV-Ma and RYMV-Mg, respectively. For the first fusion experiment, five-week-old BALB/c mice were immunised by two intraperitoneal injections of 100 μg of purified RYMV-Ma at two week intervals. The virus was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for the second. Two weeks later, the mice were given intravenous booster injections with 400 μg virus in a saline solution four days before fusion. For the second fusion, 3 to 6-week-old BALB/c mice were immunised with isolate RYMV-Mg by three intraperitoneal injections of 100 μg of virus at two week intervals. Six weeks later, the mice were given one intravenous and one intraperitoneal booster injection of 400 μg of virus in saline solution. Three days later, cell fusions were performed (Al Moudallal et al., 1982) using the P3x63AG8 myeloma cell line (Stocker et al., 1982).

Undiluted hybridoma culture supernatants were screened for the presence of specific antibodies to RYMV in antigen-coated plate indirect ELISA. The microtitre plates were coated with a purified preparation of the homologous antigen in 0.05 M carbonate buffer pH 9.6 at a virus concentration of 5 μg/ml using healthy sap as a control. An alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma Chemical Co, St. Louis, MO) was used as the detecting antibody. Selected positive cultures were cloned by limiting dilution, and by using macrophages as feeder cells. Each MAb was typed in double-diffusion tests (described below) using 10 μl anti-antibody (Sigma) of classes 1, 2a, 2b and 3 in the centre wells and 10 μl of hybridoma culture supernatants ten times concentrated in the peripheral wells. MAbs were used in Western blots to detect the capsid protein (Harlow and Lane, 1988).

Agarose-gel double-diffusion tests

The 19 RYMV isolates from Côte d'Ivoire and the isolate from Madagascar used to raise the RYMV-Mg antiserum were tested. Tests were performed on microslides (26 × 76 mm) with 0.7% agarose Type I: Low EEC (Sigma) in PBS buffer pH 7.2. The experimental pattern consisted of six peripheral wells 6 mm apart and 7 mm from the central well. Antiserum and antigens were placed in the central and outside wells, respectively, which were 4 mm diameter and 3 mm deep. The RYMV-Ma and RYMV-CI antisera were diluted 1 : 32 in PBS buffer, the RYMV-Mg antiserum was diluted 1 : 64. A volume of 10 μl was put in the central well and the same volume of crude extract or purified antigen was placed in the peripheral wells. Precipitin lines and spur formation were observed against a dark background over a box with a light source.

Correct interpretation of patterns of double-diffusion tests is based on a balanced system of antibody and antigen concentration which depends on experimental conditions (Ball, 1990; Van Regenmortel, 1982). The following standardisation of the experiments was implemented. First, the experiments were repeated systematically several times. Second, the isolates were multiplied under similar conditions (rice variety, date of inoculation and harvest, storage). Third, homogenous concentrations of antigens were checked in TAS-ELISA with the non-differentiating MAb M (see below). Fourth, experiments were conducted in parallel with leaf extracts and with purified viral preparations adjusted to 0.1 μg/ml by spectrophotometry. As the results were consistent, later tests were conducted with leaf extracts only.

For absorption experiments, the three antisera were cross-absorbed individually with infected leaf extracts. Absorption of antisera was done by mixing 1 μl of diluted RYMV-Ma or RYMV-CI antisera with 31 μl of healthy leaf extract diluted 1 : 32, whereas RYMV-Mg antiserum was diluted 1 : 64. The preparation was incubated at room temperature for 1 h, then cooled on ice for an additional 30 min before being tested as described above.

ELISA tests

Tests with polyclonal antibodies were conducted in DAS-ELISA (Clark and Adams, 1977), with an additional blocking step by adding 3% skimmed milk in washing buffer (i.e. PBS with Tween-20) for 1 h after the coating stage which consistently reduced the background values. MAbs were used in TAS-ELISA (Thomas et al., 1986). Microtitre plates were coated with 1 μg/ml γ-globulin from the rabbit polyclonal antiserum. Leaf sap prepared as described above was used as the antigen. MAbs in tissue supernatant were used as the detecting antibodies, and bound MAbs were

detected with goat anti-mouse globulin/alkaline phosphate conjugate (Sigma) diluted 1 : 8000. Dilutions of antigens and MAbs depended somewhat on the batches of hybridoma culture supernatants but were generally 1 : 1000 and 1 : 100 for MAb C, 1 : 1000 and 1 : 10 for MAb M, 1 : 1000 and undiluted for MAb G, 1 : 100 and 1 : 100 for MAb E, 1 : 1000 and 1 : 100 for MAb F, 1 : 1000 and undiluted for MAbs B and D, 1 : 10 000 and 1 : 50 for MAb A. Absorbances (405 nm) were read after incubation with p-nitrophenyl phosphate for 1 and 6 h at room temperature and after overnight incubation at 4 °C. In DAS-ELISA, the same procedure was followed, except that alkaline phosphatase-conjugated polyclonal antibody to RYMV was used as the detecting antibody. Background values were less than 0.1 in DAS-ELISA and less than 0.05 in TAS-ELISA and were not deducted from the readings. A reading above 0.3 was considered as the positive–negative threshold.

RT-PCR tests

Genome fragments with the CP gene of the 19 isolates were transcribed and amplified by RT-PCR after extraction of total RNA from leaves. The protocol was essentially that used by Brugidou et al. (1995) and nucleotide numbers refer to the RYMV genome reported by Ngon A Yassi et al. (1994). Two pairs of primers were used to amplify regions including the 720 bp CP gene which starts with the AUG codon at position 3447 to end at a stop codon UGA at nucleotide 4166 (Ngon A Yassi et al., 1994). One pair (primers I and II) had been designed by Brugidou et al. (1995) and was based on the sequence of an isolate from Côte d'Ivoire (Ngon A Yassi et al., 1994). Primer I was complementary to the 23 nucleotides at the end of RYMV-RNA from position 4428 to 4450, primer II corresponded to the 16 nucleotides from position 3442 to 3457, and a 1008 bp product was expected. The second pair (primers A and B) was designed from conserved regions and used for isolates not amplified by primers I and II. Primer A was complementary to the 22 nucleotides from position 4300 to 4321, primer B corresponded to the 18 nucleotides from position 3451 to 3468. A 870 bp product was expected.

Biological tests

Experiments were conducted to test for biological interaction between isolates of the S1 and S2 serogroups. Rice plants of the susceptible cv. Bouaké 189 were co-inoculated simultaneously with an S1 isolate and one of the S2 serogroups. The following pairs of isolates were tested: C12 (S1) and C17 (S2), C2 (S1) and C13 (S2), C15 (S1) and C8 (S2), C6 (S1) and C1 (S2). Single inoculations of the S1 or the S2 isolate were performed as controls. Each individual treatment (single inoculation and dual inoculation) was made of five plants pooled together and each treatment was replicated six times. Virus titre of each sample (singly and dually inoculated) was tested in TAS-ELISA two weeks after inoculation with discriminant MAbs A, D, F, and with the non-discriminant MAb C. A mixture of an equal volume of sap extract from S1 and S2 singly-inoculated plants was also tested for comparison. All the experiments were repeated twice. In addition, the virus titre was also assessed two and four weeks after inoculation, and the whole experiment was performed on the partially resistant cv. ITA 212. Different patterns of inoculation were tested: simultaneous S1 and S2 co-inoculation, and a one week time lag between S1 and S2 or between S2 and S1 inoculations.

In another experiment, 5 S1 and 10 S2 isolates were inoculated on cv. Bouaké 189 and cv. ITA 212, respectively. Each individual treatment (cultivar × isolate) was made of five plants pooled together and each treatment was replicated three times. Symptoms were evaluated on individual plants 7, 14 and 21 days after inoculation following the 1–3 scale adapted from John and Thottapilly (1987). Virus titre was tested 14 days after inoculation in TAS-ELISA using the RYMV-Mg polyclonal antiserum as primary antibody and the non-discriminant MAb C as secondary antibody.

Results

Polyclonal tests

The 19 isolates from Côte d'Ivoire fell into two distinct serogroups designated S1 (five isolates) and S2 (14 isolates). The serogrouping was not dependent on the antiserum used. Within isolates of each group, the precipitin lines coalesced whatever the antiserum used. By contrast, spurs spread from the five S1 isolates to the 14 S2 isolates with the RYMV-Ma antiserum. Conversely, spurs spread from S2 isolates to S1 isolates with the RYMV-CI antiserum. Response with the RYMV-Mg antiserum was similar to that obtained with the RYMV-CI antiserum, with spur formation only observed from S2 to S1 isolates. Absorption tests confirmed the separation of these isolates into

Table 1. Reactions in diffusion tests of RYMV isolates with strain-specific polyclonal antisera after cross-absorption with serotypes***

Isolates tested		Polyclonal antisera					
Serotype	Number*	RYMV-Ma (S1)**		RYMV-CI (S2)**		RYMV-Mg (S4)**	
		Serotype absorbed		Serotype absorbed		Serotype absorbed	
		S2	S1	S2	S1	S2	S1
S1	5	+	−	−	−	−	−
S2	14	−	−	−	+	−	+
S4	1	+	−	−	+	+	+

*Number of isolates tested.
**Polyclonal RYMV-Ma was raised against an S1 isolate from Mali, RYMV-CI against an S2 isolate from Côte d'Ivoire, and RMYV-Mg against an S4 isolate from Madagascar.
***'+' indicates a discernible precipitate fusion line, '−' no discernible precipitation.

two groups (Table 1). After absorption of RYMV-Ma antiserum with S2 isolates C1 or C9, the five S1 isolates, but none of the 14 S2 isolates, were detected in double-diffusion tests. Such specific detection of S1 isolates was expected assuming that antibodies directed towards S2 epitopes in antiserum RYMV-Ma had been precipitated, leaving only antibodies specific to S1 in the antiserum. Conversely, no reaction occurred after absorption with an S1 isolate as RYMV antibodies had been precipitated. After absorption of RYMV-CI antiserum and RYMV-Mg antiserum with S1 isolates C11 or C15, all S2 isolates, but none of S1, were detected (Table 1). Such specific detection of S2 isolates was also expected, assuming that antibodies directed towards S1 epitopes in RYMV-Mg and RYMV-CI antisera were precipitated during the absorption phase, leaving only antibodies specific to S2 in the antisera. Conversely, no reaction occurred after absorption with an S2 isolate as RYMV antibodies had been precipitated.

Isolates C6 (S1) and C1 (S2) were multiplied and purified under similar conditions (see Materials and methods). After adjusting the virus concentration of each isolate preparation to 0.1 mg/ml by spectrophotometry at 260 nm, serial dilutions of the purified preparations were tested in DAS-ELISA using the RYMV-Ma antiserum. Higher absorbances were obtained with S1 isolate C6 than with S2 isolate C1 either with purified virus preparations (Figure 1) or with crude extracts (data not shown). Such higher detection of the S1 isolate was expected assuming that RYMV-Ma antiserum contained antibodies specific to S1 epitopes.

Isolate RYMV-Mg was also tested. It did not react like the S1 or S2 serotypes. In double-diffusion tests

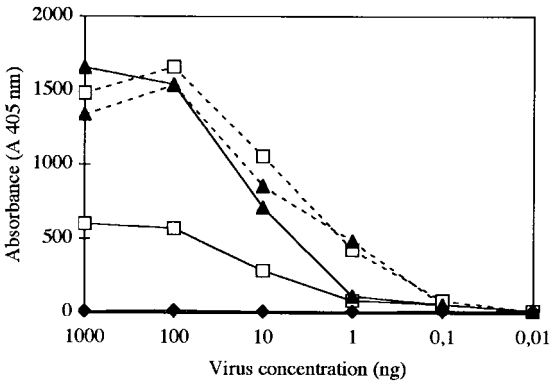

Figure 1. Absorbance (405 nm) of serial dilutions of a purified virus preparation of S1 isolate C6 (triangle) and S2 isolate C1 (square) detected in DAS-ELISA with RYMV-Ma antiserum (plain lines) and with RYMV-Mg antiserum (dotted lines). Control was with healthy leaf extracts (diamond).

with the RYMV-Mg antiserum, there were spurs from the RYMV-Mg isolate towards each of the isolates of Côte d'Ivoire. Moreover, the RYMV-Mg isolate was detected by RYMV-Mg antiserum after cross-absorption with either an S1 or an S2 isolate (Table 1). This indicated that the RYMV-Mg isolate contained additional epitopes and represented another serotype. Such serological differences between RYMV-Mg and both S1 or S2 serotypes would explain why, despite S2 specific antibodies in RYMV-Mg antiserum (see above), DAS-ELISA detection of the S2 isolate C1 was similar to that of the S1 isolate C6 (Figure 1). Only indirect assumptions can be made on the serotypes of the isolates used to raise the RYMV-Ma and RYMV-CI antisera (Fauquet and Thouvenel, 1977; Ngon A Yassi, 1993) as no information on their source was available,

other than country of origin (Mali and Côte d'Ivoire, respectively). However, the presence of S1 specific antibodies in RYMV-Ma antiserum and S2 specific antibodies in RYMV-CI antiserum would be explained if the isolates from Mali and from Côte d'Ivoire were of the S1 and the S2 serotypes, respectively.

Monoclonal tests

The three MAbs prepared against the RYMV-Ma isolate detected all RYMV isolates in TAS-ELISA similarly and could not be used to discriminate between samples. So only one of them (identified as MAb M) was kept in the tests as a non-discriminant MAb (Table 2). Monoclonal antibodies prepared at the International Institute of Tropical Agriculture (IITA) against an isolate from Nigeria recognised one epitope or few linked ones in a collection of isolates from Burkina-Faso and Mali (Konaté et al., 1997). The same seven IITA MAbs (referenced 6C71, 6C73, 6C75, 6C78, 4A95, 4A98, 4A912) were also non-discriminant in our experiments, as they all detected all isolates of Côte d'Ivoire and elsewhere (data not shown). By contrast, MAbs prepared against the RYMV-Mg isolate (identified as A, B, C, D, E, F, G) recognised seven different epitopes when tested against a range of isolates from various origins in Africa: each of these seven MAbs had a specific reaction pattern with the isolates (Table 2). This higher diversity of MAbs may reflect the specific immunological properties of the isolate from Madagascar used to prepare the MAbs, distinct from both S1 and S2 isolates in polyclonal tests (see above), or the longer immunisation protocol adopted with the RYMV-Mg isolate (see Materials and methods). The eight MAbs were immunoglobulins of IgG class. Only MAb E detected the CP in Western-blot tests (data not shown). The other MAbs failed to detect it, whatever the hybridoma supernatant fluid or antigen concentrations. MAb B belonged to the subclass 1, MAbs A, F and E to the subclass 2a and MAbs G and D to the subclass 2b.

These eight MAbs were used to assess the response of isolates from Côte d'Ivoire. MAbs M, C, G, and E reacted similarly with any of the 19 isolates from serogroups S1 or S2 (Table 2). By contrast, a clear differential reaction pattern was observed with MAbs D, F and A. Consequently, S1 and S2 isolates were distinguished readily by these differentiating MAbs. MAb D detected all S2 isolates, but none of S1. Inversely, MAbs F and A detected all S1 isolates, but failed to detect S2 isolates (Table 2). This differential pattern was consistent, although the optical density (OD) for a positive reaction with MAbs D, F and A generally ranked 4 ($OD > 1.8$), but were sometimes as low as 2 ($0.6 < OD < 1.2$), depending on the virus titre of the isolates and on the antibody titre in the hybridoma supernatant. Although MAb B always reacted strongly with the reference isolate RYMV-Mg and weakly with S1 or S2 isolates, other reactions were less consistent between repeats and more difficult to interpret.

Twenty-four additional RYMV isolates from Côte d'Ivoire were tested with the full range of MAbs. Twenty-three had serological profiles typical of the S2 serogroup. Only one isolate belonged to the S1 serogroup. Altogether, the S2 serogroup was numerically predominant in Côte d'Ivoire with 14 (vs. 6 S1) of the 20 isolates collected in 1994/1995, and 22 (vs. 1 S1) of 23 isolates collected in 1996. We never had a clear

Table 2. Serological profiles of RYMV isolates assessed with MAbs in TAS-ELISA* tests

Isolates			Monoclonal antibodies (MAbs)							
Serotypes	Countries**	Number***	M	C	G	E	B	F	D	A
S1	CI, BF, Gh, Ma, Ni	17 (7)	4	4	4	4	1	3–4	0	4
S2	CI	36 (36)	4	4	4	4	1	0	3–4	0
S3	SL, Ni	3 (0)	4	4	0–2	4	0	0	4	0
S4	Mg, Ta, Ke	8 (0)	4	4	4	4	4	4	4	4
S5	Ta	2 (0)	4	4	4	0	0	4	4	4

*Absorbances were coded as follows: '0' ≤ 0.30, $0.30 \leq$ '1' ≤ 0.60, $0.60 <$ '2' ≤ 1.20, $1.20 <$ '3' ≤ 1.80, $1.80 <$ '4'.

** Abbrevations of Côte d'Ivoire (CI), Burkina-Faso (BF), Ghana (Gh), Kenya (Ke), Mali (Ma), Nigeria (Ni), Sierra-Leone (SL), Tanzania (Ta).

***Total number tested; the number in brackets indicates the number of samples from Côte d'Ivoire.

Figure 2. Map of Côte d'Ivoire showing the distribution of the isolates of S1 (open circles) and S2 (dark circles) and position of the nearest town.

evidence of S1 and S2 in mixture, as no isolate of Côte d'Ivoire reacted simultaneously with MAbs D, F and A. There were pronounced geographical differences in the distribution of the 7 S1 and the 36 S2 of isolates from Côte d'Ivoire (Figure 2). With 3 S1 and 33 S2 from the forest vs. 4 S1 and 3 S2 from the Savannah, the geographical distribution was contrasted with a significant relationship between serotype distribution and spatial distribution between forest and Savannah zones (χ^2 test with Yates correction for small samples $= 7.15$, $p < 0.01$).

Twenty-three samples from other countries in Africa were tested. The serological pattern of S1 was found with isolates from neighbouring countries Burkina-Faso, Ghana, Mali and further away in Nigeria. In Mali, the S1 pattern was found with isolates from both *O. sativa* and the wild perennial species of rhizomatous rice *O. longistaminata*. By contrast, the S2 pattern was not found with isolates from outside Côte d'Ivoire (Table 2). Additional serological patterns were detected outside Côte d'Ivoire. Serogroup S3 includes two isolates from Nigeria and one from Sierra-Leone and shared a specific combination of reactions positive with MAbs C, D, E and M, and negative with MAbs A, B, F and G. The lack of reaction with MAb G distinguished specifically this serotype from S2 or from any S1 and S2 mixture. Serogroup S4 includes the five isolates from Madagascar, two from Tanzania and one from Kenya, and reacted strongly with all MAbs, including MAb B (Table 2). This indicated that this serological pattern was specific and did not result from an S1 and S2 mixture, but reflected that the MAb series had been raised against an isolate from Madagascar. Serogroup S5 includes two other isolates from Tanzania and failed to react with MAbs B and E whereas giving strong reactions with MAbs A, D, F and G (Table 2). Its serological pattern differed from the others and could not result from mixtures of isolates with the other serotypes. Altogether, Côte d'Ivoire was not the only country with isolates with different serological patterns as two serotypes were also found in Nigeria and in Tanzania.

Then, MAbs E and G – like MAbs A, B, D and F – were discriminant for RYMV isolates, whereas MAbs C and M which detected all RYMV isolates are likely to be directed to conserved epitopes, possibly the same one. A few isolates from Côte d'Ivoire and from other countries reacted with polyclonal antisera, with non-discriminant MAbs C and M, but failed to react with any specific MAbs, even when tested with more concentrated hybridoma culture supernatants, and were not classified.

RT-PCR tests

RT-PCR amplification of the genome region with CP gene was contrasted, depending on the serotype of the isolate tested and on the pair of primers used. With primers I and II, amplification of the targeted region of any S2 isolates was successful and the expected 1 kb band was obtained (Figure 3). Differences in band intensity were observed, possibly reflecting differences in starting RNA titre, but PCR re-amplification of these bands was always achieved. Most isolates from Madagascar, Kenya and Tanzania with the additional serological patterns were also amplified successfully (data not shown).

By contrast, amplification of S1 isolates was less consistent. S1 isolates C2, C11 and C15 from Côte d'Ivoire consistently failed to be amplified with primers I and II whatever the protocol (Figure 3). With isolates C6, C12 and C25 amplification was sometimes possible, but only when $MgSO_4$ was included in the PCR reaction mixture. Experiments with purified viral preparations from S2 isolate C1 (2.6 mg/ml) and S1 isolate C6 (3.2 mg/ml) isolates confirmed the differences

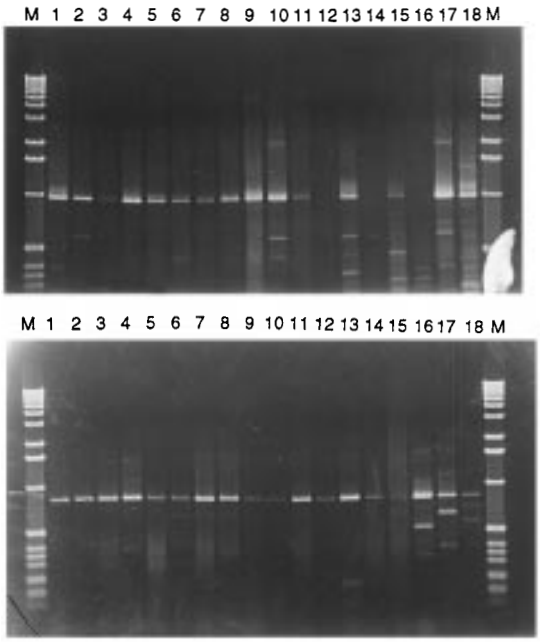

Figure 3. Ethidium bromide stained 1% agarose gel of RT-PCR products amplified with primers I and II (top), and with primers A and B (bottom) from leaf extracts of plants infected with S2 isolates (lanes 2–11: C1, C3, C4, C5, C7, C8, C9, C13, C16, C17), S1 isolates (lanes 12–17: C2, C6, C11, C12, C15, C25). An isolate from Mali was in lane 1 and one from Madagascar in lane 18. The 1 kb DNA ladder from Gibco are in lanes M.

of responses: i.e. amplification was always successful with the purified preparation of the isolate C1 (S2), but often failed with that of C6 (S1) (data not shown). A new pair of primers (A and B), was designed on the basis of the conserved sequences of isolates amplified successfully and sequenced with primers I and II. With these primers, all S1 isolates were readily amplified to give the expected 870 bp band (Figure 3). The S2 isolates were also amplified (Figure 3), as well as the isolates with the additional serological patterns from Madagascar, Kenya and Tanzania (data not shown).

Biological tests

RYMV-Mg polyclonal antiserum was used as primary antibody in indirect DAS-ELISA as it detected S1 and S2 isolates similarly in DAS-ELISA (see above). MAbs A (and F), specific for S1, and MAb D specific for S2 were used as secondary antibody to assess virus titre of S1 or S2 isolates. The non-discriminant MAb C double inoculations obtained with isolate couples C6 vs. C1, C2 vs. C13, C15 vs. C8, C12 vs. C17 were similar. Only C12 (S1) vs. C17 (S2) details which are quantified the overall virus titre, independently of the serological properties of the isolates.

A similar pattern of reactions was observed among isolates of the same serogroup. The S1-specific MAbs A and F only detected RYMV in plants singly inoculated with C2, C6, C12 or C15 of the S1 serogroup. The S2-specific MAb D only detected RYMV in plants singly inoculated with C1, C8, C13 or C17 of the S2 serogroup. The patterns of results of single vs. representative and typical of all S1/S2 pairing are presented in Table 3. A specific pattern of reaction was found in plants co-inoculated with the S1 and S2 isolates; reactions with MAbs A and F were consistently lower than those of plants singly inoculated with the S1 isolate only, and even lower than that of the S1/S2 mixture. Overall, the MAbs A and F S1-specific reactions in doubly inoculated S1/S2 plants were significantly lower than of S1 singly inoculated plants ($p < 0.001$ after one-way variance analysis). This occurred both with the highly susceptible cultivar Bouaké 189 and with the partially resistant ITA 212. This was apparent also in tests 14 and 28 days post inoculation (dpi), and there was a similarly lower S1 virus titre in plants doubly inoculated at these two stages (Table 3). Even when the S2 isolate was inoculated one week later than S1, lower S1 content in doubly infected plants was apparent, although only 28 dpi (Table 4). By contrast, S2-specific reactions with MAb D in singly and in doubly inoculated plants (Tables 3 and 4) were similar (non-significant difference after one-way variance analysis).

Fifteen isolates including five S1 and 10 S2 were inoculated. Symptom score and virus content were assessed 14 dpi as described earlier. Differences of symptom expression and virus content among plants infected with different RYMV isolates were apparent both in the susceptible ITA 212 cultivar and in the resistant cultivar Moroberekan (data not shown). Virus content and symptom expression were positively correlated ($r = 0.56$, $p < 0.05$). By contrast, there was no significant relationship between serotype and virus content or symptom expression. There was no relation either between isolate severity and dominance in co-inoculation tests. For instance, the S2 isolate C13 which induces mild symptoms dominates in co-inoculation over the severe S1 isolate C15.

Table 3. Virus titre in rice cultivars singly or doubly inoculated with S1 and/or S2 serotypes of RMYV and assessed in TAS-ELISA** tests with S1-specific (A, F), S2-specific (D) and non-specific (C) MAbs

MAbs	Cultivars	dpi***	Isolates			
			S1*	S2*	S1 + S2¶	S1 − S2¶
A	Bouaké 189	14	3.83	0.20	1.89	3.90
		28	3.64	0.16	1.73	3.51
	ITA 212	14	3.27	0.18	1.18	2.58
		28	3.87	0.26	1.04	3.89
F	Bouaké 189	14	0.92	0.08	0.42	0.62
		28	1.17	0.13	0.43	0.72
	ITA 212	14	0.65	0.09	0.28	0.53
		28	1.13	0.13	0.42	0.70
D	Bouaké 189	14	0.10	0.62	0.71	0.34
		28	0.13	0.80	0.81	0.36
	ITA 212	14	0.13	0.46	0.59	0.29
		28	0.14	1.72	2.18	0.92
C§	Bouaké 189	14	2.82	3.00	3.30	2.98
		28	3.75	3.72	3.77	3.73

*Results given were obtained from inoculation with isolate C12 of S1 and C17 of S2.
**Positive–negative threshold was set to 0.3.
***Number of days post inoculation when the ELISA was done.
¶S1 + S2 indicates the ELISA responses of a co-inoculation of C12 and C17; S1−S2 indicates the ELISA response of a mixture (v : v) of C12 and C17 sap extracts.
§Only Bouaké 189 was tested with MAb C.

Table 4. Virus titre in rice singly or doubly inoculated with S1 and/or S2 serotypes, assessed in TAS-ELISA tests with S1-specific (A), S2-specific (D) and non-specific (C) MAbs, with and without a one week time lag (l) between the two inoculations

MAbs	dpi§	Isolates*				
		Single inoculation		Double-inoculation		
		S1	S2	S1 + S2**	S1 + S2 (l)***	S2 + S1 (l)¶
A	14	2.55	0.18	0.93	2.91	1.03
	28	2.44	0.28	1.71	1.51	1.33
D	14	—	—	—	—	—
	28	0.91	1.33	1.39	1.62	1.61
C	14	3.88	3.69	3.63	3.87	3.86
	28	3.97	3.89	3.99	3.89	3.98

*Results given were obtained from inoculation with isolate C12 of S1 and C17 of S2.
**Plants simultaneously inoculated with S1 and S2.
***Plants inoculated first with S1, and one week later with S2 (l).
¶Plants inoculated first with S2, and one week later with S1 (l).
§Number of days post the first inoculation when the ELISA test was done.
— not tested.

Discussion

In Côte d'Ivoire, immunological diversity of the RYMV isolates assessed with polyclonal and monoclonal tests was apparent with consistent grouping into serogroups designated S1 and S2. First, isolates fell into two groups in double-diffusion tests with each of the three polyclonal antisera used. Second, this grouping was confirmed in double-diffusion tests after absorption of the antiserum, where isolates of one serogroup were recognised by the homologous antiserum after absorption with an isolate of the other serogroup. Third, S1 isolates were preferentially recognised in DAS-ELISA using an antiserum with antibodies specific of S1 epitopes. Fourth, this S1/S2 split was confirmed in TAS-ELISA using MAbs as secondary antibodies. The MAbs F and A readily detected S1 isolates, but not S2 isolates, whereas the reverse was true with MAb D which detected S2 but not S1 isolates. Overall, these results suggested that serogroups S1 and S2 differed by a specific combination of epitopes. The S1 isolates had epitopes specifically detected by antibodies of the RYMV-Ma polyclonal antiserum and by MAbs F and A. Conversely, they lacked epitopes present in S2 isolates detected by antibodies of RYMV-CI and RYMV-Mg antisera and by MAb D. Although most isolates from Côte d'Ivoire could clearly be assigned to serotypes S1 or S2, a few samples failed to be classified because of weak reactions with the discriminating MAbs. This was possibly due to low virus content in the leaf extracts or, alternatively, reflected specific serological properties of these isolates undetected by these MAbs.

The results of polyclonal and monoclonal tests were consistent and complementary. The clear-cut S1/S2 differences apparent in polyclonal tests suggested that the RYMV serological diversity in Côte d'Ivoire did not form a continuum. Conversely, tests with serotype-specific monoclonal antibodies were more precise and easier to implement for isolate classification, and were the only practical approach when testing many samples or several serotypes. Non-specific MAbs were also used successfully to quantify RYMV titre independently of the serotype used, an important feature for crop breeding programmes. The ability of specific MAbs to detect different serotypes was also useful to monitor simultaneously the replication of the two serotypes within doubly-inoculated plants (Halk and De Boer, 1985).

The S2 serotype predominated in Côte d'Ivoire. The geographical pattern of the distribution of the serogroup was not random, S2 isolates were more prevalent in the centre and the south of Côte d'Ivoire, and S1 in the north although the S1 serotype was a minority overall in the country. Preferential distribution of the S1 serogroup in the north of Côte d'Ivoire was consistent with detection of S1 isolates from Mali and Burkina-Faso to the north. Three additional serological patterns were detected, one (S3) in Nigeria and Sierra-Leone (West-Africa) and two (S4 and S5) in East-Africa (Madagascar, Tanzania and Kenya) which showed that the serological diversity of RYMV was not limited to that found in Côte d'Ivoire. Furthermore, as in Côte d'Ivoire, several serotypes can co-exist in the same country.

There were links between the immunological responses of the S1 and S2 isolates from Côte d'Ivoire and some of their molecular and biological properties. Contrasted amplifications of S1 and S2 isolates were found in RT-PCR tests. Amplification of S1 isolates generally failed with the first pair of primers (I and II) developed against an S2 isolate, whereas it was successful with both S1 and S2 isolates with the second pair of primers (A and B). This indicated that the sequences targeted by primers I/II differed between S1 and S2 isolates and reflected differences in molecular properties between isolates of the two serogroups. More generally there was a good correspondence between the serological typing and the molecular typing through sequencing of the CP gene of RYMV isolates (N'Guessan, 1999) which indicates that strain-specific MAbs are useful tools in epidemiological studies to assess strain identity and interaction.

Within a set of isolates of related strains, many possibilities of interaction exist including reciprocal cross-protection of varying degrees of completeness, unilateral cross-protection and no cross-protection (Matthews, 1991). S1/S2 interaction is clearly unilateral as S1 virus titre decreased whereas S2 remained stable after co-inoculation. This was apparent through immuno-detection of the CP, and also through sequencing of RT-PCR products from S1/S2 co-inoculated extracts which resulted in the S2 consensus sequence (A. Pinel and D. Fargette, unpublished results). In our experiments, S1 persisted in the co-inoculated plants albeit at a low level. Nevertheless, successive transmission by beetles of such mixtures with S2 prevalence may progressively lead to S1 elimination. This would explain why, although S1 and

S2 isolates were sometimes found in nearby sites of Côte d'Ivoire, we did not find any isolate reacting serologically like an S1/S2 mixture with simultaneous reactions against MAbs A, D and F. This may also explain the present S2 prevalence in Côte d'Ivoire. It is not known whether the higher proportion of the S2 serotype in surveys conducted after 1995 compared to those made earlier was due to an increased incidence of S2 or only reflected sampling variation. It is not excluded however that, because of S2 dominance over S1, the S2 serogroup is currently spreading in the country at the expense of S1. As S1 decrease occurred even when it was inoculated first, S1/S2 interaction cannot be assimilated to cross-protection which implies that the strain inoculated first prevents further infection from the challenging strain (Urban et al., 1990). This did not reflect either faster virus multiplication or higher severity of S2 isolates *per se* in the *O. sativa indica* varieties tested. Interactions with other strains such as S4 found in East-Africa was also suggested as symptoms of S1/S4 or S2/S4 doubly-infected plants were sometimes more severe than those induced by a single strain (D. Fargette and A. Pinel, unpublished results). Differences in thermosensitivity between strains is well documented (Matthews, 1991) and experiments are currently conducted to assess whether this contrasted south/north distribution of S1 and S2 isolates could reflect such differences, with lower temperature of the south and centre of Côte d'Ivoire favouring S2 and hotter temperatures in the north favouring S1.

Acknowledgements

We thank M. Bousalem, M. Peterschmitt, J.M. Thresh and M.H.V. Van Regenmortel for reviews of the manuscript, C. Brugidou for helpful advice, J. Aribi, J. Guignot, S. Corgier and H. Virelizier for technical assistance, J.L. Notteghem, J. d'A Hughes, Y. Pinto, F. Kimmins and F. Ali for providing isolates from Madagascar, Nigeria, Sierra-Leone, Tanzania, respectively, and G. Thottapilly for samples of MAbs.

References

Al Moudallal Z, Briand JP and Van Regenmortel MHV (1982) Monoclonal antibodies as probes of the antigenic structure of tobacco mosaic virus. EMBO Journal 1: 1005–1010

Awoderu VA (1991) Rice yellow mottle virus in West Africa. Tropical Pest Management 37: 356–362

Awoderu VA, Alam MS, Thottapilly G and Alluri K (1987) Rice yellow mottle virus in upland rice. FAO Plant Protection Bulletin 35: 32–33

Bakker W (1971) The new beetle vectors of rice yellow mottle in Kenya. Netherland Journal of Plant Pathology 77: 201–206

Bakker W (1974) Characterisation and ecological aspects of rice yellow mottle virus in Kenya. Agricultural Research Reports, Wageningen 829: 1–152

Ball EM (1990) Agar double diffusion plates (Ouchterlony): viruses. In: Hampton R, Ball E and De Boer S (eds) Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens (pp 111–115) APS, St Paul

Brugidou C, Holt C, Ngon A Yassi M, Zhang S, Beachy R and Fauquet C (1995) Synthesis of an infectious full-length cDNA clone of rice yellow mottle virus and mutagenesis of the coat protein. Virology 206: 108–115

Clark MF and Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 475–483

Fauquet C and Thouvenel JC (1977) Isolation of the rice yellow mottle virus in Ivory Coast. Plant Disease Reporter 61: 443–446

Halk ED and De Boer SH (1985) Monoclonal antibodies in plant-disease research. Annual Review of Phytopathology 23: 321–350

Harlow E and Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, 726 pp

Hull R (1988) The sobemovirus group. In: Koenig R (ed) The Plant Viruses. Polyedral Virions with Monopartite RNA Genomes, Vol 3 (pp 113–146) Plenum Press, New York

John VT and Thottapilly G (1987) A scoring system for rice yellow mottle virus disease. International Rice Research Newsletter 12: 3–26

John VT, Thottapilly G and Awoderu VA (1984) Occurrence of rice yellow mottle virus in some sahelian countries in West Africa. FAO Plant Protect Bulletin 32: 86–87

Konate G, Traore O and Coulibaly M (1997) Characterisation of rice yellow mottle virus isolates in Sudano–Sahelian areas. Archives of Virology 142: 1117–1124

Mansour AN and Baillis KW (1994) Serological relationships among rice yellow mottle virus isolates. Annals of Applied Biology 125: 133–140

Matthews REF (1991) Plant Virology. Third Edition. Academic Press, Inc., 815 pp

Ngon A Yassi M (1993) Caractérisation moléculaire du virus de la panachure jaune du riz (RYMV) et production de riz transgéniques. PhD of the Université des Sciences et Techniques du Languedoc Montpellier II, 143 pp

Ngon A Yassi M, Ritzenthaler C, Brugidou C, Fauquet C and Beachy RN (1994) Nucleotide sequence and genome characterisation of rice yellow mottle virus RNA. Journal of General Virology 75: 249–257

N'Guessan P (1999) Le virus de la panachure jaune du riz: diversité moléculaire et sérologique en Afrique et aspects épidémiologiques en Côte d'Ivoire. PhD de l'Ecole Nationale Supérieure Agronomique de Montpellier

N'Guessan K, Sy A and Fargette D (1995) Caractérisation biologique et sérologique d'isolats du virus de la marbrure du riz (rice yellow mottle virus-RYMV) en Côte d'Ivoire. Proc. First Inter. Symposium on Rice Yellow Mottle Virus (RYMV), 18–22 September, 19 pp

Raymundo SA and Buddenhagen IW (1976) A rice virus disease in West Africa. International Rice Commission Newsletter 25: 58

Raymundo SA and Konteh IK (1980) Distribution, screening methods and varietal reaction to rice pale yellow mottle disease. International Rice Commission Newsletter 29: 51–53

Rochebrun M and Sablayrolles J (1993) Atlas du continent africain. Jeune Afrique Press, Paris, 175 pp

Stocker JW, Foster HK and Miggiano V (1982) Generation of two new myeloma cell lines 'PAI' and 'PAI-O' for hybridoma production. Research Disclosure 217: 155–157

Taylor DR, Fofie AS and Suma M (1990) Natural infection of rice yellow mottle virus disease (RYMV) on rice in Sierra Leone. International Rice Research Newsletter 15: 5–19

Thomas JE, Massalski PR and Harrison BD (1986) Production of monoclonal antibodies to African cassava mosaic virus and differences in their reactivites with other whitefly-transmitted geminiviruses. Journal of General Virology 67: 2739–2748

Urban LA, Sherwood JL, Rezende JAM and Melcher (1990) Examination of mechanisms of cross protection with non-transgenic plants. In: Fraser RSS (ed) Recognition and Response in Plant–Virus Interactions, NATO ASI series, Vol H 41 (pp 416–426) Springer-Verlag, Berlin, Heildelberg

Van Regenmortel MHV (1982) Serology and Immunochemistry of Plant Viruses. Academic Press, New York, 302 pp