EVIDENCE OF NON-TRANSMISSION OF *RICE YELLOW MOTTLE VIRUS* THROUGH SEEDS OF WILD HOST SPECIES

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SUMMARY

Rice yellow mottle virus (RYMV) is a widespread and damaging rice pathogen in Africa. The presence, infectivity and transmissibility of RYMV in seeds of six wild host species were investigated. In serological analyses by enzyme-linked immunosorbent assay (ELISA), each of four RYMV isolates used was detected in individual seeds of wild rice species (*Oryza barthii* and *O. longistaminata*) and the standard susceptible rice BG 90-2 (*O. sativa*). Virus detection in seeds of other host species (*Dactyloctenium aegyptium*, *Eragrostis ciliaris*, *E. tenella* and *E. tremula*) was unsuccessful in single seeds but positive reactions were obtained for extracts made from several seeds. RYMV was infectious in freshly-harvested seed extracts whatever the plant species or the virus isolate. However, most infectivity was lost in dried seeds, possibly due to virus inactivation following dehydration of the seeds. Despite the presence of RYMV in dry seeds, no evidence was obtained for transmission of the virus through seeds of rice or wild host species, whatever the plant species-virus isolate combination. RYMV is unlikely to be transmitted through seeds of its host species and, therefore, virus dissemination or epidemics of rice yellow mottle do not originate from infected seeds.

Key words: *Rice yellow mottle virus*, RYMV, rice, wild host species, seed-transmission.

INTRODUCTION

Rice yellow mottle virus (RYMV) (genus *Sobemovirus*, Hull and Fargette, 2005) causes the most important viral disease of rice in Africa. RYMV was first reported in Kenya (Bakker, 1970) and now occurs in all major rice-producing areas of Africa (Kouassi *et al.*, 2005). Typical symptoms induced by RYMV are yellow discoloration and mottling of the leaves of infected rice plants. Additional symptoms may include stunting, reduced tillering and poor panicle exertion (Bakker, 1974; Awoderu, 1991). As a consequence, yield may be dramatically reduced by 10% to 100% (Calvert *et al.*, 2003; Kouassi *et al.*, 2005). Yield losses are affected by virus-host interactions in which both host and virus variants are key factors (N'Guessan *et al.*, 2001). RYMV isolates belong to one of five serotypes referred to as ser1 to ser5 (Fargette *et al.*, 2002a). The occurrence of isolates capable of overcoming available resistance has also been reported (Fargette *et al.*, 2002b; Traoré *et al.*, 2006).

Experimentally, RYMV is easily transmitted mechanically and several mechanisms are involved in its spread under field conditions. Transmission is mainly by chrysomelid beetles, but can also be by wind or mammals such as cows, rats or donkeys (Bakker, 1974, Sarra and Peters, 2003; Sarra *et al.*, 2004). Abiotic transmission through soil and irrigation water has been also reported (Abo *et al.*, 2000; Sarra, 2005). Several studies demonstrated that RYMV was not transmitted through rice seeds (Bakker, 1974; Fauquet and Thouvenel, 1977; Konaté *et al.*, 2001; Abo *et al.*, 2004) but transmissibility through seeds of wild hosts has not been investigated. Wild host plants may play a major role in the local and long-distance spread of viruses, especially when seed transmission occurs (Bos, 1981). Apart from cultivated rice (*Oryza sativa* L. and *O. glaberrima* Steud.), the natural host range of RYMV includes a few wild rice species (*O. barthii* A.Chev. and *O. longistaminata* A.Chev. & Roehr.) and other members of the Poaceae (*Echinochloa colona* (L.) Link., *Ischaemum rugosum* Salisb. and *Panicum repens* L.) (Konaté *et al.*, 1997). Among all the natural host species, the widespread *O. longistaminata* seems to be of prime epidemiological importance because it is also a perennial rhizomatous plant that can act as a permanent reservoir for the virus (John *et al.*, 1984; Abo *et al.*, 1998). In addition, several wild *Poaceae* species have been identified as experimental hosts, most of which belong to the tribe *Eragrostideae* (Bakker, 1974; Allarangaye, 2003). However, knowledge of alternative host plants remains insufficient, resulting in their role in RYMV epidemiology being poorly understood.

In this study, we investigated the invasion of seeds of seven host species (six wild host plants and the standard

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susceptible rice cultivar BG90-2) by four RYMV isolates. Virus infectivity in freshly harvested or dried seeds was assayed and seedborne infections were also assessed. The implications of the findings on RYMV epidemiology and evolution are discussed.

MATERIALS AND METHODS

Virus isolates. Four RYMV isolates, hereafter designated as Bf5, Td3, Td20 and Mi532 from our virus collection were used. These isolates were recovered from field samples collected in three African countries, namely Burkina Faso (Bf), Chad (Td) and Mali (Mi).

Apart from their geographical diversity, virus isolates were also chosen to cover most RYMV serological and pathogenic diversity because seed transmission of viruses may depend on such properties (Johansen *et al*., 1994). The isolates differed in their serological and pathogenic properties. Isolate Mi532 was of serotype Ser2 whereas all other isolates were of serotype Ser1. Moreover, isolate Bf5 was of RYMV pathogroup B, which overcomes resistance of rice cultivars Tog5681 and Gigante (Konaté *et al*., 2001). By contrast, all other isolates belonged to pathogroup A. Preliminary studies indicated that isolate Td3 had particular pathogenic properties as it was the only one that could infect the wild host species *Dactyloctenium aegyptium* (L.) P. Beauv.

Plant inoculation. Virus isolates were first propagated by mechanical inoculation to the standard susceptible rice cultivar BG90-2. Leaf samples were ground in inoculation buffer (0.1 M phosphate buffer pH 7.0) at a ratio of 1:10 (w/v) using sterile mortars and pestles. Carborundum (600 mesh) was added to the extracts, which were subsequently rubbed onto leaves of rice seedlings two weeks post-germination. Symptoms developed fully 14 days post-inoculation (dpi) and infected leaves were collected to serve as inoculum sources. Inoculum was prepared from these leaves as indicated above and the virus content in extracts was adjusted following ELISA tests of aliquot fractions. Extracts were mixed with carborundum and applied to leaves of 35 to 40-day-old plants of the wild host species *Eragrostis ciliaris* (L.) R.Br, *E. tenella* (L.) P.Beauv. ex Roem & Schult., *E. tremula* Hochst. ex Steud., *Oryza barthii, O. longistaminata* and *D. aegyptium*. Plants of the standard susceptible rice cultivar BG90-2 were also inoculated as controls. All plants were maintained in an insect-proof greenhouse at 25-30°C and relative humidity of 80-90%.

RYMV detection in seed samples. To test virus migration into progeny seeds, seeds from at least fifteen RYMV-infected plants were harvested, pooled and dried for one month in the open air. Only seeds from symptom-bearing and ELISA-positive shoots were collected. Seeds were tested by ELISA, essentially as described by Clark and Adams (1977). The coating and detecting antibody was a polyclonal antibody (Pab) raised against a mixture of RYMV serogroups (Konaté *et al.*, 1997). This antibody reacted strongly with all serogroups giving similar detection limits of 1/500,000 in ELISA using RYMV-infected BG90-2 sap dilutions (Traoré, 2006).

The virus was detected in extracts prepared from single seeds of the three *Oryza* species. Virus presence was also investigated in seed parts (glumellas and caryopses) of individually husked seeds. Because single seeds of other plant species were ELISA-negative (see results), samples consisting of increasing numbers of seeds were tested for virus detection. For all ELISA tests, seed extracts were obtained by grinding the seeds with a mortar and pestle in phosphate-buffered saline, pH 7.4 (PBS) containing 0.05% Tween 20 and 2% polyvinylpyrrolidone (PVP-40). Each seed or seed lot was ground in 300 µl of extraction buffer. Seed parts from individual seeds were first weighed and also ground in 300 µl of buffer. Extracts were decanted and dilutions were adjusted in relation to recorded weights before analyses. Absorbance readings (A405nm) were recorded with a S960 microplate reader after 2-3 h of substrate incubation. A mean of A405nm values from seeds of healthy plants plus three times the standard deviation was taken as the negative-positive threshold.

RYMV infectivity in seed extracts and seed transmission tests. RYMV infectivity in seeds was determined at harvest maturity and after drying the seeds for one month in the open air. Depending on plant species, single seeds or groups of seeds were ground in 300 µl of inoculation buffer. Carborundum was added to each extract which was then applied to leaves of two 14-day-old plantlets of susceptible standard rice BG90-2.

RYMV transmissibility through seeds was tested by sowing seeds from diseased plants in plastic pots containing sterilised soil. In total, 2,000 seeds from every *Oryza* species (500 seeds per virus isolate) and ca. 4,000 seeds (1,000 seeds per virus isolate) from each of the other species were tested. Emerged seedlings were monitored during 45 days for the appearance of symptoms. Then, for every plant species-virus isolate combination, the last fully-expanded leaves of the main shoots were collected from 25 random plants. Samples were made by pooling leaves from groups of five plants and were assayed by ELISA.

Statistical analysis. Proportions of infected seeds or seed parts were compared by means of either the z test for the difference between proportions, or the Chi-square (χ^2) test in the case of more than two treatments (Fleiss, 1981). Because data from virus detection in single seeds and in batches of seeds could not be com-

pared, corresponding proportions were analyzed separately. Significances of the differences between mean virus titres in seed parts of the *Oryza* species were tested by analysis of variance.

RESULTS

Symptomatology. Except for *D. aegyptium*, inoculated plants showed characteristic yellow mottle symptoms between 10 and 15 dpi, regardless of the virus isolate. Only isolate Td3 induced symptoms on *D. aegyptium* plants. Other isolates were unable to do so throughout the growing cycle and inoculated plants were ELISA-negative. Symptoms were most pronounced at 21 dpi and were systemic in all plant species. They also persisted on the leaves of infected plants for most species. However, symptoms became inconspicuous in older leaves of *E. tenella* plants, although they remained clearly visible in younger leaves.

Detection of RYMV in seeds samples. RYMV could not be detected in individual seeds of *D. aegyptium* and the three *Eragrostis* species. All four plant species, which belong to the *Eragrostideae* tribe, produced very tiny seeds having diameters of 0.73 ± 0.05 mm (mean ± std) and 1.30 ± 0.17 mm, respectively for *Eragrostis tremula* and *D. aegyptium*. *E. ciliaris* and *E. tenella* had similar elongated seeds of 0.57 ± 0.1 x 0.28 ± 0.05 mm. Therefore, the virus was tested in batches of increasing sizes.

Positive detections were obtained only if the minimum number of seeds in samples was 10 or 50 seeds, respectively for *D. aegyptium* or *Eragrostis spp*. Using these threshold numbers, RYMV was detected in lots of seeds infected by the different virus isolates (Table 1). As revealed by the χ^2 test, seed infection depended on plant species and virus isolates (χ^2=54.88; df=11; P<0.001). On the one hand, higher proportions of infected seed lots were observed in *E. tenella* compared to *E. tremula* and *E. ciliaris*. On the other hand, only 14 of 60 seed lots were infected by isolate Mi532 whereas levels of seed lots infected by isolates Bf5 and Td3 were 37 out of 60 and 26 out of 60, respectively.

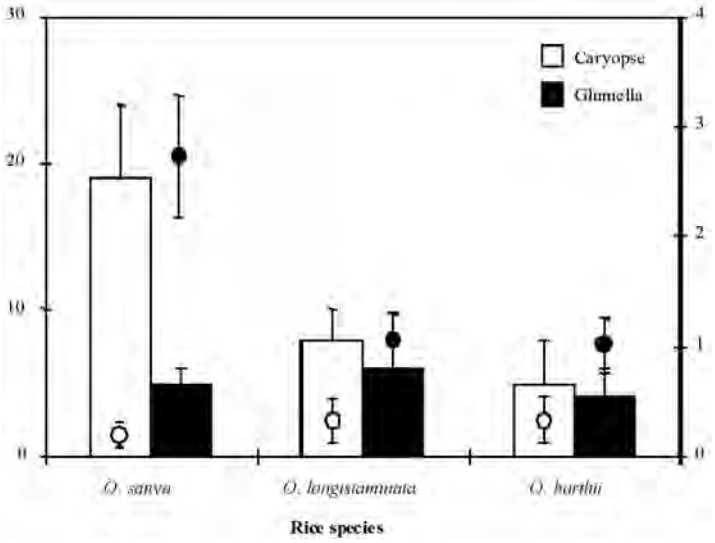

Fig. 1. Virus titre in glumellas (black dots) and caryopses (open dots) from infected seeds of cultivated (*Oryza sativa* cv. BG90-2) and wild (*O. longistaminata* and *O. barthii*) rice in relation to the weight (histograms) of both seed parts. Absorbance readings ($A_{405\ nm}$) from healthy controls varied between 0.01 and 0.07. All plants were inoculated with RYMV isolate Td3.

Unlike in seeds from plants of the *Eragrostideae* tribe, RYMV detection was successful in individual seeds from all *Oryza* species. In most cases, virus incidence in seeds was greater than 60% (Table 1). However, levels of seed infection differed significantly between virus isolates and plant species (χ^2=489.41; df=11; P<0.001). Isolate Mi532 was detected less in seeds of *O.*

Table 1. Proportions of seed infection by four RYMV isolates in rice and wild host species[a].

Poaceae	Virus isolate			
	Bf5	Mi532	Td3	Td20
Eragrostis ciliaris	15/20[b]	3/20	4/20	2/20
E. tenella	12/20	9/20	15/20	14/20
E. tremula	10/20	2/20	7/20	10/20
Dactyloctenium aegyptium	nt[c]	nt	18/20	nt
Oryza barthii	103/120	32/150	103/120	79/120
O. longistaminata	100/100	100/100	104/106	114/116
O. sativa cv. BG90-2 (control)	92/102	66/100	109/110	82/105

[a] Proportions were determined from tests of individual seeds from *Oryza* species and lots of seeds from all other plant species.
[b] Number of infected seeds/ number of seeds tested.
[c] nt, not tested. *D. aegyptium* could be infected only by isolate Td3.

sativa and *O. barthii* with incidences of 66% and 21%, respectively. Seeds of *O. longistaminata* were the most infected, as incidences were always close to 100%.

RYMV was also detected in glumellas and caryopses from individual seeds of the three *Oryza* species. All four isolates were detected similarly and data are presented only for Td3, the sole isolate able to infect *D. aegyptium*. Proportions of ELISA-positive glumellas from seeds of *O. sativa, O. barthii* and *O. longistaminata* were 93%, 82.5% and 98%, respectively.

The z test for difference between proportions (Fleiss, 1981) indicated significantly lower virus incidences in corresponding caryopses; the respective infection rates were only 78%, 69% and 81.5% (Table 2). In parallel to lower virus incidence, lower virus titres were also recorded in infected caryopses (Fig. 1). Analysis of variance indicated significant differences between virus titres in seed parts ($F=83.81$; $P<0.001$). Extracts from glumellas always yielded higher A405nm readings regardless of the plant species. The highest glumella/caryopse imbalance in virus titre was observed in *O. sativa*, as mean A405nm values were four times higher in glumella samples. In addition, virus titre was higher in glumellas of *O. sativa* seeds than any seed part of any other plant species. Similar virus titres were found in either glumellas or caryopses of *O. longistaminata* and *O. barthii*.

Table 2. Detection of RYMV (isolate Td3) in seed parts of cultivated and wild rice species.

Plant species	Seed part[a]		z	*P*
	Glumella	Caryopse		
Oryza sativa	186	156	4.12	<0.001
O. barthii	165	138	3.03	0.002
O. longistaminata	196	163	5.28	<0.001

[a]Figures indicate numbers of ELISA-positive seed parts out of 200 tested in each case.

RYMV transmission from extracts of infected seeds

For seeds of wild host species, whether seeds were tested just after harvest (stage 1), or after drying for one month (stage 2), RYMV could not be transmitted from extracts of individual seeds of *Eragrostis spp.* or *D. aegyptium.* Successful transmissions were obtained only if seeds were tested in lots of at least three and 20 seeds respectively for *D. aegyptium* and *Eragrostis spp*. Proportions of seed lots that gave infectious extracts are indicated in Table 3. At stage 1, infections were obtained from 10 to 20% of seed lot extracts. At stage 2, no extracts from the seeds were infectious and virus infectivity was significantly lower than that at stage 1 ($z=3.124$; $df=1$; $P=0.002$).

Inoculations of extracts from individual seed of the three *Oryza* species led to infections at both stages of seed testing (Table 3). At stage 1, RYMV infectivity differed significantly ($\chi^2=13.1$; $df= 2$; $P>0.01$) between the plant species and was highest (60%) in seeds of *O. longistaminata*. By contrast, no significant differences were observed between plant species at stage 2. However, virus infectivity decreased dramatically between the two stages, as mean proportions of infectious extracts were 48.6% at stage 1 and only 1.7% at stage 2.

Table 3. Infectivity of *Rice yellow mottle* virus (isolate Td3) in extracts from seeds of rice and wild host species at harvest (stage 1) and after dehydration (stage 2)[a].

Plant species	Stage 1	Stage 2
Eragrostis ciliaris	3/20	0/20
E. tenella	2/20	0/20
E. tremula	2/20	0/20
Dactyloctenium aegyptium	4/20	0/20
Oryza barthii	44/120	0/120
O. longistaminata	72/120	4/120
O. sativa	59/120	2/120

[a]Seeds were dried for one month in the open air. Proportions were determined from tests of individual seeds from *Oryza* species and batches of seeds from all other plant species.

Absence of RYMV transmission through seeds

Whatever the plant species or the virus isolate inoculated, seeds collected from RYMV-infected plants gave seedlings that did not develop any symptoms 30 days after sowing or even at the flowering stage. In addition, leaf samples taken from plants at these two periods were all ELISA-negative. Pots containing the rhizomatous wild rice *O. longistaminata* continued to be watered after harvest and numerous new shoots emerged from the ground. At least one thousand of them were examined and all were found to be healthy.

DISCUSSION

Wild poaceae species reacted like rice to infection by all four virus isolates. Infections were systemic and the symptoms induced were characteristic yellow mottle symptoms (Bakker, 1970). However, the non-persistence of symptoms on older leaves of infected *E. tenella* plants, regardless of the virus isolate was noteworthy. This plant species was reported to be a symptomless host for RYMV in Kenya (Bakker, 1974). In this case, the symptomless infection obtained was probably due to specific interactions between *E. tenella* and the virus isolate used. The contrasting behaviour of *E. tenella* revealed by our work is unlikely to be related to differences in the origins of isolates used in either studies or their phylogeny. Isolates used in this study originated from west and

central Africa and belonged to different phylogenetic strains (Traoré *et al.*, 2005). Moreover, in a recent study, Sorho *et al.* (2005) did not find any relationship between pathogenicity (either virulence or aggressiveness) of RYMV isolates and their origin or phylogeny.

Interactions between RYMV isolates and *D. aegyptium* confirmed that this plant species was infected only by isolate Td3. Therefore, it is a differential host that can distinguish between Td3 and the other isolates. However, the mechanism of the relationship between Td3 and *D. aegyptium* remains unexplained. This does not seem to be related to the serological or phylogenetic properties of isolate Td3 nor to its inability to break down resistances in rice. Other isolates such as Td20 also shared similar properties (Traoré *et al.*, 2005; Traoré *et al.*, 2006) but were not able to infect *D. aegyptium*.

RYMV could be detected by ELISA in individual seeds only for plants of the genus *Oryza* and seeds of other plant species had to be pooled into batches for successful virus detection. ELISA is a suitable technique for RYMV detection in vegetative or reproductive organs of rice (Konaté *et al.*, 2001; Thottappilly and Hughes, 2001). The absence of virus detection in individual seeds of some species indicates low virus content in these seeds, which is below the detection threshold (Bar-Joseph and Garnsey, 1981). Similar results were obtained in attempts to detect *Tobacco mosaic virus* or *Turnip yellow mosaic* virus in single seeds of *Arabidopsis thaliana* (de Assis Filho and Sherwood, 2000). The low virus contents are probably related to the tiny sizes of the seeds. This may explain why less seeds of *D. aegyptium* than of other plant species were needed for seed lots to be ELISA-positive. It was beyond the aim of this study to detect RYMV in individual seeds of all plant species; more sensitive detection techniques like RT-PCR might have given more positive results (Gillaspie *et al.*, 2001). Overall, our results indicated the presence of RYMV in seeds of all plant species tested. Proportions of infected seeds or seed lots depended on both virus isolate and plant species, indicating some specific plant-virus interactions.

RYMV incidence in whole seeds of cultivated and wild rice rarely reached 100%. Moreover, virus incidence in husked seeds was always higher in glumellas than in caryopses. These results indicated that diseased plants produced three kinds of seeds: (i) non-infected seeds that the virus fails to invade; (ii) partially-infected seeds in which the virus is detected in glumellas but not in caryopses (Abo *et al.*, 2004); (iii) fully-infected seeds in which both glumellas and caryopses are invaded by the virus. It is likely that there is some blockage of virus migration into the seeds or the seed parts (Maule and Wang, 1996). Such blockage may have resulted in the lower accumulation of the virus in caryopses compared to glumellas. Possibly, given the high accumulation of virus in glumellas from *O. sativa* seeds compared to *O. longistaminata* and *O. barthii* despite similar virus titres in glumellas (Fig. 1), host species play an important role in this process.

RYMV was infectious in mature seeds at harvest but, as for virus detection, no infection was obtained from single seeds of species from the *Eragrostideae* tribe. However, the infectivity test was more sensitive than detection by ELISA because lower number of seeds were required in lots for infection to occur. As previously reported for the RYMV-rice pathosystem (Konaté *et al.*, 2001), RYMV infectivity in seed extracts decreased dramatically when the seeds were dehydrated. Even if the presence of the virus in dry seeds was clearly shown by ELISA tests, loss of infectivity may result on the one hand from total or partial degradation of virus particles but a retention of sufficiently intact epitopes, and on the other hand, degradation may not occur but infectivity is lost following virus inactivation. In most instances, virus inactivation in seeds occurred during seed maturation (Mandahar, 1981) but post-harvest inactivation has been also reported (Mayee, 1977; Bailiss and Offei, 1990).

Dehydration of seeds has been considered as an underlying factor responsible for virus inactivation (Johansen *et al.*, 1994). In line with the loss of virus infectivity during the process of dehydration of the seeds, the absence of seed-borne transmission of RYMV was expected. Early studies of seed transmission of RYMV led to the conclusion that the virus was not seed-transmitted in rice (Bakker, 1974; Fauquet and Thouvenel, 1977). Similar results were found by Konaté *et al.* (2001) and Abo *et al.* (2004). Our data also confirmed the non-transmission of RYMV through rice seeds. Moreover, non-transmission of RYMV through seeds of the wild rice species *O. barthii* and *O. longistaminata* and of four wild host species (*D. aegyptium*, *E. ciliaris*, *E. tenella* and *E. tremula*) are reported for the first time. Therefore, as for rice seeds, seeds of wild host species are unlikely to be involved in the dissemination of RYMV, especially to new cultivated areas. Transmission of viruses through vegetative organs or other plant materials like seeds usually results in uniform genetic structures of respective virus populations (Garcia-Arenal *et al.*, 2001). This happens because differences in populations that are related to geography are often blurred by traffic of infected plant material over closed or distant areas. In many instances, populations of RYMV showed clear-cut phylogeographic structures (Abubakar *et al.*, 2003; Fargette *et al.*, 2004; Traoré *et al.*, 2005). The marked geographic structures of RYMV populations are consistent with the non-transmission of the virus through seeds of rice or wild host species.

ACKNOWLEDGEMENTS

We sincerely acknowledge Dr D. Fargette for constructive criticism of the manuscript and Mr. M. Koutou

for helpful discussions. This study was supported by a fellowship of the French Government through the French Ministry for Cooperation to M.D. Allarangaye.

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Received March 16, 2006
Accepted June 15, 2006

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