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Abiotic Transmission of *Rice yellow mottle virus* Through Soil and Contact Between Plants

[1]M.D. Traoré, [2]V.S.E. Traoré, [3]A. Galzi-Pinel, [3]D. Fargette, [2]G. Konaté,
[4]A.S. Traoré and [2]O. Traoré
[1]Institut d'Economie Rurale (IER), CRRA Sikasso BP 16 Sikasso, Mali,
[2]Institut de l'Environnement et de Recherches Agricoles (INERA),
01 BP 476 Ouagadougou 01, Burkina Faso
[3]Institut de Recherche Pour le Développement (IRD), BP 64501 34394 Montpellier cedex 5, France
[4]CRSBAN,UFR-SVT, Université de Ouagadougou, 03 BP 7021 Ouagadougou 03, Burkina Faso

Abstract: The roles of guttation fluid, irrigation water, contact between plants and transplantation into contaminated soil in the transmission of *Rice yellow mottle virus* (RYMV) were assessed. RYMV presence and infectivity were tested by Enzyme-Linked Immunosorbent Assay (ELISA) and by inoculation to susceptible rice cultivar BG90-2. The virus was readily detected in guttation fluid collected from infected rice plants. Transmission tests from this fluid led to high disease incidence (86.6%). Irrigation water collected at the base of infected plants growing in pots was less infectious, as inoculations led to disease incidences below 40%. No virus was detected and could be transmitted from field-irrigation water. Up to 44% healthy rice plants whose leaves were in contact with those of infected plants became infected but, no transmission occurred through intertwined roots. Transplantation of rice seedling into virus-contaminated soil also led to plant infection. However, virus survival in the soil decrease rapidly and infectivity was completely lost 14 days after soil contamination. Altogether, these results indicated that high planting densities of rice are likely to favour secondary spread of rice yellow mottle disease. Transplantation of rice seedlings not earlier than 2 weeks after soil preparation should prevent soil transmission of the virus. Although guttation fluid is highly infectious its contribution to virus infectivity in irrigation water is negligible as field-irrigation water was not found to be an infectious source for RYMV.

Key words: RYMV, *Sobemovirus*, survival in soil, virus sources, guttation fluid, irrigation water

INTRODUCTION

Rice (*Oryza sativa* L. and *O. glaberrima* Steud.) is a staple crop in most countries in Africa. Since the early 1990s, rice production is severely affected by rice yellow mottle which is one the most damaging disease of rice in the continent. The disease is caused by *Rice yellow mottle virus* (RYMV) first reported in Kenya 40 years ago (Bakker, 1970). RYMV is now widespread all over Africa south of the Sahara (Kouassi *et al*., 2005). Common symptoms induced by RYMV are yellow discoloration and mottling of the leaves of infected rice plants. Additionally, symptoms may also include stunting, reduced tillering and poor panicle exertion (Hull and Fargette, 2005; Kouassi *et al*., 2005). Therefore, yield may be dramatically reduced by 25 to 100% (Konaté *et al*., 1997; Calvert *et al*., 2003).

RYMV belongs to the genus *Sobemovirus* of plant viruses (Hull and Fargette, 2005). It is a stable and highly infectious virus which is easily transmitted by mechanical inoculation. The longevity *in vitro* is 56 days and the dilution end point fluctuates between 10^{-6} and 10^{-9} depending on the inoculum source (Hull and Fargette, 2005). RYMV induces systemic infections in rice and wild host species and can invade the seeds produced by infected plants. However, it has been demonstrated that seed transmission does not occur (Konaté *et al*., 2001; Allarangaye *et al*., 2006).

Current control measures against RYMV are mainly directed to breeding for resistance. Sources of resistances to the virus have been identified in a limited number of cultivars from the two cultivated rice species *O. sativa* and *O. glaberrima* (Ndjiondjop *et al*., 1999; Ioannidou *et al*., 2000). Unfortunately, RYMV isolates

Corresponding Author: Dr. Oumar Traoré, Institut de l'Environnement et de Recherches Agricoles (INERA), 01 BP 476 Ouagadougou 01, Burkina Faso Tel: +226 50319276 Fax: +226 50340271

capable to overcome the identified resistances have been reported at relatively high frequencies (Traoré *et al*., 2006a), which is a threat to the success of genetic control of rice yellow mottle disease. Consequently, in addition to using resistant rice cultivars, RYMV control should also include other means such as phytosanitary measures in an integrated management strategy. However, phytosanitary measures themselves are not fully known due to limited knowledge concerning the epidemiology of the disease.

RYMV transmission in the field involve a few insect species, mainly chrysomelid beetles (Bakker, 1974), mammals such as cows, donkeys and rats (Sarra and Peters, 2003) and man himself through some cropping practices (Traoré *et al*., 2006b). Wind-mediated transmission of RYMV has been also reported by Sarra *et al*. (2004). Several other means of transmission and sources of infection are suspected. These, include irrigation water flowing from infected fields to healthy ones, contact between plants, using dung from cows that fed upon infected stubble and planting rice in contaminated soil (Abo *et al*., 2000; Calvert *et al*., 2003).

In this study, we studied the involvement of irrigation water, contact between plants and contaminated soil in the abiotic transmission of RYMV. Implications of the findings in the epidemiology and management of rice yellow mottle disease are discussed.

MATERIALS AND METHODS

Plant inoculation: This study was conducted during two consecutive years 2003-2004 at Kamboinse agricultural research station (Burkina Faso). In all experiments, RYMV isolate Bf5 from our virus collection and rice cultivar BG90-2 highly susceptible to RYMV, were used. The virus isolate first was propagated by mechanical inoculation. Inoculum was prepared by grinding 1 g of frozen infected rice leaves in 10 mL of 100 mM phosphate buffer pH 7. Carborundum (600 mesh) was added to the inoculum and the mixture was rubbed onto leaves of 14 day-old plants. Inoculations were done in an insect-proof screenhouse with temperature between 25 and 30°C and 80-90% relative humidity. Symptoms developed fully at 21 days post-inoculation (dpi) and infected plants were used as source of inoculum in the experiments.

Testing the presence of RYMV in guttation fluid and irrigation water: Guttation fluid was collected from infected rice plants. To ease fluid collection, the plants were previously covered with a plastic bag overnight. Samples of standing water were collected from pots in which infected plants were grown. Five days before collecting the samples, the plants were irrigated either by sprinkling the water from top (top-irrigation) or by pouring it directly into the pots (pot-delivered water). Field samples of irrigation water were also collected in plots where rice yellow mottle incidence was 80 to 100%. Altogether, irrigation water was sampled in 20 mL vials from 10 fields.

To test the presence of RYMV, samples of guttation fluid and irrigation water were analyzed by double antibody sandwich enzyme Linked-immunosorbent assay (DAS-ELISA) (Clark and Adams, 1977). A high titre polyclonal antibody prepared in rabbit (Traoré, 2006) was used for coating microtitration plates. The same antibody was coupled to alkaline phosphatase and used as conjugate. All samples were tested in triplicates and were considered positive if absorbance readings (A405 nm) from each replicate were more than the negative/positive threshold (mean A405 nm readings from healthy controls plus three times standard deviations). Biological tests were also conducted to diagnose RYMV in samples of guttation fluid and irrigation water. Each sample was mixed with carborundum (600 mesh) and rubbed onto leaves of 10 two-week-old rice seedlings. Appearance of symptoms was monitored for 45 dpi and leaves were taken from inoculated plant for serological confirmation tests.

Testing RYMV transmissibility through contact between leaves and between roots: RYMV-infected rice plants grown in plastic pots in the greenhouse were pruned to get rid of all symptomless leaves. The effect of contact between leaves on transmission of the virus was tested by displaying 20 groups of five pots containing healthy rice seedlings in circles around an infected plant. The close positions of the pots allowed the leaves of all healthy seedlings to be in contact with those of diseased plants.

To test RYMV transmissibility by contract between roots, three to five rice seeds were sown in plastic pots. At 14 days post-germination, the plants were thinned to two per pot. A transparent plastic tube was placed over one of the plants to isolate its above-ground part from that of the second plant. In this way, the two plants were in contact through the roots only. Therefore, RYMV was inoculated to the non-isolated plant. The experiment was done in 100 replicates and isolated plants were observed during 45 days for symptom development. RYMV detection performed by ELISA and bioassays was positive in leaves, stems and roots of inoculated plants when tested at 21 days post-inoculation.

Tests of RYMV transmission through contaminated soil: Infected leaves were pooled and cut into small pieces from which inoculums were prepared. In each case, four

extracts were prepared by grinding leaf pieces (100, 10, 1 and 0.1 g, respectively) in one litre of tap water. Extracts obtained were referred to as E1, E2, E3 and E4, respectively. Corresponding healthy leaf extracts were also prepared in the same way to be used as negative controls. Each extract including plant debris was mixed with 5 kg of moistened sterile soil in a bucket and all soil preparations were done the same day. Then, the two following experiments were performed:

In experiment 1, the transmissibility of RYMV by sowing rice seeds in contaminated soil was tested. Seeds were sown in the buckets containing contaminated and healthy soil (dilution E1), respectively. Twenty seeds were put per bucket and treatments were run in triplicates for each leaf extract.

Experiment 2 was conducted to assess the effect of time of transplantation of rice seedlings into contaminated soil on the transmission of RYMV. All seedlings were transplanted at 14 days after sowing. The experimental design was composed 100 buckets arranged into four blocks of 25 buckets each (one block per leaf extract). In every block, buckets were further arranged into sub-blocks of five, three of which contained soil mixed with the adequate dilutions of RYMV-infected leaf extracts whereas the two remaining contained soil mixed with healthy leaf extracts. At day zero (when leaf extracts were incorporated into the soil), rice seedlings were transplanted in a first group of four sub-blocks (corresponding to extracts E1, E2, E3 and E4, respectively). A total of 10 seedlings were transplanted per bucket. Four other transplantations were done similarly at days 2, 4, 8 and 14, respectively. During the whole experiment, rice plants were watered once a day, all buckets receiving similar amounts of water.

Statistical analyses: Data on disease incidence (proportions of infected plants) were analysed by using the χ^2 test for difference between proportions (Fleiss, 1981). Differences in mean disease incidence were compared using analysis of variance after angular-transformation of the data to take into account the correction for normality (Zar, 1999).

RESULTS

Detection and infectivity of RYMV in guttation fluid and irrigation water: ELISA detection of RYMV in guttation fluid and irrigation water samples was successful in guttation fluid only. The virus was readily detected in guttation fluid samples with absorbance readings (A405 nm) higher than 1.2 after 2 h of substrate incubation (Table 1). By contrast, all irrigation water samples gave A405 nm readings that were below the positive/negative threshold, indicating the lack of virus detection. In mechanical inoculation tests, guttation fluid and all water samples, except irrigation water collected from the fields, were found to be infectious. Inoculated rice seedlings showed mottle symptoms between 8 and 12 dpi. As indicated by the χ^2 test, proportions of infected plants depended on virus inoculum sources ($\chi^2 = 44.98$, $p<0.01$, $df = 4$). Guttation fluid was the most infectious as disease incidence reached 86% and was more than twice that of any other case. None of the inoculated plants became infected when irrigation water collected from the field was used.

Table 1: Detection and infectivity of RYMV in guttation fluid and irrigation water

Virus sample[a]	ELISA (A405 nm)[b]	Infectivity[c]
Guttation fluid	1.220-1.301	13/15
Top-irrigation water	0.034-0.059	8/20
Pot-delivered irrigation water	0.053-0.068	3/20
Field-irrigation water	0.045-0.071	0/20
Tap water (negative control)	0.058-0.069 (0.08)	NT
Infected rice leaf extract	2.186-2.254	NT

[a]: Irrigation water was collected from the field and from pots in the greenhouse one day after irrigating rice plants by sprinkling or delivering the water directly into pots, [b]: Lowest and highest absorbance readings (A405 nm) after 2 h of substrate incubation. The positive/negative threshold for virus detection is indicated in parenthesis, [c]: No. of infected plants/total number of susceptible rice plants inoculated in each case; NT: Not Tested

Transmission of RYMV by contact between plants: Up to 44% of the healthy rice plants which were in contact with RYMV-infected plants through the leaves became infected and showed clear yellow mottle symptoms. First symptoms appeared 2 weeks after the plants were brought into contact. Serological tests conducted on the remaining symptomless plants (56%) were all negative. When diseased and healthy plants were put into contact through their roots only, no virus transmission occurred. Despite the fact that the roots were strongly intertwined, none of the 100 plants tested became infected, as indicated by the absence of symptoms and the lack of virus detection by ELISA.

Transmission of RYMV through contaminated soil: Sowing rice seeds in soil contaminated with RYMV-infected leaf extracts did not result in any infection of the emerged seedlings. All seedlings remained symptomless throughout the time of the experiment. Therefore, one leaf was taken from each seedling and the batch was used to prepare an extract. This extract appeared to be serologically negative, confirming the absence of RYMV in the plantlets.

On the contrary, rice yellow mottle infections occurred clearly following transplantation of rice seedlings in soil contaminated with infected leaf extracts

Table 2: *Rice yellow mottle* incidence in plots where rice seedlings were transplanted into contaminated soil

Date of rice seedling transplantation[a]	Virus extracts[b]			
	E1	E2	E3	E4
Day-0	43.3	8.4	0	0
Day-2	19.6	5.3	0	0
Day-4	7.9	3.5	0	0
Day-8	4.2	1.7	0	0
Day-14	0.0	0.0	0	0

[a]: Leaf extracts (E1, E2, E3 and E4) were prepared by grinding infected rice leaves (100, 10, 1 and 0.1 g, respectively) in 1 litre of water. Control (E1) was prepared as E1 but with uninfected leaves and all extracts were mixed with soil in pots, [b]: Rice seedlings were transplanted into contaminated soil at different times: same day (Day-0) and two, four, eight and fourteen days later (Day-2, Day-4, Day-8 and Day-14, respectively)

(Table 2). Typical yellow mottle symptoms appeared on transplanted plantlets one week after transplantation. As revealed by analysis of variance, there were significant effects of leaf extract ($F = 71.92$, $df = 4$, $p<0.0001$) and time of transplantation ($F = 28.20$, $df = 4$, $p<0.0001$). Especially, on the one hand, the disease was induced only if rice seedlings were transplanted in soil contaminated with infected extracts E1 and E2. On the other hand, disease incidence dropped sharply from Day-0 to Day-14 at which time no disease could be induced.

DISCUSSION

Guttation fluid from RYMV-infected plants was first reported to be a source inoculum by Bakker (1974). But its role in the spread of rice yellow mottle disease in the field was not known. Recently, Sarra *et al.* (2004) indicated that guttation fluid had no significant effect on wind-mediated transmission of RYMV. Present results are consistent with those of Bakker (1974). Guttation fluid reacted strongly in serological tests and also a high disease incidence was obtained when it was used as source of inoculum. Infectiousness of top-irrigation and pot-delivered irrigation waters is probably due to the fact that they contained some guttation fluid. It is likely that top-irrigation water contained more guttation fluid swept from the leaves by irrigation water coming from above the plants. Pot-delivered irrigation water was expected to contain less guttation fluid which self fell from the leaves. As a result, a higher disease incidence was obtained upon inoculation of top-irrigation water (Table 1). Although the disease was induced by inoculating both types of irrigation water, serological tests conducted on these waters were negative. This indicated that the biological test of RYMV is more sensitive that serological detection of the virus. Like top-irrigation and pot-delivered irrigation water, field-irrigation water probably contained some infectious guttation fluid fallen from infected plants. However, although collected from highly infested plots, field-irrigation water was ELISA negative and no infection was induced from it (Table 1). This result is consistent with the view that the virus from infectious guttation fluid was likely too much diluted beyond its dilution end point. Consequently, irrigation water flowing from infested plots is not to be considered as inoculum source for the infection of plants in downstream plots.

RYMV was transmitted at a relative high rate (44%) though contact between leaves of infected and healthy rice plants. This is consistent with earlier reports by Abo *et al.* (1998, 2000) and Sarra *et al.* (2004). The latter authors found virus transmission through contact between leaves can increase by five to eightfold when rice planting densities were doubled from 16 to 33 plants m^{-2}. In contrast to contact between leaves, no transmission was obtained through intertwined roots of diseased and healthy rice plants. This result does not support the findings of Abo *et al.* (2000). In order to induce any infection, the roots needed to be wounded. In present experiments, such wounds were not produced by the intertwining of the roots and they could not be produced by any other factor as rice plants were grown in sterile soil. In field conditions, root-feeding organisms such as nematodes can produce wounds, thus leading to possible infection, even if nematodes themselves are not reported to be vectors of RYMV (Bakker, 1974).

Sowing rice seeds into contaminated soil did not result in any infection of the emerged seedlings. Therefore, direct seeding rice fields instead of using the seedbed system is a way to reduce primary infections by RYMV. It was recently reported that the process of setting rice seedbeds up and transplantation of seedlings into the fields is a major cause of RYMV dissemination (Traoré *et al.*, 2006b). Unfortunately, direct seeding is not advisable in irrigated rice system because it causes lower yields compared to transplantation of rice seedlings and also because of more difficulties in controlling the weeds.

Transplantation of rice seedlings into contaminated soil can lead to the establishment of rice yellow mottle disease. Notably, this occurs with soil contaminated with highly concentrated inoculum such as E1 and E2. This may happen in the field if highly infected rice stubble is incorporated to the soil during ploughing (Reckhaus and Andriamasintseheno, 2001). Sarra (2005) also reported that contamination of the soil can result from the use of dung taken from cattle which fed upon infected plant material. Fortunately, our results indicated that the survival of the virus in the soil decreased rapidly (Table 2). At two weeks after the soil was contaminated, transplantation of rice seedlings did not result in any infection. Consequently, as a prophylactic control measure, transplantation of rice seedlings should be done at least two weeks after the field has been ploughed.

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