Evidence of Non-Transmission of *Rice yellow mottle virus* (RYMV) through Rice Seed

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Summary

An indexing of the organs (radicle and plumule) and components (husk, endosperm and embryo) of rice seeds using Enzyme Linked Immunosorbent Assay (ELISA) was carried out to detect Rice yellow mottle virus *(RYMV) and establish the exact location of the virus in the rice seed. RYMV was detected only in the husk (seed coat) but not in the endosperm, plumule, radicle, nor embryo. None of the seedlings raised from the seeds expressed RYMV symptoms. No virus particle was detected by the ELISA test in the leaves of the screenhouse-reared plants obtained from seeds of infected plants. The results indicate that RYMV is apparently not transmitted through rice seed probably because the virus is seed-borne in the husk (seed coat) of mature rice seeds.*

Résumé

Évidence de la non-transmission du virus de la panachure jaune du riz par la semence du riz

Un indexage des organes (radicule et plumule) et composants (coque, endosperme et embryon) des semences de riz par le test immunoenzymatique (ELISA) a été effectué pour détecter le virus de la panachure jaune du riz (RYMV) et établir la localisation exacte du virus dans la graine de riz. Le virus RYMV n'a été détecté que dans la balle mais pas dans l'endosperme, ni dans la plumule et l'embryon. Aucune plante provenant des semences infectées n'a exprimé des symptômes de RYMV, et le test ELISA n'a détecté aucune particule virale dans les feuilles. Ces résultats montrent que la RYMV n'est pas transmise par la semence bien que le virus ait été retrouvé dans les graines.

Introduction

Rice (*Oryza* species) is the principal staple food for millions of people in most Asian countries, parts of Africa and Latin America (25). Today, rice is produced in more than 110 countries in the world (6) and in every country in West Africa (41).

There are now over 30 viruses reported to infect rice through experimental tests and in nature (3). However, only two rice viruses are seed-borne (37). In Africa so far only *Rice yellow mottle virus* (RYMV) (11) is of economic importance to rice production. RYMV has been reported in several countries in Africa where rice is grown (4, 40). The virus is transmitted mechanically through sap inoculation and by insect vectors (2, 10). Very low rates of seed transmission and spreading by insect vectors can lead to widespread incidence of virus diseases (35).

Some seed-transmitted viruses have been shown to be present in the embryo (22). Thus, *Bean common mosaic virus* that is transmitted through seed was detected in the blossoms, young pods, cotyledons, embryo, but not in seed coat (husk) of Phaseolus bean (*Phaseolus vulgaris*) (19). It has been reported by Matthews (34) that viruses, which are confined to vascular tissues that have no connection with the parent, may be unable to enter the ovule.

Anonymous (7), Bakker (11), Fauquet and Thouvenel (20) and IITA (27) in their separate studies reported the inability of RYMV to be transmitted through rice seed, while Awoderu (9) obtained evidence that point to the possible seed-borne nature of RYMV. These studies did not propose any reason why RYMV could not be transmitted through rice seed. Awoderu (9) called for a re-examination of the role of rice seeds from infected plants in the transmission of RYMV. This investigation was carried out to determine the seed-borne nature of the virus and its transmission through rice seed.

Materials and methods

Virus indexing and Enzyme Linked Immunosorbent Assay (ELISA) procedure:

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Four rice varieties were used for this analysis. These varieties are highly susceptible to RYMV and their continuous cropping and preference by farmers in Ivory Coast (Bouake 189), Mali (BG90-2) and Niger (IR 1529-680-3) are linked to the outbreaks of RYMV in those countries (1, 4). Four lots of rice with each lot containing about 25 seeds harvested from naturally and artificially RYMV infected rice plants were drawn from these varieties at random, weighed and subjected to the Antigen Coated Plate (ACP) form of Enzyme Linked Immunosorbent Assay (ELISA). About 0.2 g of each lot was ground with mortar and pestle in 2 ml of extraction buffer (8 g sodium chloride, 0.2 g monobasic potassium phosphate, 1.15 g diabasic sodium phosphate, 0.2 g potassium chloride, 0.2 g sodium azide dissolved in 900 ml H_20 adjusted to pH 7.4 with HCl to make up (1 l) + 0.5 ml Tween 20 per liter and 2% polyvinyl pyrollidone (PVP). The homogenate was squeezed through cotton wool and collected in eppendorf tubes. A further 4 lots from the samples each containing 25 seeds were taken and germinated in petri-dishes containing moistened filter paper (Whatman student grade) for 7 days on benches at a room temperature of 28 $\pm$ 3 ^{o}C. Emerging seedlings were excised with sterile blades into endosperm, plumule and radicle. Again 0.2 g of the rice organs from each petri dish was ground with mortar and pestle in 2 ml of extraction buffer and 2% PVP. The homogenate was also squeezed through cotton wool to obtain clear extracts. The extracts were analyzed according to the method of Clark and Adams (16). Alkaline phosphatase (ALP) enzyme was conjugated to antiglobulin (29) and antigen was directly trapped on the microtitre plate and detected by the conjugate against the RYMV antibody introduced after the antigen. The blocking solution contained phosphate buffered salin (8 g sodium chloride, 0.2 g monobasic potassium phosphate, 1.15 g diabasic sodium phosphate, 0.2 g potassium chloride, 0.2 g sodium azide dissolved in 900 ml H_20 adjusted to pH 7.4 with HCl to make up (1 l) and 3% of 99% Marvel Fat free milk. The working dilution for both the antibody and conjugate was 1:1000. Each sample was replicated in two wells of microtitre plate. About 100 µl of 0.6 mg/ml of 4-nitrophenyl phosphate buffers at pH 9.8 were dispensed into each well of the plate and incubated at 37 ^{o}C for 30 minutes. Colour change was measured with METERTECH Σ960 ELISA Plate Microreader. Absorbance values (A 405 nm) were accepted as positive when the reading was greater than twice the mean absorbance of the virus-free control sample.

The seed sample lots that gave a consistently positive result in ELISA were again used for further indexing for the location of RYMV in the rice seed. The husk (seed coat) was separated from each other with a dehusker while the embryo and endosperm were separated apart with a pin (needle). About 0.2 g of each rice component was weighed and ground with mortar and pestle in 2 ml of extraction buffer and 2% VP and analysed by ELISA.

Biological test

Rice seeds harvested from artificially infected plants were seeded in sterilized soil in a greenhouse and visually monitored on standard evaluation scale (28) and by ELISA (16, 29) as described above to detect RYMV symptoms and possible infection respectively. Fifty seedlings were raised per variety from which seeds were selected at random as presented in table 3. ELISA test was carried out on the leaf samples at 63 days after seeding (DAS).

Results and discussion

The results of RYMV indexing of rice organs and components are presented in tables 1 and 2. RYMV was not detected in the plumule, radicle, endosperm and embryo but in the husk (seed coat).

Table 1

Detection of RYMV in the organs of germinating rice seeds from infected plants using enzyme linked immunosorbent assay (ELISA) test

Rice cultivars	Mode of RYMV infection	Source of seeds	ELISA OD values of germinated seeds tested for RYMV (A 405 nm)		
			Endosperm/Husk	Plumule	Radicle
BG 90-2	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.4(++)	0.1(-)	0.1(-)

Rice cultivars	Mode of RYMV infection	Source of seeds	ELISA OD values of germinated seeds tested for RYMV (A 405 nm)		
			Endosperm/Husk	Plumule	Radicle
Serberang MR	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)

(+)= Positive values at A 405 nm, virus particle present

(-)= Negative values at A 405 nm, virus particle absent

Table 2

Detection of the presence of RYMV by ELISA in the rice components of the rice seeds from infected plants in nature and from screen house

Rice cultivars	Mode of RYMV infection	Source of seeds	ELISA OD values of rice components tested for RYMV (A 405 nm)		
			Husk (seed coat-	Endosperm	Eembryo
BG 90-2	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Serberang MR	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.2(+)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.2(+)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	0.4(++)	0.1(-)	0.1(-)

(+)= Positive values at A 405 nm, virus particle present

(-)= Negative values at A 405 nm, virus particle absent

When plumule and radicle were excised from the endosperm and husk, the latter two tested positive by ELISA when analyzed together as one sample unit. But when the endosperm was separated from the husk (seed coat) it was only the latter that tested positive. The result was consistent in all the tests and across the varieties tested.

None of the seedlings raised from seeds harvested from the infected plants of Bouake 189, BG 90-2 and IR 1529-680-3 showed any visual symptoms of RYMV and infection was not detected by ELISA in leaves of these plants until at 63 DAS (Table 3). The non-expression of RYMV was similar in all the varieties screened.

Table 3

Results of visual assessment and ELISA values of leaf samples of screenhouse reared plants raised from seeds of RYMV infected rice plants

Seeds from Rice cultivars	Mode of RYMV infection	Source of seeds	Location	ELISA OD values (A 405 nm)		
				21 DAS	42 DAS	63 DAS
BG 90-2	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
BG 90-2	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
IR 1529-680-3	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
IR 1529-680-3	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
IR1529-680-3	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
IR1529-680-3	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
IR1529-680-3	Naturally	Field	Mali	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Naturally	Field	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)
Bouake 189	Mechanically	Screenhouse	Ivory Coast	0.1(-)	0.1(-)	0.1(-)

- = Negative values at A 405 nm, virus particle absent

DAS= Days after seeding. No single rice plant became infected after 63 days.

This study shows that RYMV is located in the husk (seed coat) of rice seeds. Although it was established that RYMV was present in samples drawn from seeds of infected plants, no evidence of transmission of the virus was obtained when they were planted. This result indicates that RYMV is unlikely to be transmitted through the seed probably because it is located in the husk (seed coat) and not in the embryo. However, studies by Konate *et al*. (30) also established that RYMV is seed-borne but not seed-transmitted. Konate *et al*. (30) explained the non-transmission of the virus by the rice seeds to be due to the inactivation of the virus as the seed matures and begins to loose water after harvest. Bailiss and Offei (10) also gave a similar explanation involving the non-transmission of *Alfalfa mosaic virus* in lucerne. Cheo (15) and Crowley (17, 18) demonstrated that both seed coat and embryos of immature bean seeds contained *Southern bean mosaic virus* but only seed coat of mature seeds contained the virus. And thus, the virus was not transmitted by seed. They concluded that the virus was inhibited or inactivated as the seed matured and dried. Seed transmission of *Tobacco ring spot virus* was associated with the presence of the virus in embryonic tissue of the seed but not in the seed coat (8, 24, 31). Filho and Sherwood (21) reported that absence of seed transmission might be due to the activity of the vascular tissue and the location of the virus within the seed that hampers seedling infection. Earlier reports elsewhere involving sowing of rice seeds from RYMV-

infected plants indicated lack of evidence for the transmission of the virus through rice seed although such reports did not provide reasons why the virus is not transmitted through the seed (e.g. 7, 20, 27). The lack of transmission of this virus through the seed could as well be due to the fact that viruses, which are confined to vascular tissues, may be unable to enter the ovule which is a pathway through which possible virus transmission can take place (23, 34). Lack of plasmodesmata or plasmodesmata breakdown (14) between embryos and surrounding seed and mother plant tissues were suggested as possible reasons for inability of viruses to invade embryos directly. Many workers have reported that the plasmodesmata harboured virus-like particles (32, 38, 42). Viruses that were restricted to the vascular tissues had not been shown to be seed-borne (12). However, Hollings and Huttinga (26) and Taylor *et al*. (39) reported non-embryonic transmission of *Tomato mosaic virus* (TMV) in seeds of tomato plant. But the majority of seed-transmitted viruses have been shown to be through embryonic tissues (5, 33, 36).

It is therefore likely that RYMV is seed-borne but not transmitted through rice seed because virus is probably located in the husk (seed coat) and not in the embryo, or due to the combination of other factors. This finding and that of Konate *et al*. (30) are important to enable us to focus on other sources of infection of RYMV in order to develop durable management strategies for the disease in Africa.

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