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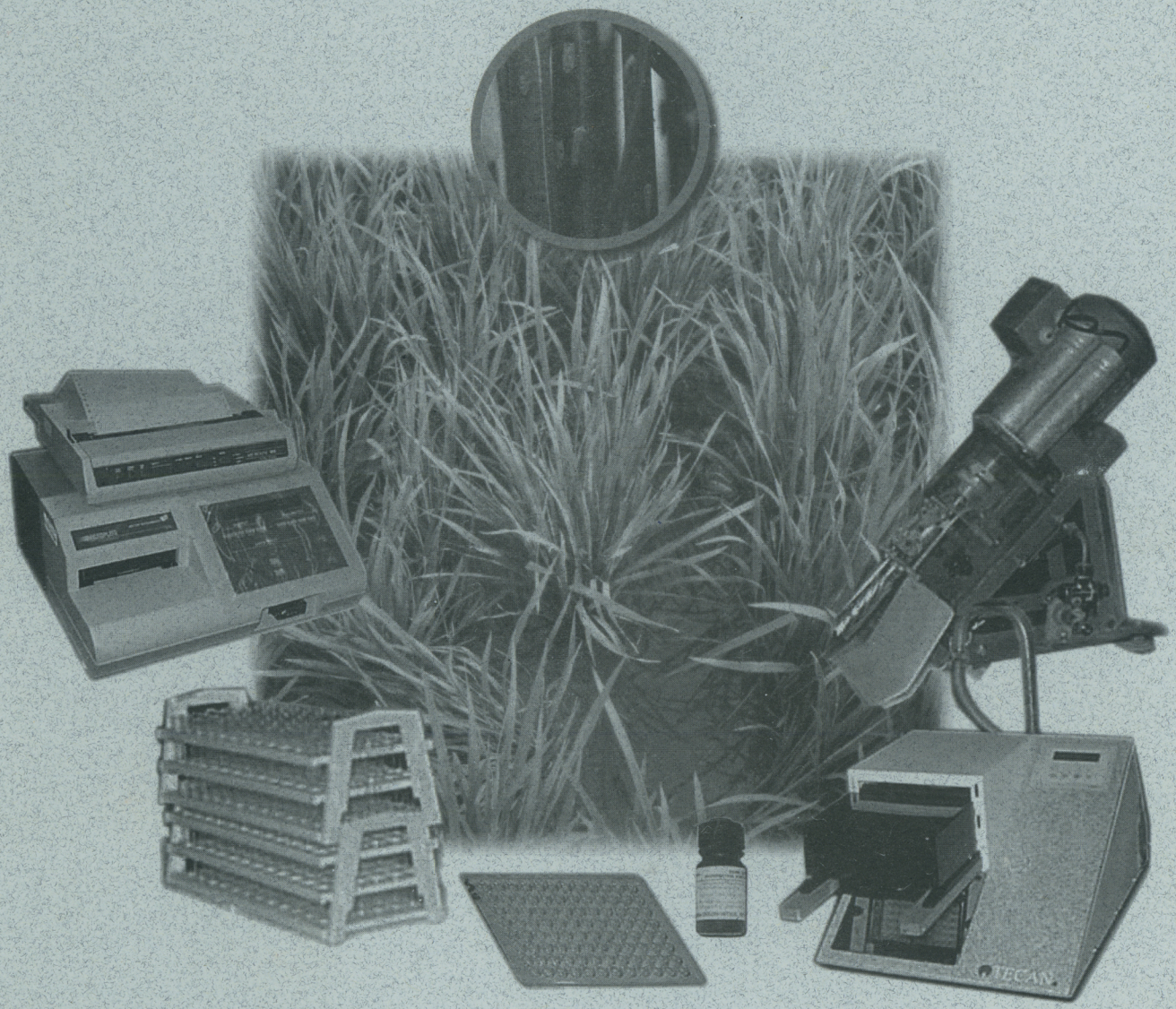
Methods for Evaluating Resistance to Rice Tungro Disease

Ossmat Azzam, Rogelio C. Cabunagan, and Tim Chancellor, Editors



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Contents

Preface

This manual represents the efforts of many rice scientists from different countries who have contributed their expertise in trying to reduce the rice tungro disease problem in Asia during the past thirty-five years. It is an attempt to provide researchers currently involved in tungro resistance breeding programs with a practical guide on the most efficient methods for germplasm evaluation. Some of the techniques discussed are described in other publications, and references to these works are indicated in the manual. Protocols for other activities have been developed at the International Rice Research Institute (IRRI) in collaboration with various partners and are being used in the IRRI breeding program for tungro resistance. We hope that, by including protocols for each of the steps needed in a resistance breeding program in a single volume, the manual will be a useful and practical reference book.

Thanks are due to the Department for International Development (DFID) of the United Kingdom for providing financial support for this manual. Support was given through the Crop Protection Programme under DFID's Renewable Natural Resources Knowledge Strategy. Special thanks go to R. Angeles and G. Khush for their contributions to the breeding methods and procedures for tungro resistance, and to Ma. L.M. Yambao and E. Coloquio for compiling the original protocols used in tungro screening. We are in debt to the efforts of the many people who contributed to this publication.

Introduction

High-yielding varieties with "field" resistance to rice tungro disease have been bred at the International Rice Research Institute (IRRI) and at various national agricultural research institutes and grown widely in South and Southeast Asia. Most of these varieties, which show a low level of tungro disease in the field, have resistance to the major virus vector, green leafhopper (GLH) *Nephotettix virescens* (Khush and Virmani 1985). A few varieties, such as IR20 and IR26, have resistance to rice tungro spherical virus (RTSV), although this was not known at the time they were released because suitable methodologies were not yet developed for distinguishing between the vector and virus resistance.

Resistance to *N. virescens* has been a cornerstone of tungro management in many areas. After a few years of intensive cultivation, however, some of these varieties succumb to tungro infections. Such examples were found in Indonesia (Manwan et al 1985), the Philippines (Hibino et al 1987), Thailand (Inoue and Ruay-Aree 1977), and Malaysia (Nemoto and Habibuddin 1992). The "breakdown" of resistance in the Philippines was attributed to adaptation among populations of *N. virescens* (Dahal et al 1990) and this is also thought to have occurred in other countries.

Developing varieties with resistance to rice tungro viruses in order to produce more durable resistance to the disease is now a major objective of IRRI's breeding program and is also being pursued by several national agricultural research institutes. Useful progress has been made with conventional plant breeding methods. Also, the emergence of new laboratory techniques such as gene mapping and marker-aided selection has created new opportunities for the introgression of viral resistance genes into elite lines.

Currently, varieties ARC11554 (accession no. 21473), Utri Merah (accession no. 16680), Utri Rajapan (accession no. 16684), Habiganj DW8 (accession no. 11751), and some wild rice are being used as resistant donors in the breeding program at IRRI. New breeding lines have been developed using these promising sources of resistance. There are many sources of resistance to RTSV but we still need to identify new sources of resistance for rice tungro bacilliform virus (RTBV) because few sources of tolerance are available (Koganezawa and Cabunagan 1997).

It is important to use efficient methods both to identify new sources of resistance and to evaluate new breeding lines to confirm that resistance has been successfully transferred from the donor line or variety. In this manual, we describe protocols for each of the germplasm screening and evaluation methods for use in a breeding program for tungro disease resistance. The mass-screening method has undergone several revisions in recent years to improve its efficiency (Colloquio et al 1998). The two more commonly used mass-screening methods are described here, with emphasis on the water tray method, which offers several advantages. We outline the forced-tube inoculation method, which provides a stronger test for tungro resistance and is used to evaluate lines classified as resistant in the mass-screening method. We describe the two standard methods for identifying vector resistance and summarize the two techniques for establishing the level of antibiosis for GLH resistance in the test materials. Proto-

cols are included for mass rearing of the green leafhopper and for maintaining virus-infected plants in the greenhouse.

This manual describes various methods of evaluating tungro disease resistance in the field. Presented as an example is the protocol involved in conducting the International Rice Tungro Nursery (IRTN). It is important to clearly establish the reaction of a resistant line in a particular locality before it is released as a variety. Recent studies have shown that certain varieties react differently to tungro disease in different locations because of the presence of specific subpopulations of tungro viruses (Dahal et al 1992, Cabauatan et al 1995, Arboleda and Azzam 1999, Azzam et al 1999). We therefore describe field screening techniques that allow the reaction of a breeding line or variety to be thoroughly assessed.

Samples of data sheets used to record data in the mass screening for tungro viruses and GLH at IRRI are presented. Also, a field layout for tungro field screening and seed box layout for GLH mass screening and scheme of the protocols for the conduct of ELISA and the latex test are presented for easy reference.

The use of serology is of major benefit to a tungro resistance-breeding program as it enables resistance to tungro viruses to be established and quantified. This manual describes the two most reliable and practical serological techniques, along with the tungro screen kit B for RTBV diagnosis.

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Rice tungro viruses, disease symptoms, and transmission

Rice tungro disease is caused by two viruses: rice tungro bacilliform virus (RTBV), 100–300 nm in length and 30–35 nm in width, containing a circular double-stranded DNA; and rice tungro spherical virus (RTSV), 30 nm in diameter and containing single-stranded RNA (Hibino et al 1991, Jones et al 1991). Both viruses multiply independently in rice plants. RTSV particles are restricted to the phloem tissues, whereas RTBV particles are present in both phloem and xylem cells (Sta. Cruz et al 1993).

Rivera and Ou (1965) described the following symptoms of tungro:

1. Stunting of plants and yellowing of leaves are the most conspicuous symptoms.
2. Yellowing of the leaves appears first as partial yellowing or striping with diffused chlorotic mottles.
3. When infected at an early stage of growth, plants produce few tillers.
4. Root development is poor.
5. Panicles formed on infected plants are small, sterile, and incompletely exserted.
6. Grains have dark brown blotches.

The use of serology, however, enabled Hibino et al (1978) to establish that symptoms are dependent on the virus present:

1. When both RTBV and RTSV are present, infected plants generally exhibit yellowing and stunting.
2. With RTBV alone, infected plants exhibit mild yellowing and stunting.
3. With RTSV alone, infected plants exhibit almost no symptoms.

Hasanuddin (1987) reported that symptoms vary depending on varieties infected. For example:

1. Balimau Putih and Utri Rajapan showed only mild stunting even when infected with both RTBV and RTSV.
2. Palasithari and Sigadis showed relatively severe symptoms at the early growth stage, but symptoms tended to be mild at later stages.
3. FK135 and ASD7, even when infected with RTBV alone, showed severe stunting and yellowing until the late stage of growth.

Tungro viruses are transmitted by adults and nymphs of *Nephotettix virescens, N. cincticeps, N. nigropictus, N. malayanus, N. parvus,* and *Recilia dorsalis* (Ling 1972). The transmission efficiency differs depending on the vector species and the populations, but *N. virescens* is the most efficient vector. Tungro viruses are transmitted in a semipersistent manner. It is reported that the minimum acquisition and inoculation feeding periods for *N. virescens* are 5 min and 7 min, respectively, but the acquisition and transmission efficiency increase with time. *Nephotettix virescens* retains tungro infectivity for 2 to 6 d but nymphs lose it after molting. Leafhoppers readily acquire RTSV from plants infected with RTSV alone, but do not acquire RTBV from plants infected with RTBV alone. Leafhoppers acquire RTBV only when exposed to RTSV-infected plants before feeding on RTBV-infected plants (Hibino et al 1979).

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Resistance to rice tungro disease

Growing resistant varieties has been considered essential for suppressing outbreaks of tungro disease and controlling its spread in South and Southeast Asia (Sogawa 1976). Thus, varietal resistance to tungro is an important breeding objective for rice improvement in this area (Khush and Virmani 1985). Screening for tungro resistance at IRRI started in 1963. In the early period, selected resistance was to the vector because resistance to *N. virescens* is abundant in rice germplasm and is easy to identify. Most IRRI crosses made after 1969 had at least one parent with resistance from Ptb 18, Gam Pai 30-12-15, or Ptb 33 and most IR varieties, except for IR22, were rated green leafhopper-resistant at the time of release.

Vector-resistant varieties escape from tungro infection in the field under light to moderate disease and vector pressure. Undoubtedly, the introduction of vector-resistant varieties reduced tungro incidence because disease levels declined drastically in several areas after the introduction of these varieties. At some locations, however, the resistance ceased to be effective after a few consecutive seasons of intensive cultivation of designated resistant varieties. In recent years, research has shifted to virus resistance, which may lead to varieties with more durable resistance.

About 30% of the total IRRI Rice Germplasm Center (IRGC) accessions have been screened for tungro resistance and results revealed that most of the resistant accessions originated in Bangladesh, India, Pakistan, and Indonesia (Cabunagan and Koganezawa 1993, Azzam et al, unpublished data).

Many accessions have been reported as resistant to RTSV infection (Hibino et al 1990, Koganezawa and Cabunagan 1997, Azzam and Coloquio 1999). Some of these RTSV-resistant accessions also have vector resistance and it is difficult to separate the two types of resistance using the present inoculation method at IRRI. Generally, RTSV-resistant varieties show high infection rates with tungro disease in artificial inoculation tests because of their susceptibility to RTBV infection, but disease development is expected to be slow in the field. This is because RTBV cannot be acquired from plants infected with this virus alone, unless the vectors had prior access to an RTSV-infected source (Hibino 1983). When the RTSV-resistant variety IR26 was exposed to infection in the field, the distribution of infected plants was scattered (Cabunagan et al 1989, Satapathy et al 1997). In contrast, infected plants were more numerous and appeared in patches in the comparable plots of susceptible varieties in which secondary spread occurred.

The variety TKM6 reacted differently to RTSV isolates from India and the Philippines. In the Philippines, a virulent strain of RTSV designated "Vt6" was found infecting TKM6 at Midsayap, Cotabato, in Mindanao. TKM6 is still resistant to the IRRI glasshouse strain of RTSV, which is designated as the "A" strain. When rice accessions previously identified as resistant to strain A were tested against Vt6, 52 accessions were found resistant to Vt6 (Azzam and Coloquio 1999) and 18 of these are susceptible to the vector (Table 1). These may be used as sources of resistance to both strains of RTSV.

Currently, no improved rice variety is resistant to RTBV infection. Two varieties (ARC11554 and Katijan) had less than 30% RTBV infection, but these varieties are also

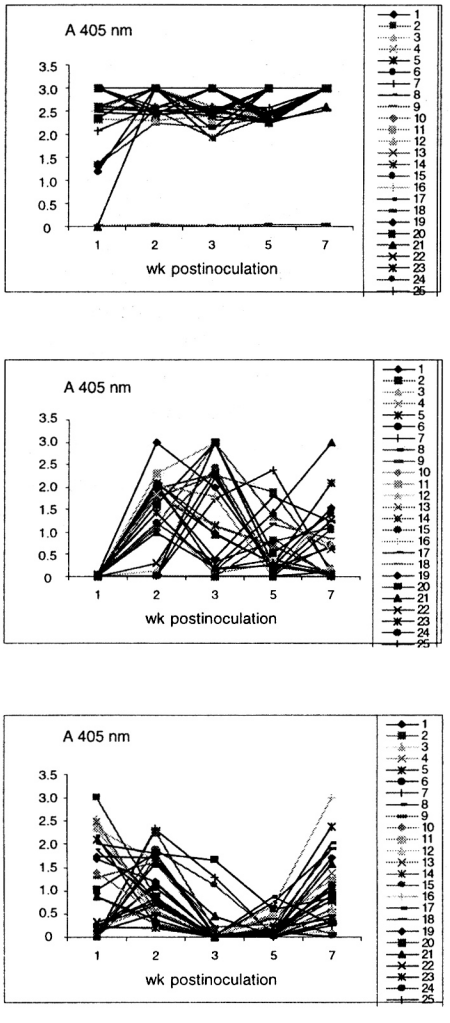
Table 1. Accession lines that showed 0% infection when inoculated with the avirulent (A) populations and virulent on TKM6 (Vt6) populations of RTSV at the IRRI greenhouse (1999).

	ACC. no.	Variety name[a]	Scores[b]		RTSV-A			RTSV-Vt6		
			SS[c]	GLH	n	B (%)	S (%)	n	B (%)	S (%)
1	681	Tilakkacharry	2	1	31	84	0	34	0	0
2	20260	ARC5842	2	3	43	88	0	38	0	0
3	**20352**	**ARC6128**	**3**	**9**	**38**	**95**	**0**	**36**	**3**	**0**
4	**20359**	**ARC6147**	**2**	**9**	**38**	**95**	**0**	**35**	**3**	**23**
5	**20368**	**ARC6177**	**2**	**9**	**38**	**92**	**0**	**35**	**3**	**0**
6	**20377**	**ARC6202**	**2**	**9**	**38**	**76**	**0**	**36**	**6**	**6**
7	**22630**	**ARC13827**	**2**	**9**	**42**	**95**	**0**	**31**	**0**	**0**
8	**22641**	**ARC13299**	**2**	**9**	**36**	**81**	**0**	**37**	**0**	**0**
9	**22644**	**ARC13303**	**1**	**9**	**39**	**92**	**0**	**35**	**0**	**0**
10	**27608**	**ARC13302**	**2**	**9**	**38**	**89**	**0**	**34**	**0**	**0**
11	28548	Lajyabati	1		38	100	0	36	0	0
12	33976	AC7030	2	3	38	68	0	32	0	0
13	**41274**	**ARC13874**	**3**	**9**	**41**	**49**	**0**	**36**	**0**	**0**
14	**41336**	**ARC13951**	**1**	**9**	**41**	**56**	**0**	**28**	**0**	**0**
15	**41475**	**ARC14212**	**1**	**9**	**45**	**62**	**0**	**34**	**0**	**0**
16	**41509**	**ARC14320**	**1**	**9**	**40**	**8**	**0**	**31**	**0**	**0**
17	**41567**	**ARC14455**	**2**	**9**	**42**	**21**	**0**	**15**	**7**	**0**
18	41608	ARC14564	3	5	42	24	0	33	36	0
19	**41643**	**ARC14624**	**2**	**9**	**33**	**85**	**0**	**30**	**3**	**0**
20	**41714**	**ARC14719**	**3**	**9**	**34**	**97**	**0**	**36**	**17**	**0**
21	41830	ARC14944	3	3	32	34	0	20	0	0
22	42192	ARC15990	2			10	0	37	8	0
23	42198	ARC18004	2			46	0	24	8	0
24	42609	ARC10970	1			66	0	13	0	0
25	42797	ARC13953	2			67	0	20	0	0
26	42830	ARC13995	2			48	0	18	0	0
27	43025	ARC14934	2			54	0	19	0	0
28	44906	Ajan	3			86	0	15	0	0
29	45116	Begubbichi	1			24	0	18	0	0
30	45518	Dhalamukhi	1			42	0	15	0	0
31	45548	Dhuli	3			92	0	17	0	0
32	45580	Dudhikalma	2			72	0	19	0	0
33	46870	Unnamed	2			2	0	10	0	0
34	52195	Matikiba	3			25	0	13	0	0
35	52510	Allur Sanna	3			31	0	17	0	0
36	55142	PTB				16	0	18	0	0
37	55196	1371				29	0	16	0	0
38	62470	NCS752				38	0	18	0	0
39	77506	Gudhari	2		40	53	0	11	0	0
40	31746	Bish Katari	3	3	38	68	0	37	5	0
41	37257	Pakkiraj	3	3	40	70	0	35	14	0
42	37856	Cylindrical 30-617	3	5	14	86	0	33	15	0
43	**37928**	**Deep Straw 24-521**	**2**	**9**	**40**	**60**	**0**	**32**	**16**	**0**
44	38191	Rajasail 2-14	1	3	39	13	0	38	0	0
45	38371	Straw Fine 22-378	2	3	36	44	0	38	8	0
46	38448	Straw 23-463	2	3	40	5	0	35	3	0
47	49165	Butubalam	2		35	54	0	25	0	0
48	49196	Holdemethi	1	5	37	95	0	27	0	0
49	**49208**	**Kalaaman**	**3**	**9**	**17**	**71**	**0**	**38**	**0**	**0**
50	**48686**	**Bidara**	**2**	**9**	**0**	**97**	**0**	**34**	**100**	**0**
51	42979	ARC14743	2			37	0	11	0	0
52	50232	Vellai Gundu Samba	3			68	0	18	0	0

[a]Varieties in bold are susceptible to the vector.
[b]On a scale of 1 to 9, where 1 = no symptom observed, 3 = 1–10% reduction, no distinct yellow to yellow-orange leaf discoloration, 5 = 11–30% height reduction, no distinct yellow to yellow-orange leaf discoloration, 7 = 31–50% height reduction, no distinct yellow to yellow-orange leaf discoloration, 9 = more than 50% height reduction, with distinct yellow to yellow-orange leaf discoloration.
[c]SS = severity score, GLH = green leafhopper, B = RTBV + (RTBV + RTSV), S = RTSV + (RTBV + RTSV), n = number of plants inoculated.

resistant to the green leafhopper vector. Although sources for resistance to RTBV infection have not been found in rice germplasm, several varieties showed symptomatic resistance or tolerance (Koganezawa and Cabunagan 1997). Tolerant varieties do not show conspicuous symptoms and yield losses are low. Virus multiplication is suppressed in some tolerant varieties such as Utri Merah, Balimau Putih, and Utri Rajapan. In these varieties, RTBV concentration is low by enzyme-linked immunosorbent assay (ELISA) (Cabunagan et al 1993, Sta Cruz and Azzam 1999).

Recently, using a time-course experiment, we screened 14 of the lines most tolerant of RTBV infection using the new agroinoculation method that distinguishes RTBV resistance from resistance to RTSV or to the vector (Azzam and Sta Cruz, unpublished results) and Figure 1 shows the typical three groups of infection cycles detected for RTBV in these lines. Varieties in group III have a potential to be used in breeding programs. These varieties seem to be good sources of resistance because virus spread in the field is expected to be slow. Among varieties already tested in several regions, Utri



Germplasm

Group I
TN1
ARC14320
ARC7140
ARC11554
Ghigos

Group II
Adday Sel
Adday LS
ARC5905
Bish Katari
Sham Rosh

Group III
Utri Merah
Balimau Putih
Kashia Binni
ARC10963
ARC14455
Kaisha Binni

Fig. 1. The three groups of RTBV infection cycles determined by agroinoculation for the 14 lines tolerant of RTBV as assessed by ELISA in a time-course experiment (1999).

Merah, which originated in Indonesia, always showed tolerance. So far, RTBV strains that can induce severe symptoms on tolerant varieties have not been reported. Efforts to transfer the RTBV tolerance gene from Utri Merah (which also has resistance to RTSV) to improved varieties are being made at IRRI.

Advanced breeding lines with resistance to tungro infection had been identified (Angeles et al 1998) and had been tested in multilocation trials in the Philippines, Indonesia, and India (Cabunagan et al 1998).

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Breeding methods and procedures

The most logical and practical way of controlling rice tungro disease is by using resistant varieties. One of the major objectives, therefore, in the rice improvement program at IRRI and in many national rice breeding programs is to develop improved varieties with resistance to rice tungro viruses or their vectors, the green leafhopper (GLH). Rice varieties with tungro resistance are almost completely protected against infection by the disease. On the other hand, varieties with green leafhopper resistance may escape disease infection in the field, but when forced-tube-inoculated with the virus, they become infected. Such resistance, also known as field resistance, is observed in the earlier released IR varieties (Khush 1992).

The breeding methods and procedures used at IRRI in breeding rice varieties with multiple insect and disease resistance including tungro (Khush and Coffman 1977, Khush 1978, 1981, 1989) are briefly reviewed here.

Sources of resistance

Success in breeding for tungro resistance depends largely on the availability of sources of resistance. Fortunately, many rice varieties from IRRI's germplasm collection showed a resistant reaction to the disease. Of the 20,384 accessions tested for tungro reaction, 1,368 were reported to be resistant (Jackson 1997, Azzam et al, unpublished data).

Many varieties reported as resistant to tungro usually have vector resistance only and few have resistance to the viruses. Utri Rajapan (Acc. 16684), Utri Merah (Acc. 16680), ARC11554 (Acc. 21473), and Habiganj DW8 (Acc. 11751) are among the varieties with resistance to the virus. These varieties, along with the wild rice *Oryza longistaminata* and *O. rufipogon* (Acc. 105908, 105909, and 105910), have been used as sources of resistance to the virus (Angeles et al 1998).

Improving sources of resistance

Most of the sources of resistance to the virus have poor plant type, typical of traditional varieties of the tropics. As a first step, the genes for resistance to the virus are transferred into improved plant-type varieties. This is achieved by crossing the tall donor parents with an improved plant-type parent and selecting improved plant-type segregants with resistance to the virus. The selected lines are evaluated for two or three generations for plant type, grain quality, and resistance traits. In the case of wild rice parents with an extremely poor plant type such as *O. longistaminata* and *O. rufipogon*, 2–3 backcrosses are made using the improved plant-type lines as recurrent parents.

Many IRRI-released varieties can be used as parents in the transfer of virus resistance genes. These varieties, aside from having good agronomic traits, carry resistance to several insects and other diseases of rice. Presently, IR64 and IR1561-288-3-3 are extensively used as parents in improving sources of resistance to the virus. IR64 is popular because of its good grain quality, whereas the line IR1561-288-3-3 is being used because of its good plant type and early maturity.

Combining tungro resistance with other resistance genes

Varietal resistance to the virus is not enough. Modern rice varieties must have multiple

resistance to most of the pests prevalent in the areas where they are to be grown. Thus, improved breeding lines with virus resistance are intercrossed with other improved breeding lines with one or more genes for resistance to insects and other diseases. From numerous topcrosses and double crosses, segregating populations are thoroughly screened to identify lines with combined resistances to as many as four diseases and four insects, including resistance to the tungro virus.

Breeding methods

Since the inception of the breeding program at IRRI, the pedigree and backcross methods of breeding have been used to develop germplasm with multiple resistance. The bulk method was not employed because it does not permit concurrent screening for reactions to insects and diseases.

The pedigree method is most suited to combining traits governed by major genes such as resistance to insects and diseases. Thus, this method is almost exclusively used in developing improved germplasm with multiple resistance. With this method, selection is based on comprehensive records of the disease and insect reactions of each line. F_4 and later-generation lines are also selected based on the reaction of the ancestral lines.

The backcross method is commonly used to transfer tungro resistance from wild rice donors. For instance, tungro resistance in *O. longistaminata* and *O. rufipogon* was transferred into the background of IR1561-288-3-3 through 4–5 backcrosses. IR1561-288-3-3 lines carrying tungro resistance from the two wild rice parents are now being used as donors for resistance in the breeding program for developing lines with multiple resistance.

The backcross method has been used to transfer tungro resistance into popular varieties that gained wide acceptance in many countries. A good example is IR64, which is now used as one of the recurrent parents in the transfer of tungro resistance from Utri Merah, Habiganj DW8, and *O. rufipogon*. This variety, in addition to all the desirable traits of IR36, has higher yield potential, horizontal resistance to blast, and superior grain quality. Several IR64 lines with tungro resistance from the aforementioned sources are now available and are being evaluated for yield and tungro resistance in multilocation trials conducted in the Philippines, Indonesia, and India.

Breeding procedures

Based on many years of experience in disease and insect resistance breeding, IRRI plant breeders have developed procedures for handling donor parents, making crosses, growing and screening the segregating populations, and evaluating the progeny materials for agronomic traits and grain quality. These procedures are as follow:

Planting of donor parents

Each season, donor parents are planted in a hybridization block (HB) consisting of 250 to 300 entries, including both newly identified unimproved donors and breeding lines with specific pest resistance. The HB entries are planted 5 times per season at an interval of 2 wk. This schedule ensures availability of donor materials for crossing at different times in the crossing season.

Hybridization

To produce single-cross F_1 hybrids, each donor parent or breeding line is crossed with several other breeding lines. Thus, a set of single-cross F_1 progenies is available for double-crossing or topcrossing the next season. All the F_1s involving the same donor parent or breeding line are grown together in the F_1 nursery. The best are used for making topcrosses or double crosses. Topcross parents are selected to complement deficiencies of the single-cross F_1 hybrids. Thus, if one parent of the single-cross F_1 is resistant to brown planthopper (BPH) and green leafhopper (GLH) and the other is resistant to tungro, the topcross parent should be resistant to blast and bacterial blight. The use of topcross parents that are homozygous for resistance is desirable so that all of the F_1 plants will inherit the trait. The use of improved breeding lines or varieties as a topcross parent is also desirable. If the unimproved tall donor is used as a topcross parent, all

the F_1 progenies will be tall and only 25% of the F_2 progenies will be short.

F_1 seeds (300 to 400) are obtained for each topcross or double cross to be able to sample gametic variability of the single-cross F_1 hybrids. Screening begins at the F_1 generation. Consider a double cross between four parents: A is resistant to bacterial blight, B to tungro, C to BPH, and D to GLH. All these traits are governed by dominant genes that segregate independently of each other. About 400 seeds from the double cross A/B//C/D are grown and inoculated with the tungro virus in the greenhouse. About 50% of the seedlings are susceptible so they are eliminated. The remaining 200 seedlings are transplanted in the field and inoculated with bacterial blight at about 60 d of age; about 50% are susceptible and these are discarded. The remaining 100 plants are harvested individually and progeny-tested for BPH and GLH resistance. Those carrying BPH resistance genes (50%) and those carrying GLH resistance genes (50%) are identified. The F_2 populations are grown only from those carrying both genes (25–30 plants). Thus, by judicious and timely screening, the original F_1 sample of 400 plants is reduced to 25 or 30. All the F_2 populations grown from those plants segregate for the four resistance genes.

F_2 populations

F_2 populations are grown without insecticide protection to expose them to leafhopper and planthopper infestation. Tungro-inoculated TN1 plants are planted around the borders of F_2 plots to ensure tungro infection. Most F_2 populations are also inoculated with bacterial blight in the field. Plants susceptible to tungro or bacterial blight are immediately destroyed.

F_2 populations from the individual F_1 plants of topcrosses or double crosses are grown separately and selections are made only from agronomically suitable populations. From each cross combination, 2,000–5,000 F_2 plants are grown and 100–500 plants are selected from promising combinations to grow in the pedigree nurseries.

Pedigree nurseries

F_3 seeds (25–30 g) are obtained from each plant selection in the F_2. This sample is divided into 5 or 6 sets of 5 g each. One set is used to plant the pedigree nursery, where each plant selection is planted to one single 5-m-long row consisting of 25 plants. The remaining seed sets are tested for resistance to blast, grassy stunt, GLH, and BPH, grain quality, and sometimes for other traits such as drought or salinity tolerance. The pedigree rows are inoculated with bacterial blight in the field. The pedigree nursery is grown without insecticide protection to allow the buildup of GLH populations. To ensure tungro infection, tungro-inoculated TN1 plants are planted around the borders of the pedigree nursery. Diseased plants from the previous nursery are also maintained and occasionally transplanted around the nursery borders to provide a source of infection. Thus, screening for tungro and bacterial blight is done in the field. Data on blast are obtained from blast nursery screening. Resistance to grassy stunt, GLH, and BPH is evaluated in the greenhouse. The data from various screening trials are recorded in the pedigree nursery book before making selections in the field. The main selection criteria, besides agronomic characteristics and grain quality, are insect and disease resistance ratings.

Three plants from each promising row are individually harvested. Seeds from the remaining plants of each selected row are bulk-harvested. Seeds obtained from each selected plant are again divided into 5 or 6 sets of 5 g each. One set is replanted in the pedigree nursery, the others are used for disease and insect screening. The three plant selections from each common source are planted together for comparison. Data from the ancestral row of the previous generation are transferred to the new pedigree book where the data for each row's reaction are recorded. Thus, in the F_4 and later generations, data from two seasons are available for each pedigree row to facilitate selection. These procedures are repeated for several generations until the breeding lines become uniform in maturity, height, and other traits.

Yield trials

Selected F_5, F_6, or later-generation lines are evaluated for yield in replicated trials. Entries in this trial are also screened for resistance to major diseases and insects. All yield trial entries are inoculated with sheath blight in the field and evaluated for stem borer resistance in the screenhouse. Promising materials with high yield, good grain quality, and multiple pest resistance are supplied to various national programs and can be released as varieties.

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Mass rearing green leafhopper *Nephotettix virescens*

The main requirements for mass rearing the rice green leafhopper (GLH) *Nephotettix virescens* are a greenhouse, rearing and oviposition cages, and metallic trays for the propagation of TN1 plants. A detailed description of the system developed at IRRI for rearing rice leafhoppers and planthoppers is provided by Heinrichs et al (1985). Information is provided about the construction of a greenhouse and the fabrication of cages as well as the initiation and maintenance of the insect cultures. It is important to periodically introduce into the insect cages a new batch of GLH collected from the field to minimize the risk of inbreeding depression. This may be done once a year. A summary of the main steps in mass rearing GLH is given below.

Materials

Insect cages (53 cm × 53 cm × 90 cm)
Sweep net
Adult green leafhopper (GLH)
TN1 seeds
Clay or plastic pots
Plastic or metallic trays

Procedure

Before mass rearing the GLH, prepare the TN1 plants to be used for feeding the insects and for oviposition. Sow TN1 seeds in pots and grow them until the one-leaf stage. Transplant the seedlings in pots at 5 seedlings per pot and do this at weekly intervals. When the first batch of plants is about 45 d old, mass rearing of the insects can be started.

1. Collect adult GLH from rice fields with a sweep net. Confine 500–1,000 adult GLH in a cage with 45-d-old TN1 plants for 2–3 d oviposition.
2. Transfer the plants used for oviposition to another cage and replace with a fresh batch for oviposition. Clean the plants for oviposition, remove the old leaves and leaf sheaths, and wash the plants before introducing them into the cage to remove ants and predators.
3. Repeat the process in step 2 until no insects remain.
4. Maintain plants used for oviposition until the nymphs emerge. Put in more feeding materials (45-d-old TN1 plants) as needed until the nymphs develop into adults.
5. Transfer the newly emerged adults to the oviposition cage. Repeat steps 2 and 3. This time a new batch of adult insects is available every 2–3 d. Replace dead insects with additional adult insects for egg laying.
6. Continue the process to maintain a constant supply of insects. The number of egg cages used may be adjusted according to the number of GLH required.

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Maintenance of virus-infected rice plants

Rice tungro bacilliform virus and rice tungro spherical virus

A supply of rice plants infected with both RTBV and RTSV is needed in mass screening for tungro resistance. It is essential to maintain and use the same source of inoculum when evaluating breeding. If multiple sources are used, different phenotypes may result. Doubly infected rice plants are also used to produce plants infected separately with RTBV or RTSV for use in other studies. This section describes the procedure for maintaining virus-infected plants as a disease source. In areas where a GLH colony cannot be maintained throughout the year, the disease source can be maintained through the cooler period by ratooning or by tiller propagation (Anjaneyulu 1996).

Materials

TN1 seeds
Clay or plastic pots
Insect cages (53 cm × 53 cm × 90 cm)
Metallic trays
GLH colony

Procedure

If there are no tungro sources in the greenhouse, identify diseased plants in the field, uproot them, and transplant in pots. To confirm whether plants are infected with RTBV and RTSV, test them by enzyme-linked immunosorbent assay (ELISA). If facilities to conduct ELISA are not available, plants showing symptoms of severe stunting and yellowing can be assumed to be infected with both viruses. Select doubly infected plants and use them as a source to propagate tungro-diseased plants.

Tungro viruses can be transmitted from diseased plants to healthy TN1 plants, or other susceptible rice varieties, by viruliferous insects. The number of virus source plants to maintain will vary depending on the particular requirements of the program. In the procedure outlined here, 32 pots of tungro-diseased plants are initially maintained. This will allow *c.* 9,000–10,000 adult GLH to feed on the plants for 4 d to acquire the viruses. Since doubly infected plants are being maintained, it is not necessary to ensure that GLH are nonviruliferous before use.

1. Transfer adult GLH to tungro-diseased plants for 4-d acquisition access.
2. Inoculate 16 pots (2 seedlings per pot) of 45-d-old TN1 plants inside the insect cages for 4 h (16 pots in the morning and 16 pots in the afternoon) with the viruliferous GLH (3 insects per seedling).
3. Confine the inoculated TN1 plants in screen cages and observe for symptom development for 2–3 wk.
4. Test inoculated plants by ELISA to determine presence of RTBV and RTSV.
5. Select doubly infected plants and keep as virus source.
6. Repeat steps 1–5 to maintain RTBV and RTSV doubly infected source.

Rice tungro spherical virus

If RTSV-alone-infected plants are needed for inoculations to further test the resistance of varieties and lines identified in mass screening against RTSV, single sources of the inoculum can be maintained. At IRRI, two RTSV-alone sources are currently available, one for the

RTSV avirulent source on TN1 and the other for the virulent source on TKM6 (Cabauatan and Hibino 1988).

Materials

TN1 or TKM6 seeds
Insect cages (53 cm × 53 cm × 90 cm)
Test tubes (18 mm × 150 mm) with cap in test tube racks
Mylar cages
Clay or plastic pots
RTSV-infected plant
GLH colony
Aspirator
Forceps

Procedure

Identify a rice plant infected with RTSV alone by ELISA. If none is available, produce one as follows. Allow adult GLH to feed on rice plants infected with both RTBV and RTSV and transfer to 5–6-d-old TN1 seedlings in test tubes for overnight inoculation feeding at the rate of 1 insect per seedling. Two weeks after inoculation, discard plants with symptoms of yellowing and stunting (these plants are generally infected with both RTBV and RTSV). Test the remaining healthy-looking plants by ELISA to identify those that are infected with RTSV alone. This will now serve as the virus source.

Nonviruliferous GLH must be used to make sure only RTSV is transmitted. Ensure that GLH are nonviruliferous by allowing them to feed on fresh virus-free rice seedlings daily for 5 d.

1. Transfer adult GLH to RTSV disease source plant for 3-d acquisition access.
2. Collect 2–3 viruliferous GLH with an aspirator and transfer to a test tube with a single 5–6-d-old TN1 seedling for 24-h inoculation access period.
3. Remove inoculated seedlings from test tubes and transplant them in clay or plastic pots (1–4 seedlings per pot) inside screen cages. Maintain cages GLH-free.
4. Collect leaf samples from each plant and test by ELISA for RTSV infection 2–3 wk after inoculation.
5. Select RTSV-infected plants that could be used as a virus source when they are 30–45 d old.
6. Repeat steps 1–5 to maintain RTSV-diseased plants.

Rice tungro bacilliform virus

RTBV cannot be transmitted alone. It requires a "helper factor" from RTSV for its transmission. Therefore, propagation of RTBV-infected plants requires that GLH feed first on rice plants infected with RTSV or with both RTSV and RTBV. The methodology outlined below was developed to propagate TN1 plants infected with RTBV only. TN1 may be used as a source of RTBV for purification purposes. However, if RTBV-infected plants are only required as a virus source, a variety susceptible to RTBV and resistant to RTSV may be used. Inoculate plants of an RTSV-resistant variety such as IR26 with RTBV + RTSV. Select plants infected with RTBV alone 2–3 wk after inoculation (Yambao et al 1993).

Materials

TN1 or IR26 seeds
Insect cages (53 cm × 53 cm × 90 cm)
Test tubes (18 mm × 150 mm) with cap in test tube racks
Mylar cage
Clay or plastic pots
RTSV-diseased source plant
GLH colony
Aspirator
Forceps

Procedure

1. Confine GLH on doubly infected (RTBV + RTSV) plants for 3 d.
2. Transfer GLH to RTBV-alone-infected plant for 2-d acquisition access.
3. Confine 2 viruliferous GLH per 5–6-d-old seedling in a test tube for 24-h inoculation access. Transplant inoculated seedlings in pots and maintain GLH-free inside a screened cage.
4. Test inoculated plants by ELISA at 45–60 d after inoculation and select RTBV-infected plants.

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Mass screening for tungro resistance

Water tray method

Mass screening for resistance to rice tungro disease at IRRI was developed in 1964, modified in 1974 (Ling 1974), and revised again in 1987. Since that time, several modifications to the procedure have been made. The biggest change involved the introduction of the water tray method, which has several advantages over the caged-pot method used previously (Coloquio et al 1998). Inoculation efficiency is greater in the water tray method and there is less variability in the results. In addition, the same batch of viruliferous GLH can inoculate three sets of materials in one day (compared with only two sets for the caged-pot method). The procedure for the water tray method is as follows (Fig. 2):

Materials

TN1 seeds
Clay or plastic pots
Pot labels and marking pens
Petri dishes
Insect cages (53 cm × 53 cm × 90 cm)
Seed boxes (69 cm × 26 cm × 9 cm)
Water tray (97 cm × 66 cm × 30 cm)
Screen cage with cover (100 cm × 70 cm × 46 cm)
Test seedlings
Adult GLH
Black cloth

Procedure

Ensure that adequate sources of tungro virus-infected plants and adult GLH are available. Refer to the sections on the maintenance of virus-infected plants and the mass rearing of GLH in this manual.

Preparation of plants to be inoculated

a) Soak seeds overnight in petri dishes. If you are testing seeds of wild species, seeds may need to be dehulled, scarified, or treated in some other way. Check with seed providers for the appropriate germination requirement of the seeds to be tested.
b) Sow 20 seeds per row in seed boxes, with 26 entries per seed box, including TN1 as the control. Label the test entries and prepare the data sheets.
c) After 9–10 d, test seedlings are ready for inoculation. Trim the leaves of the seedlings.

Inoculation

d) Place the seed box that contains the test seedlings inside the water tray and cover with a screen cage.
e) Viruliferous insects (allowed to feed on tungro-infected plants for 4 d) are then released in the cage at an average of 6–7 insects per seedling for 2–3-h inoculation access. The inoculation cage needs to be covered with black cloth so that GLH will not be attracted to light and move away from the seedlings.
f) After inoculation, fill the trays with water until the test seedlings are submerged. Once the water covers the seedlings, the insects will transfer to the screen cage, which is then removed. Remove the seed box and drain the water. Place a second seed box inside the cage and repeat *step b*. Use the same process for the third seed box.
g) After inoculation has been completed for the third seed box, allow the insects to feed overnight on infected plants placed inside

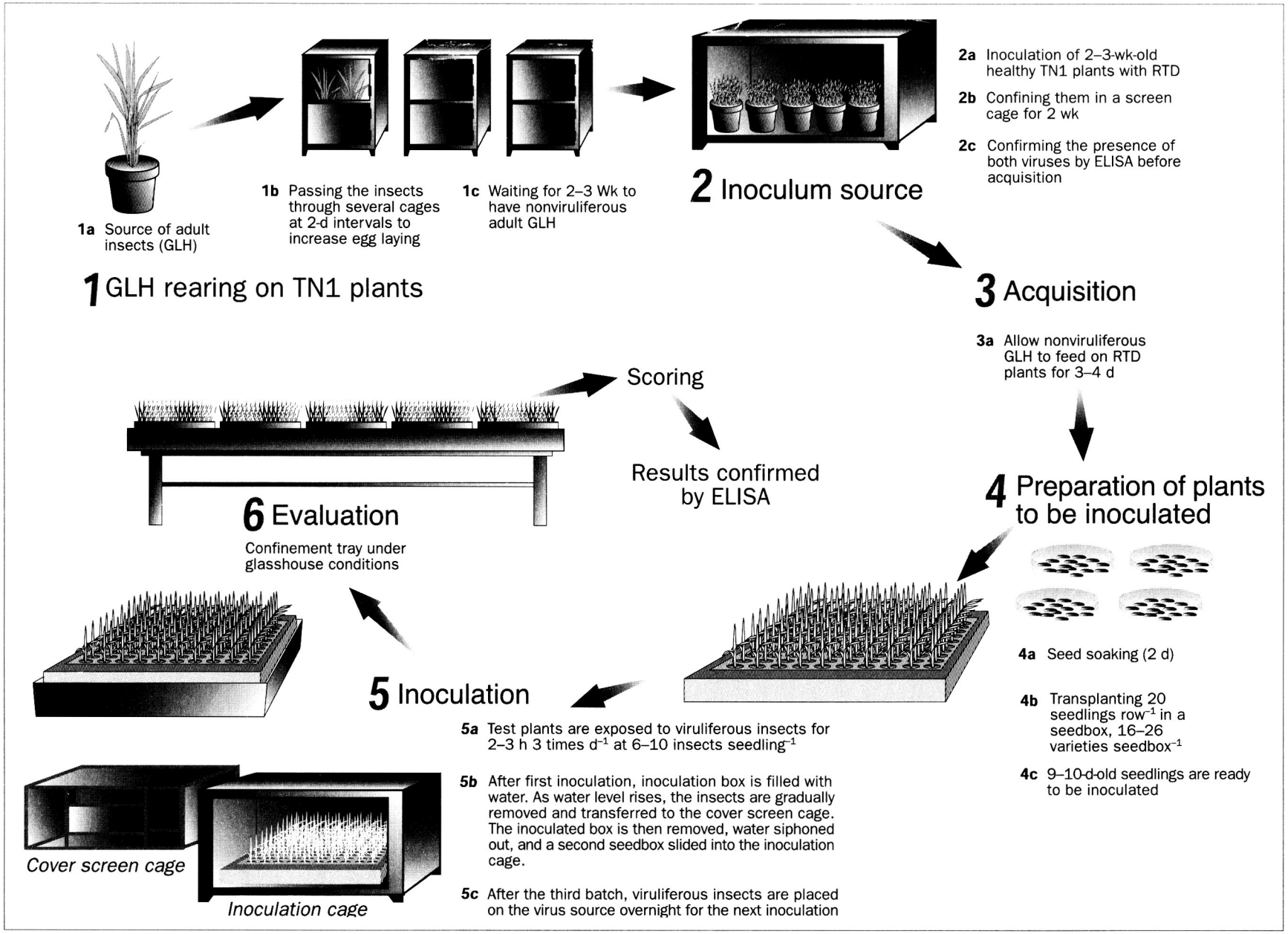

Fig. 2. Protocols involved in the water tray method of mass screening for tungro virus resistance.

the cage. In this way, the GLH reacquire the virus and are used again for inoculation the following day. The same batch of insects may be used for up to 4 d.

Evaluation

h) One month after inoculation, score seedlings individually using the standard evaluation system (SES) for rice (INGER 1996) based on the following scale:

Scale	Description
1	No symptom observed
3	1–10% height reduction, no distinct yellow to yellow-orange leaf discoloration
5	11–30% height reduction, no distinct yellow to yellow-orange leaf discoloration
7	31–50% height reduction, with distinct yellow to yellow-orange leaf discoloration.
9	More than 50% height reduction, with distinct yellow to yellow-orange leaf discoloration

i) Calculate the disease index (DI) for each entry using this formula:

$$DI = \frac{3(A3) + 5(A5) + 7(A7) + 9(A9)}{tn}$$

where A3…A9 = number of plants in scale 3, 5, 7, and 9 and tn = total number of plants inoculated.

The resulting DI can be classified as:

Disease index	Reaction
0–3	Resistant/tolerant
4–6	Moderate
7–9	Susceptible

Determine the percentage of infection for each test entry using the following formula:

$$\% \text{ Inf.} = \frac{\text{number of plants infected}}{\text{number of plants inoculated}}$$

Use the average % infection of two replicates to evaluate the reaction as follows:

0–30%	Resistant
31–60%	Intermediate
61–100%	Susceptible

Data sheet

Shown on the next page is the sample data sheet.

Reevaluation

Those lines that show an average score of 1–3 are subjected to further evaluation (see section on forced-tube inoculation method).

Caged-pot method

In the caged-pot method, 16 pots with 20 seedlings (9–10-d-old) are each confined in a cage with 6–10 viruliferous adult GLH per seedling for a 2.5-h inoculation access period. The pots are then removed from the cage and diseased source plants introduced in the cage for 2.5 h to allow the leafhoppers to reacquire the viruses. The source plants are then removed and a new set of pots placed in the cages for inoculation. Source plants are then reintroduced into the cages and the leafhoppers are allowed to feed on them overnight to reacquire the viruses (Fig. 3). Scoring of inoculated plants is done in the same way as in the water tray method.

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Data Sheet for Mass Screening

Test materials _____

Dates:

- Soaking _____
- Seeding _____
- Acquisition _____
- Inoculation _____
- Reading of infection _____

Code	Test entry	Plant height	No. of plants with symptom score					Disease index	Infection		
			1	3	5	7	9		No. tested	No. infected	% infected
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											
21											
22											
23											
24											
25											
TN1											

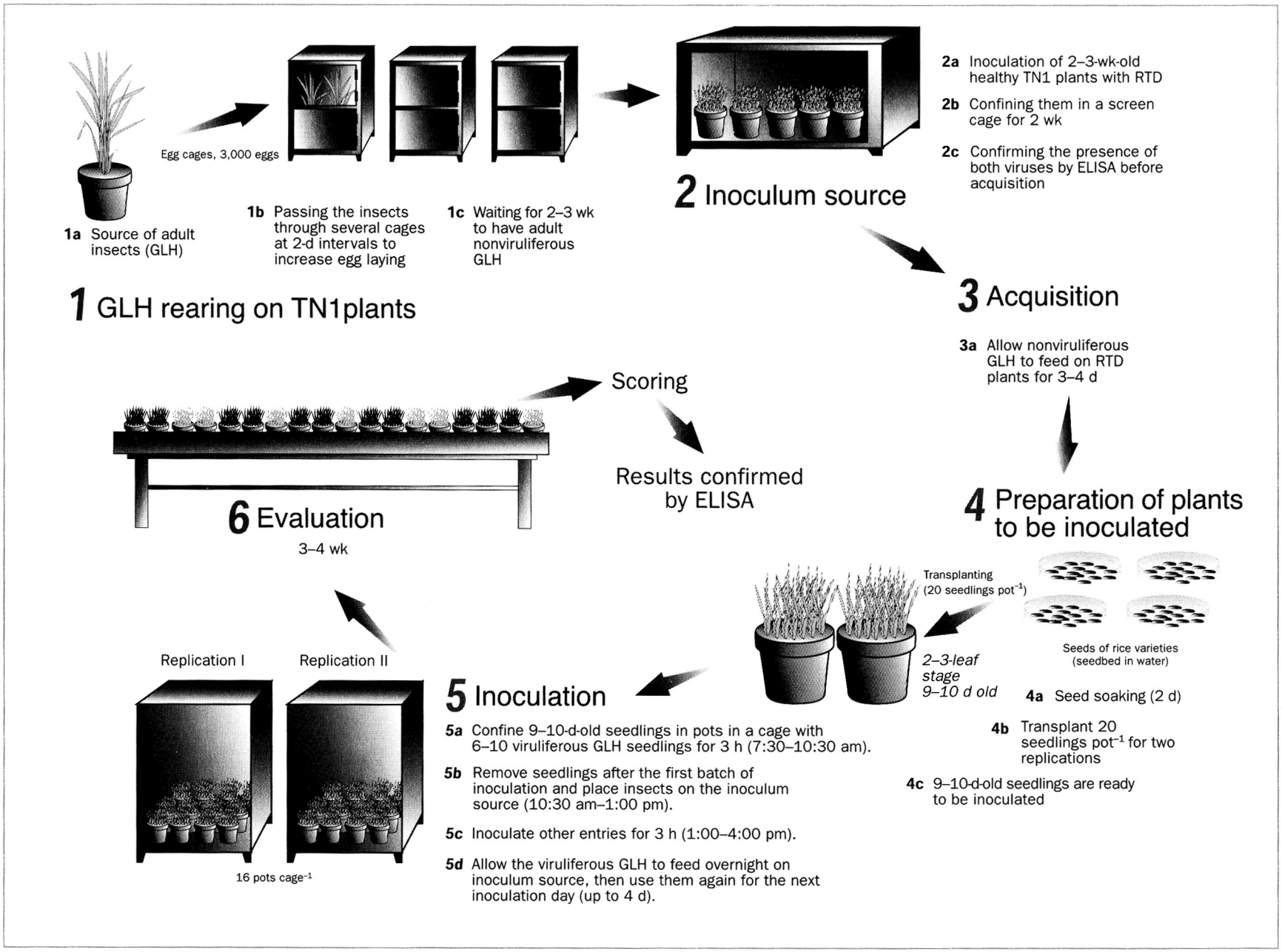

Fig. 3. Protocols involved in the caged-pot method of mass screening for tungro virus resistance.

Forced-tube inoculation

Following initial mass screening of germplasm for tungro resistance by the water tray method, test entries that show a resistant reaction (scores 1–3) are evaluated further by forced-tube inoculation. This test provides a more reliable measure of the resistance status of test entries as insects have no choice but to feed on the single seedling placed in each tube. Shahjahan et al (1991) compared the efficiency of the mass inoculation and forced-tube inoculation methods. In their study, only 1.4% of test seedlings escaped infection with tungro viruses using forced-tube inoculation compared with 17.4% for mass inoculation. The forced-tube inoculation method is described by Hibino et al (1990). The initial test is done using doubly infected (RTBV + RTSV) plants as a disease source. Entries with RTSV infection (tested by serology) of less than 10% will be retested using RTSV alone as a source to further confirm resistance against RTSV.

Materials

Test tubes (18 mm × 150 mm) with cap in test tube racks
Clay or plastic pots
Pot labels
Aspirator
Forceps
Screen cage
TN1 seeds
RTBV + RTSV-infected plants
RTSV-alone-infected plants

Procedure

1. Place a single 5–6-d-old seedling of the test entry in a test tube containing about 0.5 mm water. Use 40 seedlings for each test entry. Use TN1 as the control. Label test entries properly (same label to be used when transplanted in pots).
2. Introduce into each test tube 3 GLH adults that have fed for 4 d on doubly infected TN1 plants. Allow insects to feed overnight.
3. Transplant inoculated seedlings in pots, at the rate of five seedlings per pot, and place pots in a screen cage in the greenhouse. Maintain GLH-free by observing nymphs that emerge 8–10 d after inoculation and spray with selective insecticides.
4. Four weeks after inoculation, score seedlings for disease index based on the standard evaluation system (SES) for rice (INGER 1996) scale of 1–9 (see section on mass screening for tungro resistance by the water tray method). Calculate the disease index score for each entry to be used as the severity index. Low severity scores might be caused by low infection rates or by tolerance for RTBV. Use serological analysis to determine the resistance to RTSV and RTBV.
5. Index seedlings for infection with tungro viruses by DAS-ELISA (see section on detection methods for the procedure to follow).
6. Testing should be done twice for each test entry.
7. The data sheet for recording results is shown on the next page.

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Data Sheet for Forced-Tube Inoculation

Test material _____
Dates:
Soaking _____
Sowing _____
Acquisition _____
Inoculation _____
Reading of infection _____
Sampling _____
ELISA test _____

Code	Test entry	Plants with score					Disease index	ELISA test (no.)			
		1	3	5	7	9		Tested	BS[a]	B	S
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
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29											
30											

[a]BS = RTBV + RTSV, B = RTBV alone, S = RTSV alone.

Screening for resistance to green leafhopper

Conventional seed box test

The conventional seed box test is used to evaluate levels of resistance to rice leaf- and planthoppers. This test may be used to establish resistance to the green leafhopper (GLH), *N. virescens*, as part of a tungro resistance breeding program (Heinrichs et al 1985). It is a rapid method for screening large numbers of entries for qualitative resistance. The method is described below.

Materials

Standard seed boxes (60 cm × 40 cm × 10 cm)
Wooden row marker (to mark 14 rows widthwise)
Row labels
Screen cage
GLH colony

Procedure

1. Place soil in seed box to a level depth of 3 cm. Use the row marker to mark rows. These rows will serve as a sowing guide.
2. Sow 39 test entries in 12-cm rows running the width of the seed box, with 3 entries in each row. Sow 25 seeds of each test entry in the 12-cm row. In the middle row, sow seeds of a resistant and susceptible check. See Figure 4 for the layout.
3. At 7 d after sowing, place the seed boxes in a water pan inside a screened room. Thin seedlings to about 20 per row. Fill the pan with water to a depth of 5 cm.
4. Infest the seedlings with 2nd- and 3rd-instar GLH nymphs at a density of 3 per seedling. A higher density may be used if older seedlings are tested or if the susceptible check is not being damaged as rapidly as usual.
5. Grade the entries for plant damage using the standard evaluation system (SES) for rice scale (INGER 1996) when about 90% of the seedlings of the susceptible check are dead.

Scale	Description
0	No damage
1	Very slight damage
3	First and 2nd leaves yellowing
5	All leaves yellow; pronounced stunting or both
7	More than half the plants are dead; remaining plants wilting; severely stunted
9	All plants dead

Entries should be replicated three times, if possible. In this case, the damage grading is based on the average of the replications (see data sheet).

R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
R	T	T	T	T	T	T	T	T	T	R	T	T	T	T	T	T	T	T	T	R
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
S	T	T	T	T	T	T	T	T	T	S	T	T	T	T	T	T	T	T	T	S
R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	R

Compartment seed box

Fig. 4. Layout of the test materials (T) and resistant (R) and susceptible (S) check varieties in the conventional seed box test for GLH resistance mass screening.

DATA SHEET FOR GLH RESISTANCE MASS SCREENING

Test insect ____________ Date sown ____________ Test material ____________
Tray no. ____________ Date infested ____________ ____________________
Batch no. ____________ Date scored ____________ No. tested ____________

Entry no.	R1	R2	R3	Ave	Entry no.	R1	R2	R3	Ave

References

Heinrichs EA, Medrano FG, Rapusas HR. 1985. Genetic evaluation for insect resistance in rice. Los Baños (Philippines): International Rice Research Institute. p 92-102.

INGER Genetic Resources Center. 1996. Standard evaluation system for rice. 4th edition. Los Baños (Philippines): International Rice Research Institute. 52 p.

Modified seed box test

Some entries rated as susceptible in the conventional seed box test may show moderate field resistance at later stages of plant growth. The modified seed box test was developed to detect this field resistance in the greenhouse. It was primarily designed for evaluating resistance to the brown planthopper, but may also be used for GLH (Velusamy et al 1986). In this test, the seed is sown as for the conventional test, but the seedlings are infested with four 2nd-instar nymphs per seedling at 20 d after sowing.

Reference

Velusamy R, Heinrichs EA, Medrano FG. 1986. Greenhouse techniques to identify field resistance to the brown planthopper, *Nilaparvata lugens* (Stäl) (Homoptera: Delphacidae), in rice cultivars. Crop Protect. 5:328-333.

Population growth and nymphal development studies

Two further tests may be conducted on materials identified as resistant in the seed box tests. Both tests are conducted in the greenhouse and provide a measure of the level of antibiosis in test entries.

1. *Population growth study.* Although this test takes several weeks to complete, it gives the most accurate evaluation of the level of antibiosis in a test entry and provides a guide as to how it will perform in the field (Heinrichs et al 1985). Plants are placed in mylar cages and infested with 5 pairs of 3-d-old *N. virescens* adults. The size of the F_1 generation is recorded 30 d later. Ideally, the tests are carried out on rice plants at different growth stages. For example, plants may be infested at 30, 45, and 60 d after sowing to detect any effects of plant age on resistance.
2. *Nymphal development study.* This test takes less time to conduct than the population growth study. However, since it involves only nymphs and not adults, it may not give an accurate indication of the level of antibiosis in the test entry. The nymphal development study also involves the use of caged plants over a range of different growth stages that are each infested with 10 1st-instar nymphs. Observations of the numbers of surviving nymphs are made daily and the number of insects surviving to the adult stage is recorded. The growth index can be calculated using the formula

$$\text{Growth index} = \frac{\%\text{ adult survival}}{\text{mean nymphal duration (days)}}$$

Reference

Heinrichs EA, Medrano FG, Rapusas HR. 1985. Genetic evaluation for insect resistance in rice. Los Baños (Philippines): International Rice Research Institute. p 114-117.

Field screening for resistance to tungro

Certain characteristics of field screening for tungro resistance need to be considered before deciding on the appropriate methods to use. Numbers of leafhopper vectors and local sources of inoculum vary both within and between seasons. They also vary between locations, an important point when multilocation testing is being conducted. Virus sources can to some extent be manipulated in the field by using spreader rows and introduced sources of inoculum. At some locations, the planting of trials can be timed to coincide with the appearance of the peak vector population as practiced in Cuttack, India (Anjaneyulu et al 1982). In some areas, however, the peak abundance of the vector is difficult to predict.

In designs where only a few plants of each test entry are evaluated, field screening gives a greater chance of disease escape. Leafhoppers can easily move to adjacent lines after encountering rice plants that are not suitable feeding hosts. This problem can be reduced by using designs with small plots, the size of which is determined by the specific objective of the screening and the resources available. In general, plots should be at least 3 m × 3 m in size and replicated if yield data are to be collected. Ideally, larger plots should be used to evaluate the performance of test lines or varieties in relation to secondary plant-to-plant spread of tungro as opposed to primary infection being brought into plots from outside sources.

1. Various methods are used to field-screen large numbers of test entries using rows of test plants flanked by spreader rows to increase disease pressure. The International Rice Tungro Nursery (IRTN) uses a standard methodology that is replicated across several sites in different countries to assess any location differences in reaction to tungro (see next section for details). The "field screening method" described by Anjaneyulu et al (1982) has been used at Cuttack in India for many years. In the Philippines, a modified design is used in the National Cooperative Testing Program (Department of Agriculture, Philippines). The Philippine Department of Agriculture recommends that, where facilities are available, test entries be inoculated using the forced-tube method and subsequently transplanted in the field (Rice Technical Working Group 1998). This provides a strong test of tungro resistance as seedlings are infected at an early growth stage and then exposed to inoculum for potential reinfection (see next section).
2. Small plots of promising test materials identified in initial field screening can be used to provide a more accurate assessment of disease resistance. In this system, the risk of disease escape is reduced. An example of this approach is the "field evaluation trial" described by Anjaneyulu et al (1982) in which 3 m × 3 m-plots of test materials are alternated with similar-sized plots of a susceptible variety. Three infected tillers of a susceptible variety are planted in the middle of each plot.
3. A randomized complete block design with four replications has been used in multilocation trials to evaluate advanced breeding lines for resistance to tungro viruses in India, Indonesia, and the Philippines (Cabunagan et al 1998). The plot size is 8 m × 8 m with a 2-m separation distance between plots. Two to three seedlings per

hill are transplanted at 21 d after sowing at 20 cm × 20 cm-spacing and exposed to natural infection with tungro viruses. At some locations, where disease incidence at the trial site is low, spreader rows of a leafhopper- and tungro-susceptible variety are placed between the four blocks to enhance the potential for disease spread. Tungro-diseased plants collected from nearby fields or from the greenhouse are transplanted at intervals of a few meters along the spreader rows. Care is taken to ensure the even distribution of these source plants along the rows of each block to make sure that each plot within a block is exposed to an equal amount of inoculum.

Plants are assessed for disease symptoms and leaves are sampled for detection of tungro viruses by enzyme-linked immunosorbent assay (ELISA) at 30–35 and 55–60 d after transplanting. Disease recording and leaf sampling are conducted in six quadrants of 4 × 4 hills arranged in a "W" pattern in each plot. Vector leafhoppers are collected using 10 sweeps of a 30-cm-diameter insect net in each plot on the same dates as for disease assessment. In one replicate of each treatment, disease assessment is done on each hill in the whole plot to determine the spatial distribution of diseased plants.

References

Anjaneyulu A, Singh SK, Shenoi MM. 1982. Evaluation of rice varieties for tungro resistance by field screening techniques. Trop. Pest Manage. 28:147-155.

Cabunagan RC, Angeles ER, Tiongco ER, Villareal S, Azzam O, Teng PS, Khush GS, Chancellor TCB, Truong XH, Mancao S, Astika IGN, Muis A, Chowdhury AK, Ganapathy T, Subramanian N. 1998. Multilocation evaluation of promising advanced breeding lines for resistance to rice tungro viruses. Int. Rice Res. Notes 23(1):15-16.

Rice Technical Working Group, National Seed Industry Council, Department of Agriculture, Philippines. 1998. National cooperative testing manual for rice: guidelines and policies. 113 p.

The International Rice Tungro Nursery

The International Rice Tungro Nursery (IRTN) is organized and conducted as an integral part of the International Network for Genetic Evaluation of Rice (INGER) coordinated by IRRI. The IRTN has been designed to evaluate the reaction to rice tungro disease (RTD) in promising breeding lines and varieties from various rice improvement programs over a wide range of environments. The objectives of this nursery are to (1) evaluate the reactions of rice varieties and lines to infection by tungro viruses, (2) encourage the use of tungro-resistant sources within national breeding programs, (3) study the existence of virus strains of tungro in various areas, and (4) identify varieties with durable resistance to the viruses.

Nursery composition

The IRTN test entries include varieties from the breeding programs of IRRI, the International Institute for Tropical Agriculture (IITA), and the West Africa Rice Development Association (WARDA). Included also are the standard resistant (e.g., ARC11554, Acc. 21473, ARC10343, Acc. 12437) and susceptible (e.g., IR22 and TN1) checks in addition to the local resistant and susceptible varieties, along with GLH differentials (ARC11554 and Gam Pai 30-12-15) and some entries with known sources of resistance to the disease.

Nursery management

Greenhouse screening

Resistance to the virus can be assessed in the greenhouse, where factors needed for infection can be manipulated.

Seedling establishment and maintenance

Fifty seeds of each variety are seeded in rows in either trays or pots. Seven to 10 d after sowing, seedlings in excess of 30 are removed.

Inoculation

It is critically important to use high numbers of viruliferous insect vectors during inoculation. Initially, leafhoppers are left to feed on 45–60-d-old infected plants for 2–3 d. Seedlings in trays or pots are then exposed to viruliferous leafhoppers for 1 d at the rate of at least three viruliferous leafhoppers per seedling. The leafhoppers on the seedlings are disturbed several times to ensure even distribution. Entries can be divided into 3 to 4 batches for inoculation.

Scoring based on symptoms

Four weeks after inoculation, seedlings are scored based on visual observation of the symptoms. The susceptible check should have at least 90% infection or a DI of 7–9 at this time to make the results valid. A healthy check should be used as a reference to measure plant height. For field screening, any tall plants or average of 10 healthy-looking plants in a plot should be used as a reference for height reduction. Two trials on the set of entries are desired.

Field screening

Field tests generally select for vector resistance and are not as effective in selecting for virus resistance. The tungro nursery evaluation under field conditions, however, considers the effect of the complete spectrum of epidemiological factors contributing to the field reaction. Where single-season screening is done, planting should

coincide with the location buildup of GLH populations. Inoculated seedlings of highly susceptible local varieties or TN1 should be planted around the test site 2–4 wk before planting the tungro nursery. Each test entry should be planted in two rows of at least 2 m each (see Fig. 5). Two rows of the susceptible local variety or TN1 should be planted alternately with each test entry. A moderately susceptible variety (such as IR8) should be inoculated and planted perpendicular to the test entries to ensure a long-lasting supply of inoculum.

Scoring

Scoring for infection is based on the following symptoms of tungro: yellow to yellow-orange leaves, stunting, and slightly reduced tillering. Scoring should be done at the seedling stage for the greenhouse test and at the tillering to booting stage for field screening.

1. *Greenhouse test.* Since disease incidence and symptom severity in the greenhouse test are generally not uniform, a disease index (DI) can be used as an indicator for virus resistance (INGER 1996). DI can be calculated as

$$DI = \frac{n(3) + n(5) + n(7) + n(9)}{tn}$$

where n(3), n(5), n(7), and n(9) = number of plants showing a reaction on a scale of 3, 5, 7, and 9 and tn = total number of plants scored.

Scale

- 1 = no symptom observed
- 3 = 1–10% height reduction, no distinct yellow to yellow-orange leaf discoloration

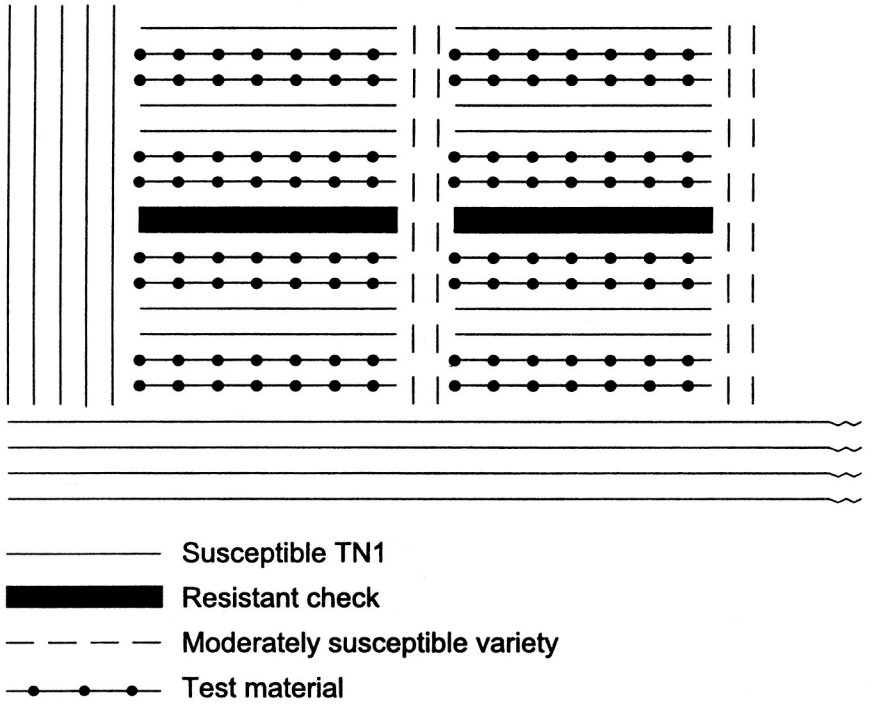

Fig. 5. Suggested layout for field screening against rice tungro disease.

- 5 = 11–30% height reduction, no distinct yellow to yellow-orange leaf discoloration
- 7 = 31–50% height reduction, with distinct yellow to yellow-orange leaf discoloration
- 9 = more than 50% height reduction, with distinct yellow to yellow-orange leaf discoloration

The resulting DI can be classified as

DI	Reaction
0–3	Resistant/tolerant
4–6	Moderate
7–9	Susceptible

For further confirmation, test materials with a DI rating of 0–3 can be tested by forced-tube inoculation using a different number of vectors, at different plant growth stages, and can be assayed serologically to differentiate between virus resistance and tolerance.

2. *Field test.* Reaction to virus infection can be assessed on a scale of 0–9 based on the percentage of infection observed.

Scale
- 0 = no symptom observed
- 1 = 1–10% infection
- 3 = 11–30% infection
- 5 = 31–50% infection
- 7 = 51–70% infection
- 9 = 71–100% infection

Reference

INGER Genetic Resources Center. 1996. Standard evaluation system for rice. 4th edition. Los Baños (Philippines): International Rice Research Institute. 52 p.

Detection methods for rice tungro viruses

Rice tungro disease can be detected by symptomatology, the iodine/starch test, and leafhopper transmission tests (Cabunagan et al 1987). To detect infection with rice tungro viruses, however, serological tests are generally used. For routine detection of RTSV and RTBV, the enzyme-linked immunosorbent assay (ELISA) is the most suitable method. Conventional ELISA is expensive and requires laboratory equipment that is not easily available in many countries. Nevertheless, more laboratories are now developing the capacity to use ELISA and the method is described below.

Some modified serological techniques have been developed for tungro detection (Cabauatan and Koganezawa 1997, Nath et al 1999). Although none of these techniques are widely used currently, work is in progress at IRRI to develop simple and robust modified ELISA screen kits (Azzam et al 1999). The latex agglutination test is described here, along with the newly developed "tungro screen kit B" for RTBV diagnosis.

Enzyme-linked immunosorbent assay: double-antibody sandwich (DAS-ELISA)

Materials and equipment

Microtiter plates, 96-well, flat-bottomed, polystyrene or polyvinyl chloride (e.g., Dynatech Lab, Inc., Immulon 2 Cat # 011-010-3450, Cat # 001-010-2801)
Cover lids for microtiter plates or parafilm membranes
Plastic bags
Micropipette, repeating pipettor (Gilson Pipetman Cat # P-200, Cat P-100, Rainin Instruments Co. Inc., 1715 64th St., Emeryville, CA 94608)
Multichannel pipette that will dispense 0–250 µL (Biohit Cat # 720230, Biohit Oy Verkkosaarenkatu 4, 00580 Helsinki, Finland)
Plate washer or wash bottle (Dynadrop SR 1 Cat # 002-955-1000, Dynatech Laboratories, Inc., 900 Slaters Lane, Alexandria, Virginia 22314)
ELISA reader or spectrophotometer (microplate autoreader, BIOTEK Instruments EL 311)
IgG of specific viruses
Alkaline phosphatase (Sigma P-552)
Conjugated IgG of specific viruses
P-nitrophenyl phosphate substrate (Sigma, phosphatase substrate Cat # 104-105)
Buffer reagents (see preparation of buffers)

Procedure

There are several types of ELISA. DAS-ELISA is the most commonly used type in the detection of rice viruses. The procedure is divided into 5 steps (see Fig. 6 for the schematic presentation).

1. *Sensitizing plate*

a) Dilute IgG to the appropriate concentration in coating buffer. The appropriate concentration should be determined in a preliminary test in which IgG is diluted to 10, 1.0, and 0.1 µL per mL and tested against known antigen samples. Recommended dilutions are listed below.
b) Add 150 µL diluted IgG to each well. Some researchers suggest that outer wells may be unreliable due to charge buildup; if desired, these outer wells can be filled with PBS and not used for samples.

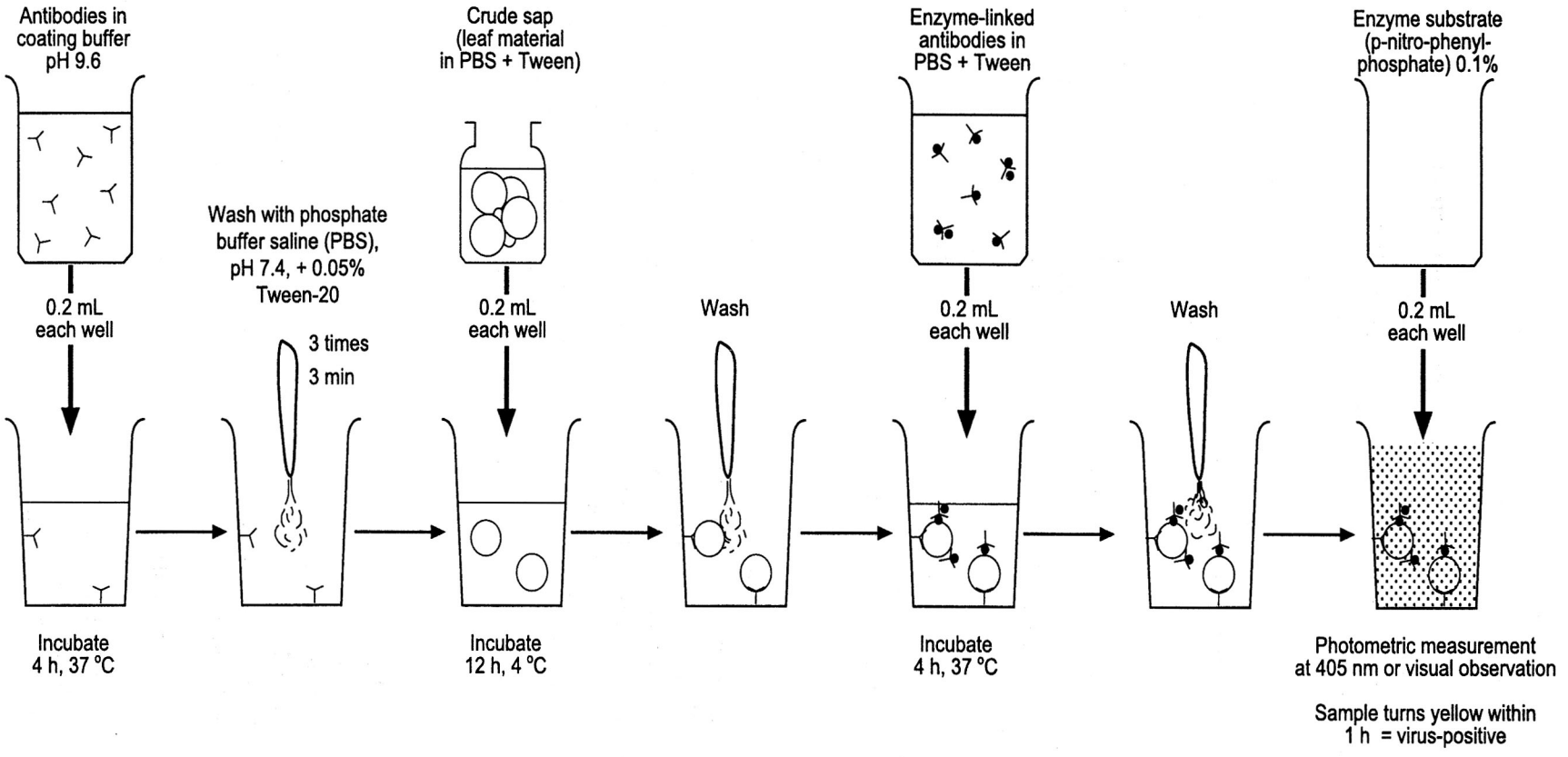

Fig. 6. Protocol for the enzyme-linked immunosorbent assay (ELISA).

c) Cover plate with lid, wrap plate in damp paper towel, put into plastic bag (or cover plate with Saranwrap), then incubate 3–4 h at 37 °C or overnight at 4 °C.
d) Plates can be used at this point, or can be stored frozen for several months (without removing IgG from wells).

2. *Homogenizing samples*
e) Homogenize leaves (0.1 g) in 1 mL of 1X PBS-T with mortar and pestle or appropriate homogenizer.

3. *Adding samples*
f) Empty contents of wells over sink and rinse with PBS-T 5 times. Each time, squirt 1X PBS-T forcibly from a wash bottle into each well (or microplate washer) and let it sit for 3 min between rinsings.
g) Add 150 µL antigen (purified sample or plant tissue ground in PBS-T) to wells. Cover, wrap, and incubate at 4 °C overnight or 3–4 h at 37 °C.

4. *Adding conjugate*
h) Empty contents of well and rinse as in 3.f.
i) Dilute enzyme-labeled antibody (conjugate) appropriately in PBS-T. (Appropriate dilutions should be determined in a preliminary test in which conjugate is diluted to, say, 1:1,000, 1:500, 1:200, 1:50. Recommended dilutions are listed below.)
j) Add 150 µL diluted conjugate to each well. Cover, wrap, and incubate at 4 °C overnight or 3–4 h at 37 °C.

5. *Adding substrate*
k) Empty contents of wells and rinse as in 3.f.
l) Dissolve substrate tablets (or powder) at 1 mg per mL in diethanolamine buffer.
m) Add 150 µL per well and incubate at room temperature for 30 min to 1 h or until reaction takes place. Positive wells will turn bright yellow.
n) Add 50 µL per well of 3 M NaOH to stop reaction.
o) Scan wells at 405 nm in ELISA reader or dilute contents of wells and read in cuvette in a spectrophotometer at 405 nm. Samples with $A_{405} > 3^X$ mean absorbance of control healthy samples can be considered positive.

Buffer preparation

1. *Coating buffer:* 0.05 M sodium carbonate buffer, pH 9.6
Dissolve in 800 mL distilled H_2O:

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g
NaN_3	0.2 g

Adjust pH to 9.6
Adjust volume to 1 L

2. *Substrate buffer:* 10% diethanolamine buffer, pH 9.8

Diethanolamine	100 mL
Distilled H_2O	750 mL
NaN_3	0.2 g

Work under hood
Adjust pH to 9.8 with concentrated HCl
Adjust volume to 1 L

3. *0.02 M phosphate buffered saline + Tween-20, pH 7.4 (PBS-T)*

NaCl (0.15 M)	87.0 g
KH_2PO_4	2.0 g
Na_2HPO_4	29.0 g
NaN_3	2.0 g

Add distilled water to 1 liter. This will be your 10X stock of PBS buffer.

For 1X stock, dilute 100 mL of 10X stock with 900 mL distilled water and add 0.5 mL of Tween-20. This will be your 1X stock of 1X PBS-T buffer.

4. *3 M NaOH*
Dissolve in 1 L distilled H_2O

NaOH	120 g

Note:
1. Recommended dilution for coating using IRRI IgG: 1 µL IgG per 1 mL buffer for both RTBV and RTSV
2. Recommended dilution for IRRI conjugates: 1 µL of conjugated IgG per 1 mL buffer for RTBV, 1.5–2 µL per mL for RTSV

Latex agglutination test

Materials

Partially purified IgG (RTBV, RTSV)
Glass slides and rod with rounded ends
Light microscope
Used microtiter plates

Procedure

In this test, purified antibody is adsorbed to the surface of polystyrene latex particles. When the homologous virus antigen is mixed with suspensions of these sensitized latex particles, clumping of the particles will occur and the aggregates can be seen visually or under a light microscope (see Fig. 7 for the schematic presentation). This reaction is referred to as agglutination.

Before you start this procedure, sensitize latex particles with the desired antibody.

Sensitizing latex particles

1. Mix 14 mL of 0.15 M NaCl and 1 mL latex particles.
2. In another tube, add 40 µL partially purified IgG to 15 mL Tris-HCl.
3. Mix 1 and 2.
4. Incubate for 1 h at room temperature.
5. Centrifuge at 10,000 rpm for 10 min.

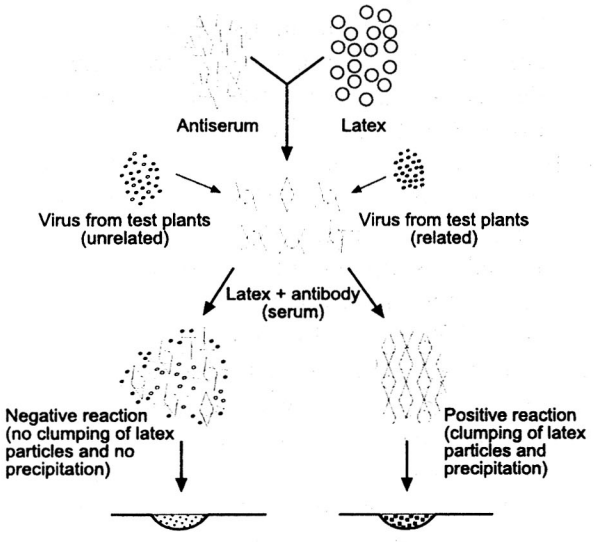

Fig. 7. Protocol for latex agglutination test.

6. Resuspend latex in 30 mL Tris-PVP[1] and centrifuge at 10,000 rpm for 10 min (repeat 3 times).
7. Resuspend latex in 15 mL Tris-PVP.
8. Add 1–5% NaN_3. Store at 4 °C.

Assay

1. Mix 30 µL each of plant sap (diluted 20 to 60^X w/v) and sensitized latex suspension in a small tube or in a well of a used but clean microtiter plate.
2. Vigorously shake the tube or plate for 30–60 min at room temperature.
3. Using a glass rod, place a small amount of the mixture on a glass slide and observe under a light microscope at 100^X magnification. A positive reaction is indicated by the clumping of latex particles and is seen as irregular black spots.

Buffer reagents

1. 0.15 M NaCl (8.77 g per L DH_2O).
2. 0.05 M Tris buffer (6.06 g Trizma base per L DH_2O). Adjust to pH 7.2.
3. Dissolve 0.2 g PVP MW 10,000 to make 1:1 Tris-PVP for washing pellets.
4. Bacto-latex 0.81 (DIFCO Lab).
5. NaN_3.

Tungro screen kit B

This kit was developed for RTBV diagnosis only. This is a simple tissue-print assay, where fresh stems of rice plants are cut transversely and the cut surface immediately "printed" onto a membrane.

Contents of the kit

Membrane (for 5 tests)	7.5 cm × 5 cm
Tube 1: 10x buffer	50 mL
Tube 2: blocking reagent	5 g (1 g per pack)
Tube 3: detection reagent	25 mL
Tube 4: blank for solution I	
Tube 5: blank for solution II	
Tube A: specific antibody	250 µL (50 µL per tube)

[1]PVP = polyvinylpyrrolidone.

Tube B: conjugated antibody	250 µL (50 µL per tube)
One box to perform the test	
Forceps and pipette (one each)	
One razor blade	
One layout paper	
One evaluation sheet	
One instruction sheet	

Procedure

1. Transfer 5 mL of solution from tube 1 to tube 4 and fill tube 4 to 50 mL with distilled water. Cover the tube and invert a few times to mix the solution. Tube 4 is now your solution I.
2. Transfer 20 mL of solution I to tube 5. Open one sachet of blocking reagent and empty contents into tube 5. Cover the tube and shake vigorously to dissolve the blocking agent completely. Tube 5 is now your solution II.
3. Take out one membrane (sandwiched between two blue sheets) and place it on a clean tissue paper or paper towel. Avoid touching the membrane with bare hands; use the forceps provided.
4. Wipe off dirt and water from fresh rice stems with tissue paper. Cut rice stems with the razor blade and press the cut surface gently on the membrane (do this at least twice). Wipe razor blade with tissue paper between each sample. Record the sample number on the supplied layout paper. Repeat the process for all samples.
5. After printing all samples, air-dry the membrane for about 10 min. Transfer the membrane inside the box cover (use as tray). Then pour 10 mL of solution II over the membrane and let stand for 1 h at room temperature.
6. Pipette about 0.5 mL of solution II from the tray and add to tube A. Cap the tube and invert several times to mix well.
7. Pipette all the contents of tube A and transfer back to the tray with the membrane. Shake the tray gently to mix the solution and let stand for 1 h at room temperature. Be sure to rinse the pipette well after each use.
8. Pour off the solution and rinse the membrane 3 times with solution I (use 10 mL each time).
9. Transfer the remaining 10 mL of solution II into the tray with the membrane.
10. Pipette out 0.5 mL of solution II from the tray and add to tube B. Mix well, as in step 6. Pour all the contents of tube B back into the tray, shake gently to mix well, and let stand for 1 h at room temperature.
11. Prepare solution I as in step 1.
12. Pour off the solution from the tray and rinse membrane as in step 8.
13. Completely decant the solution and add just enough (about 5 mL) detection reagent (tube 3) to cover the entire membrane. Cover tray with aluminum foil or put the tray in the dark and incubate for 1–2 h. Positive samples turn purple, whereas the negative ones remain unchanged.
14. Stop the reaction by washing the membrane in plenty of tap water. Dry the membrane and take a reading of the reaction immediately. Store at room temperature for future reference.

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National program on varietal evaluation: the Philippine example

The Philippine Government rice improvement program aims to develop varieties that are high-yielding and resistant to pests and diseases, have good grain quality, and are well adapted to the different rice-growing ecosystems in the country. It takes about 10 years from conception to adoption of a variety: 5 years to develop promising rice lines and another 2–3 years to test them in multilocation yield trials before they are recommended for release. From here on, the seeds of the new variety are increased from breeder seeds to certified seeds before they will be available to farmers. Furthermore, it may take another 2 more years before the new variety becomes adopted (Rice Technical Working Group 1998).

The last stage of evaluation before a promising line is recommended for release as a commercial variety is conducted in the National Cooperative Test (NCT). The promising line is tested nationwide and compared with prevalent commercial varieties as checks. Its yield performance, insect and disease resistance, and grain quality are assessed in the NCT. The Rice Technical Working Group (RTWG) evaluates the results of the NCT and identifies the most promising lines during the semiannual and annual meetings. These lines are submitted to the Technical Secretariat of the National Seed Industry Council (NSIC), which in turn recommends them to the NSIC for approval as new varieties.

Agencies involved

The members of the RTWG come from the agencies participating in the NCT. These include researchers from the primary rice breeding institutions in the Philippines: PhilRice, the University of the Philippines Los Baños, and IRRI. The other members of the RTWG come from the Bureau of Plant Industry and other Department of Agriculture stations, selected agricultural colleges and universities, Philippine nuclear research institutes, and some private individuals or agricultural corporations.

Entries for testing

Entries for testing come from the entities participating in the RTWG. They may be pure lines, hybrids, or multilines. These are accompanied by essential information such as the original source of varieties, parents and cross combinations, type of pollen control (hybrids), filial generation, preliminary yield trial data from the sponsoring agency, disease and insect pest reactions, number of days from sowing to heading, and plant height. The RTWG determines the check variety and entries to be accepted for testing. Promising selections in advanced tests or those recommended for seed increases are entered in on-farm adaptation trials.

Number of entries and test location

Entries are classified according to their intended agroecological environment (ecosystem) such as irrigated lowland (transplanted and direct-seeded rice), drought-prone or flood-prone rainfed lowland, saline-prone, upland, and cool elevated (rice terraces) areas. In addition, there are separate groups for hybrid rice and special-purpose rice. In irrigated lowlands, entries are grouped according to maturity and method of crop establishment. There can be one or more check varieties for each group. The maximum

number for each environment-maturity group is 15 entries.

Tests are conducted in the major rice-producing areas of the country. There are specific sites for different ecosystems, for testing hybrid rice, and for evaluating tolerance for salinity and cold.

Dropping of entries

The RTWG discusses dropping of entries during its regular meetings. As a general rule, entries in the irrigated lowland advanced tests with an average yield of 5% lower than the group check (or group mean) for 2 seasons are discarded. Susceptibility to disease or insect pests, poor grain quality, and poor agronomic characteristics are also used as criteria for dropping entries.

Nominations to the National Seed Industry Council

A selection may be nominated to the Technical Secretariat of the NSIC as a commercial variety for all regions (national recommendation), for specific regions of the country (regional or location-specific recommendation), and for registration.

For a national recommendation, a selection must have satisfied a minimum of three advanced yield tests and at least two seasons of farmers' field trials. For a regional recommendation, evaluation in at least two locations for at least two seasons within a particular region is needed. Recommendations for rainfed lowland, upland, saline, and cool elevated areas are regional in nature, while those for special-purpose rice are for variety registration.

A new selection may be nominated to the Technical Secretariat of the NSIC on the basis of superior yield, better agronomic and grain characteristics, or higher levels of resistance to diseases and insects than existing varieties. Those with average yield of 10% or higher than the group check or the mean may be recommended for approval. A selection not distinctly superior to existing varieties may also be recommended for release as a variety if it carries new genes for resistance against the major diseases and pests or has a different genetic background. Sufficient breeder seed (50 kg) should be available when a selection is recommended for release as a variety.

Recommended selections should have a narrative description and other information based on nursery tests and field observations. These data include grain yield, number of days from sowing to maturity, mature plant height, response to lodging, and reaction to diseases and insects. Information is also included on grain quality data derived from physical tests, physico-chemical evaluation, cooking parameters, and sensory evaluation, and agro-morphological data derived from the distinctness, uniformity, and stability test (DUST).

Naming of varieties

All rice varieties recommended to the NSIC follow the nomenclature approved by the Philippine Seed Board (PSB) in 1990. Each variety is coded PSBRc plus a number. Irrigated and rainfed lowland varieties are assigned even numbers and upland varieties odd numbers. Furthermore, a local name is assigned to each variety. Irrigated lowland and rainfed lowland varieties are given popular names of rivers and lakes and upland varieties are given names of mountains. "Stop-gap" varieties retain their breeding line numbers. Traditional and farm varieties retain their local name.

Seed production

PhilRice maintains breeder seeds of all PSB-approved rice varieties. These breeder seeds are increased to foundation seed in quantities adequate to meet the national requirement. Foundation seeds of appropriate varieties are allocated to different stations, selected member agricultural colleges and universities, and qualified seed producers to produce registered seeds.

For popularly planted rice varieties, the breeding institution maintains at least 50 kg of breeder seed and makes it available to PhilRice upon request.

Reference

Rice Technical Working Group, National Seed Industry Council, Department of Agriculture, Philippines. 1998. National cooperative testing manual for rice: guidelines and policies. 113 p.

National Cooperative Test protocol for screening for tungro resistance

Greenhouse screening

Steps	Key points
1. Prepare test plants	• Sow seeds of test entries and susceptible check IR64 at random in a compartment seed box. • Thin out seedlings into 10–15 per entry 5–6 d later.
2. Virus acquisition by green leafhoppers (GLH)	• Collect adult GLH and allow to feed on infected plants for 4 d.
3. Inoculation	• Release the viruliferous GLH into the aluminum screen-covered seed box at 3–4 hoppers per seedling. • Cover with black cloth to prevent attraction of insects to light. Allow 8–10-h inoculation feeding. • When 1–2 GLH per seedling are used, allow 24-h inoculation feeding.
4. Transplant inoculated seedlings	• Remove/retrieve the vectors. Transplant the inoculated seedlings in lowland plot.
5. Evaluation	• Evaluate at 3–4 wk after inoculation and determine percentage infection.

Symptoms

Stunted plants with mottled and yellow-orange leaves.

$$\text{Percent infection} = \frac{\text{No. of infected seedlings}}{\text{Total no. of seedlings}} \times 100$$

Index 1: 0–20% infection—resistant
Index 2: 21–40% infection—intermediate
Index 3: 41–100% infection—susceptible

Reference

Rice Technical Working Group, National Seed Industry Council, Department of Agriculture, Philippines. 1998. National cooperative testing manual for rice: guidelines and policies. 113 p.

Modified field screening

At some test sites, a susceptible variety and tungro-diseased plants are planted in spreader rows to increase disease pressure.

Steps	Key points
1. Prepare test plot	• Transplant susceptible variety (IR64) in spreader rows. • Collect and transplant tungro-infected plants within the spreader row. • If the station is capable of producing artificially inoculated plants, transplant them 1 mo before sowing the test entries.
2. Prepare test plants	• Follow the wet-bed method of raising seedlings. Transplant test entry in a row of 20 hills spaced 20 cm × 20 cm at one seedling per hill. Plant S-check (IR64) for every row of the test entry.
3. Evaluate	• Score at 45 and 60 d after transplanting. • Evaluate disease reaction by taking the percent infection.

$$\text{Percent infection} = \frac{\text{No. of infected plants}}{\text{Total no. of plants}} \times 100$$

Index 1: 0–25% infection—resistant
Index 2: 26–50% infection—intermediate
Index 3: 51–100% infection—susceptible

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