J.Natn.Sci.Foundation Sri Lanka 2013 **41**(2):147-154

RESEARCH ARTICLE

Antixenosis and antibiosis effects of *Oryza nivara* accessions harbouring bph2 gene on brown planthopper [*Nilaparvata lugens* (Stal)]

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Revised: 05 February 2013; Accepted: 13 March 2013

Abstract: Gene bph2 is one of the 21 brown planthopper (BPH), *Nilaparvata lugens* (Stal) resistant genes identified so far in several *indica* cultivars and wild relatives. The present study evaluated *Oryza nivara* accessions collected from different locations in Sri Lanka for bph2 and, antibiosis and antixenosis effects of *O. nivara* accessions harbouring bph2 gene on BPH. The five *O. nivara* accessions; WRAC 02, WRAC 04, WRAC 07, WRAC 21 and WRAC 25 amplified the resistant band (300 bp) indicating the presence of the bph2 gene in their genomes. The level of resistance observed in these accessions was high and showed negative effects on honeydew production and nymphal survival (antibiosis) and revealed antixenosis action against nymphs and oviposition. In addition, dynamic response mechanisms of BPH against *O. nivara* accessions that possess bph2 was found, which may be indicative of the complex structure of genetics of resistance of these accessions. Substantial level of resistance similar to that in Ptb 33, observed in *O. nivara* accessions WRAC 02, WRAC 04 gives a clue to the possible existence of polygenic resistance as detected in Ptb 33. Results of this study highlighted the possibility of the use of *O. nivara* accessions that possess bph2 gene in their genome as a potential source of resistance in pyramiding of genes for BPH resistance to avoid the genetic uniformity of BPH resistant rice varieties cultivated in Sri Lanka in the future.

Keywords: Antibiosis, antixenosis, bph2, brown planthopper, *Oryza nivara.*

INTRODUCTION

The brown planthopper (BPH), *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae) is one of the most destructive monophagous insect pests of rice throughout the rice growing countries in Asia. The BPH damages the rice plant directly by sucking the phloem sap and plugging the xylem and phloem with their feeding sheaths, which causes serious yield reductions (Sogawa & Cheng, 1977). BPH also serves as a vector for transmission of viruses that cause grassy stunt (Rivera *et al*., 1966) and ragged stunt (Ling *et al*., 1978) viral diseases in rice. Heavy dependence on chemical pesticides for the control of this pest leads to many adverse effects like harmful effects on natural enemies, development of resistant biotypes, environmental pollution and high cost of production (Kudagamage & Nugaliyadda, 1995). It has been reported that host plant resistance is the most economic, least complicated and environmentally friendly approach for protecting crops against insect pest damage (Pathak & Kush, 1979).

The presence of host plant resistance against BPH in rice was first reported by Pathak *et al*. (1969). It has been reported that the resistant varieties suppressed the weight gain of nymphs and maintained low BPH populations across multiple generations in a large production area (Cohen *et al*., 1997; Jung & Im, 2005). Therefore, the use of varietal resistance to manage this pest is considered as an important approach in integrated pest management. In general, resistant rice plants exhibit two strategies against BPH: antixenosis and antibiosis. Antixenosis affects insect settling, colonization or oviposition and antibiosis reduces insect feeding, growth rate or survival (Alarn & Cohen, 1998).

According to Rahman *et al*. (2009), 21 BPH resistant genes have already been identified and characterized in

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traditional rice cultivars and wild rice species. Out of 22 wild rice species, BPH resistance has been detected only in a few species, i.e. *O. officinalis* (Hirabayashi *et al.,* 1998), *O. australiensis* (Ishii *et al.,* 1994), *O. eichingeri* (Liu *et al.,* 2001), *O. minuta* (Rahman *et al.,* 2009) and *O. latifolia* (Yang *et al.,* 2002). However, due to undesirable agro-morphological traits associated with the BPH resistance in these wild relatives, transferring the resistance to high yielding varieties through conventional breeding techniques is not easy.

Out of the five wild relatives of rice, *O. nivara* is the most common and widely distributed species found in Sri Lanka (Hemachandra *et al.,* 2010a). In a previous study (Madurangi *et al*., 2011) it was reported that the high level of resistance observed in *O. nivara* accession (WRAC 04) collected from Otththapahuwa in the Anuradhapura District is mainly due to the presence of the bph2 gene in its genome. WRAC 04 accession has been already used to develop BPH resistant lines (Hemachandra *et al.*, 2010b) at the Rice Research and Development Institute (RRDI), Bathalagoda. The results of wide hybridization tests have proved that it can be incorporated into *O. sativa.* Lines derived from the cross between WRAC 04 (male parent) and Bg 380 (female parent-susceptible check) were resistant to BPH populations available in Sri Lanka (Hemachandra *et al.*, 2010b). These findings highlight the importance of screening the available *O. nivara* germplasm with a similar genome to the cultivated rice, *O. sativa* against BPH resistance in order to identify potential donors towards developing rice varieties having broadbased and durable resistance to BPH.

Gene bph2 is one of the 21 BPH resistant genes identified so far in several *indica* cultivars. It was originally identified as a recessive gene in an *indica* breeding line, Karsamba Red ADS7 (Athwal *et al*., 1971) and mapped on the long arm of chromosome 12 (Murata *et al*., 1998). Khush (1979) reported that the high level of resistance observed in Ptb 33 was due to the combination of two different resistant genes, bph2 and Bph3.

The introduction of varietal resistance to manage the BPH problem in Sri Lanka commenced with the recommendation of the first resistant variety, Bg 379/2 (variety developed using Ptb 33) in 1980 (Kudagamage & Nugaliyadda, 1982). Since then, several resistant donors have been used in developing BPH resistant rice varieties. However, only the resistance of Ptb 33 has been successfully incorporated into high yielding varieties (Kudagamage & Nugaliyadda, 1995) and as such bph2 and Bph3 genes play a major role in BPH management in Sri Lanka.

For incorporation of *O. nivara* in the rice improvement programme, it is essential to evaluate its resistance level and illustrate the biological effects, i.e. antibiosis and antixenosis on BPH. Therefore, the present study was aimed at screening the *O. nivara* genome against KAM 4 primer to detect the accessions harbouring bph2 gene and to illustrate the antibiosis and antixenosis effects of positive accessions on BPH, in order to assess the potential for using them in rice varietal improvement (gene pyramiding) in Sri Lanka.

METHODS AND MATERIALS

Seventeen *O. nivara* accessions collected from different locations in Sri Lanka and maintained by RRDI, Bathalagoda, and check varieties; Ptb 33 (resistant check), Bg 380 (susceptible check) and Bg 379/2 (moderately resistant check) were used for the molecular study (Table 1). Accessions selected from the molecular screening were used in the bioassay tests. The BPH culture maintained at RRDI, Bathalagoda on susceptible rice

Table 1: Details of *O. nivara* accessions used in the study

No.	Accession No.	Collected location	District
1	WRAC 01	Bulunawewa	Matale
2	WRAC 02	Pelbediyawa	Matale
3	WRAC 04	Otththapahuwa	Anuradhapura
4	WRAC 07	Ilakkttuwa	Puttlam
5	WRAC 11	Kabaraya wewa, Poonewa	Anuradhapura
6	WRAC 12	Sangilikulama wewa	Anuradhapura
7	WRAC 14	Yakadapotha	Kurunegala
8	WRAC 19	Ihalawewa	Anuradhapura
9	WRAC 21	Inamaluwa	Matale
10	WRAC 22	Rangirigama	Matale
11	WRAC 24	A-9 road 105 km post	Anuradhapura
12	WRAC 25	A-9 road 105 km post	Anuradhapura
13	WRAC 35	Olugaskade tank	Anuradhapura
14	WRAC 41	Paranagama	Matale
15	WRAC 46	Mahiyanganaya	Badulla
16	WRAC 62	Meegahawewa, Weerawewa	Anuradhapura
17	9864	Matara	Matara

variety Bg 380 for many years and subsequently cultured on Bg 380 for a year in a planthouse at the Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna was used for the bioassay tests.

Molecular screening

DNA was extracted from healthy tender leaves of test accessions and varieties using the protocol described by Anushka *et al.* (2008). The DNA extracted from test plants were screened for the presence or absence of the bph2 gene by PCR amplification using the STS marker, KAM 4 (F- 5' TAACTGGTGTTAGTGCGAATGC 3', R- 5' AATTCACGGCATGTGAAGCCCTAG 3'), which shows complete co-segregation with bph2.

PCR was performed following the procedure described by Murai *et al*. (2001). The PCR mixture contained 50 – 100 ng template DNA, 10 pmol of each primers, 0.2 mM dNTP's each, 1X buffer (20 mM Tris pH 8.0 including 2.5 mM $MgCl_2$) and 0.2 units of Taq polymerase in a total volume of 15 µL. PCR was performed using Master Cycler Gradient. PCR mixtures were initially denatured at 94 ^{0}C for 5 min followed by 30 cycles of PCR amplification using the following parameters: denaturation of 94 ^{0}C (30 s), primer annealing at 66 ^{0}C (1 min) and primer extension at 72 ^{0}C (1 min) with final extension at 72 ^{0}C for 10 min. PCR products were electrophoresed in 1.4 % agarose gel to separate the DNA fragments. The gel was subsequently stained by ethidium bromide and the amplified DNA fragments were visualized under UV light. The screening was repeated twice.

BPH bioassays

Evaluation of BPH resistance of O. nivara accessions

Evaluation of BPH resistance of test plants was performed following the conventional seed box test described by Heinrichs *et al*. (1985). *O. nivara* accessions selected through molecular screening (harbouring bph2 gene) and the check varieties were sown in a galvanized tray (60 × 40 × 10 cm) filled with sterilized soil up to a depth of 5 cm. The seeds were sown in 40 cm long-rows and spaced 5 cm between the rows. When the seedlings were 7 d old (at 3 leaf stage), they were infested with 2nd instar nymphs of BPH at the rate of 3 nymphs/seedling. The test was carried out with 4 replicates in a completely randomized design (CRD). The standard evaluation system for rice, developed by the International Rice Research Institute (IRRI), Laguna, Philippines in 1988 was used to score the BPH damage. Accordingly BPH damage was rated by a 0 to 9 scale. The damage score was recorded when about 90 % of the susceptible check (Bg 380) seedlings were dead. The screening was repeated twice.

Antixenosis resistance

***Estimation of non preference of BPH nymphs to settle on test seedlings*:** Antixenosis of *O. nivara* accessions carrying the bph2 gene against settling of BPH nymphs was assessed using conventional seed box test described by Heinrichs *et al*. (1985). Ten days after sowing of selected accessions, approximately 250 second instar BPH nymphs were released on to the seedlings. The number of nymphs on each seedling was counted at 12, 24, 48 and 72 h after infestation. The seedlings were disturbed after each count for re-orientation of nymphs on seedlings. Evaluation was performed following CRD with 4 replicates and repeated twice.

***Oviposition*:** Antixenosis of test plants against oviposition was assessed by the procedure described using Heinrichs *et al*. (1985). Single seedlings from 6 rice accessions were transplanted in a circular fashion about 2 - 3 cm from the edge of 20 cm clay pots as shown in Table 2. To create free choice conditions, 3 check varieties, namely Ptb 33, Bg 379/2 and Bg 380, were included in each arrangement while the *O. nivara* accessions were selected randomly for the test with the accessions that showed positive results for the bph2 gene. Five week-old plants were prepared for the test by removing all leaves while keeping the stem part up to 20 – 25 cm height. Plants were then infested with 10 gravid brachypterous BPH females and covered using mylar cages. The insects were allowed to oviposit on any variety they chose for 48 h and subsequently removed from the plants. Leaf sheaths of tested accessions were observed for determination of the

Table 2: Planting arrangement of test plants for oviposition assessment

Arrangement No.	Test plants
1	Ptb 33, WRAC 01, Bg 380, WRAC 21, Bg 379/2, 9864
2	Ptb 33, WRAC 35, Bg 380,WRAC 22, Bg 379/2, WRAC 25
3	Ptb 33, WRAC 24, Bg 380, WRAC 14, Bg 379/2, WRAC 07
4	Ptb 33, WRAC 12, Bg 380, WRAC 02, Bg 379/2, WRAC 04

number of eggs laid in each accession by observing under the light microscope (10 × 5). Preference for egg laying was assessed by following CRD and each arrangement was replicated 4 times and repeated twice.

Antibiosis resistance

***Evaluation of resistance to feeding by BPH*:** Reaction of BPH to host plants and the plant resistance to feeding by BPH were determined based on honeydew production. The *O. nivara* accessions with bph2 and the check varieties were grown singly in 15 cm clay pots and managed free of BPH infestations. Screening for resistance was performed when they reached the age of two months. A Whatman no. 2 filter paper stained with the pigment bromocresol green (2 mg/1 mL of 70 % ethanol) was placed around the base of the feeding chamber before infesting with BPH. A pair of BPH females previously starved for 4 h were placed in the feeding chamber and allowed to feed for 24 h. Honeydew excreted by BPH was absorbed by the filter paper and appeared as a blue spot. Area of the spot was proportional to the honeydew production and therefore to the amount of BPH feeding (Heinrichs *et al*., 1985). Screening was conducted following the randomized complete block design (RCBD) with 4 replicates and was repeated twice.

Evaluation of survival of nymphs on test accessions

Ten-day old seedlings of the test accessions were placed singly in test tubes (diameter = 2 cm) containing distilled water and each seedling was infested with 10 newly emerged first instar BPH nymphs. The test tubes were loosely plugged with cotton wool to prevent the insects from escaping. The seedling host was changed every 2 d and a count on the number of nymphs surviving was recorded until they became adults. Antibiosis in terms of nymphs survived on test accessions was measured following the CRD with 4 replicates and repeated twice.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means were separated by Duncan multiple range test (DMRT) using SAS 6.12 version (SAS Institute, 1998).

RESULTS

Molecular screening

PCR amplification with KAM 4 primer gave rise to products of 300 bp only with WRAC 02, WRAC 04, WRAC 07, WRAC 21, WRAC 25 and Ptb 33 (Figure 1) indicating the presence of resistance gene bph2 in their genomes.

Damage scores obtained by test accessions/varieties

Bioassay by conventional seed box screening test revealed low damage scores with *O. nivara* accessions (score: 2-3) and Ptb 33 (score: 2.5) that showed positive results for KAM 04 primer. This indicates the presence of high level of resistance to BPH in *O. nivara* accessions harbouring bph2, which is similar to that of Ptb 33 (Figure 2).

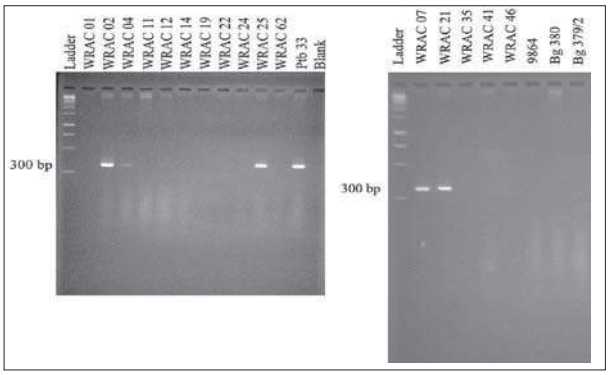

Figure 1: PCR amplification of DNA of the tested rice entries by KAM 4 primer followed by electrophoresis in 3 % agarose gel

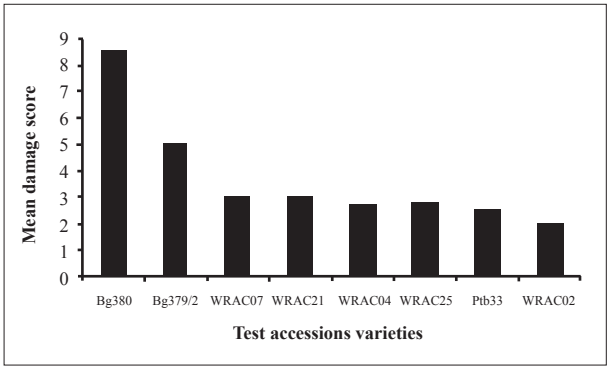

Figure 2: Variation of mean damage scores of test accessions caused by BPH

Antixenosis of *O. nivara* accessions to BPH nymphs and oviposition

Under free choice conditions, the settling response of BPH nymphs on test accessions and varieties was

significantly different ($\alpha = 0.05$). The numbers of BPH nymphs observed on Ptb 33 and *O. nivara* accessions were significantly lower than on Bg 380, the highly susceptible variety to the BPH. Also, the mean number of BPH nymphs observed on Bg 380 and Bg 379/2 significantly increased with time. However, a significant difference of insect numbers on *O. nivara* accessions was not visible during the experiment period (Table 3). These results indicate that all *O. nivara* accessions including Ptb 33 that possess the bph2 gene in their genome have antixenotic characteristics that deter or reduce the BPH nymphs colonizing on them.

Table 3: Antixenosis of BPH nymphs to settle on test accessions

Test accession / variety	Mean no. of nymphs/seedling after different times of infestation (± SD)			
	12 h	24 h	48 h	72 h
WRAC 02	2.5 (± 0.58) ^{b}	4 (± o) ^{b}	3.25 (± 0.96) ^{bc}	4.5 (± 0.58) ^{c}
WRAC 04	1.5 (± 0.58) ^{b}	1.5 (± 0.58) ^{e}	2.25 (± 0.5) ^{cd}	2.5 (± 0.58) ^{d}
WRAC 07	2.25 (± 0.96) ^{b}	1.5 (± 0.58) ^{e}	1.5 (± 0.58) ^{d}	1.5 (± 0.58) ^{e}
WRAC 21	2 (± 1.15) ^{b}	2.5 (± 0.58) ^{d}	3 (± 0) ^{bc}	2.5 (± 0.58) ^{d}
WRAC 25	2.25 (± 0.5) ^{b}	3.25 (± 0.5) ^{c}	2.25 (± 0.5) ^{cd}	1.25 (± 0.5) ^{e}
Ptb 33	1.75 (± 0.5) ^{b}	2.5 (± 0.58) ^{d}	2.5 (± 0.58) ^{cd}	2.5 (± 0.58) ^{d}
Bg 379/2	2.5 (± 0.58) ^{b}	3 (± 0) ^{cd}	4 (± 0) ^{b}	6.5 (± 0.58) ^{b}
Bg 380	9.5 (± 0.58) ^{a}	11 (± 0) ^{a}	12.5 (± 1.73) ^{a}	14.5 (± 0.58) ^{a}

Note: Means with same letter of the same column are not significantly different at 5 % level of significance (DMRT).

Under free choice conditions, the number of eggs laid by BPH in six different test plants was significantly different in each arrangement ($p < 0.05$). The presence of a large number of eggs in the stem is an indication of susceptibility as BPH causes considerable damage to rice plants, not only by feeding but also due to excessive oviposition. In all arrangements, significantly higher and average number of eggs were identified in stems of Bg 380 (variety highly susceptible to the BPH) and Bg 379/2 (variety moderately resistant to the BPH), respectively, which was indicative of their level of susceptibility for egg laying over *O. nivara* accessions except WRAC 22 and WRAC 25. In contrast, the least number of eggs were detected in the stems of Ptb 33, WRAC 04, WRAC 02, WRAC 01 and WRAC 35 indicating low preference for egg laying than in the others (Figure 3-A, B, C & D). However, the number of eggs observed in *O. nivara* accessions harbouring the bph2 gene was significantly lower with the exception of WRAC 25, which showed a difference in antixenosis action against BPH females (Figure 3-B).

Effects on honeydew production of adult BPH females

Honeydew production of BPH females on Bg 380 was significantly higher than all the other test accessions and varieties. Bg 379/2, the variety moderately resistant to the BPH produced moderate amounts of honeydew, which was significantly higher than the honeydew production in all *O. nivara* accessions tested. There was also a significant variability of honeydew produced on *O. nivara* accessions (Table 4). These results indicate that *O. nivara* accessions WRAC 21 and WRAC 02 had significant negative effects on feeding behaviour of BPH females over other test accessions with the bph2 gene in their genomes.

Effects on survival of nymphs

There was a significant difference in survival rates from the first instar nymphs to adults among test accessions and varieties ($p < 0.05$). Significantly higher survival

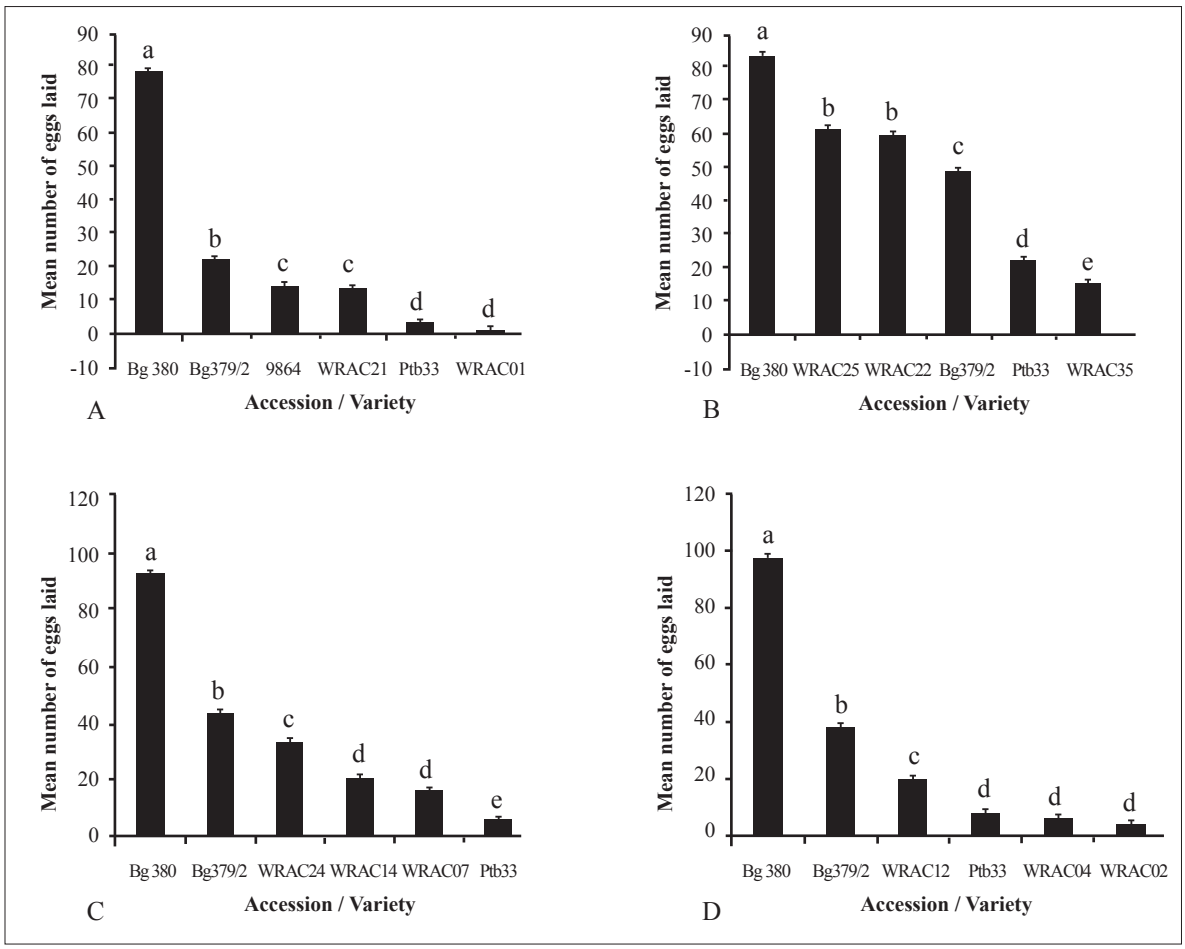

Figure 3: Mean number of eggs laid by BPH in test accessions; different graphs are used to represent the number of eggs laid by BPH females under different arrangements. (A) 1st arrangement; (B) 2nd arrangement; (C) 3rd arrangement; (D) 4th arrangement. Error bars indicate standard errors. Bars labeled with the same letter are not significantly different at 5 % level of significance (DMRT).

Table 4: Honeydew production of BPH females on different test plants

Accession / variety	Mean spot area (mm^2)(± SD)
Bg 380	395.5 (± 5.26) a
Bg 379/2	121 (± 2.58) b
WRAC 07	58.5 (± 2.52) c
WRAC 04	50.25 (± 1.26) d
WRAC 25	27.25 (± 2.22) e
Ptb 33	11.5 (± 1.91) f
WRAC 02	2.5 (± 1.8) g
WRAC 21	1.25 (± 1.5) g

Note: Means with the same letter are not significantly different at 5 % level of significance (DMRT).

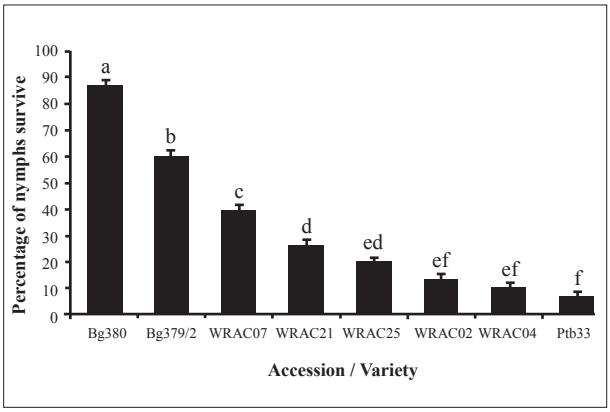

Figure 4: Survival of BPH nymphs fed on different test accessions. Error bars indicate standard errors. Bars labeled with the same letter are not significantly different at 5 % level of significance (DMRT).

rate (87 %) was observed in Bg 380, while over 50 % of adult emergence was observed in BPH nymphs fed on Bg 379/2 as an indication of their level of susceptibility. Among the tested *O. nivara* accessions, WRAC 04 and WRAC 02 showed the least adult emergence, which was not significantly different from that of Ptb 33. However, a significant variation was found in BPH nymphs that survive on *O. nivara* accessions harbouring the bph2 gene with WRAC 07 that showed a significantly higher survival rate (40 %) than in the others (Figure 4).

DISCUSSION AND CONCLUSION

Out of the seventeen *O. nivara* accessions tested, molecular screening revealed positive results with WRAC 02, WRAC 04, WRAC 07, WRAC 21 and WRAC 25 accessions indicating the existence of bph2 gene in their genomes. This work also confirmed the presence of the bph2 gene in the genome of *O. nivara* accession WRAC 04 as the key cause for the resistance (Madurangi *et al.,* 2011). As previously reported (Madurangi *et al.,* 2011), the variety Bg 379/2 developed by RRDI, Bathalagoda using Ptb 33 as a source of resistance failed to confirm the presence of the bph2 gene in its genome.

Bioassay with conventional seed box screening test revealed that *O. nivara* accessions harbouring the bph2 gene survived after BPH infestation at the seedling stage showing high resistance to the BPH. The results of antixenosis and antibiosis effects examined in this study revealed that all *O. nivara* accessions possessing the bph2 gene had substantial levels of antixenosis and antibiosis effects on BPH.

Low honeydew excretion by BPH females and low percentage survival of BPH nymphs indicated the existence of an antibiosis as the mechanism of BPH resistance in *O. nivara* accessions possessing the bph2 gene. It is possible that these accessions lack essential nutrients, which are required for insect survival. Alternatively, these accessions may possess mechanisms or factors, which prevent ingestion of the required quantity of nutrients from a given plant. Also, the reluctance to settle on *O. nivara* and the low number of eggs detected in the stems indicate either the presence of a repellent or the lack of necessary attractant for orientation on the plant.

The observed variations in antixenosis and antibiosis effects of *O. nivara* accessions that possess the bph2 gene may indicate the dynamic response mechanisms of BPH against *O. nivara* and the complex structure of genetics of the resistance of these accessions, which are still unknown. Also, substantial levels of resistance, antixenosis and antibiosis effects observed in *O. nivara* accessions WRAC 02 and WRAC 04, which are similar to Ptb 33 provide a clue to the existence of polygenic resistance in these accessions as detected in Ptb 33 (Khush, 1979). Hence, the results of this study indicated the possible use of *O. nivara* accessions harbouring the bph2 gene as a potential source of resistance and its possible use in pyramiding of genes for BPH resistance to avoid the genetic uniformity of BPH resistance in rice varieties cultivated in Sri Lanka in the future.

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