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Evaluation of Protocol for Assessing the Reaction of Rice and Wheat Germplasm to Infection by *Meloidogyne graminicola*

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Abstract: Root-knot nematode (*Meloidogyne graminicola*), an important and widespread pathogen, causes high yield losses in rice with limited information on wheat and on efficient management. Absence of uniform screening protocols is contributing to slow progress of host resistance development. To develop an efficient screening protocol, several greenhouse studies were conducted, and effects of incubation period, inoculum level, inoculation method, seedling age, and their interactions on root-galling severity (RGS) ratings and reproductive factor (RF) values of *M. graminicola* were determined. At 2 eggs/cm^3 soil, significantly lower RGS but higher RF values were observed at 60 days than at 45 days of incubation. *Meloidogyne graminicola* reproduced six times more on rice than on wheat where the RGS index in both crops increased steadily with increasing inoculum levels, but RF increased at lower levels and decreased beyond a maximum at medium inoculum levels. Inoculum level, container size, seedling age, inoculation method, and their interactions impacted nematode infection and reproduction. The protocol was verified on eleven rice germplasm lines and seven wheat cultivars using the resistance index (RI) calculated from RGS and RF, to screen rice and wheat germplasm.

Key words: Meloidogyne graminicola, screening protocol, rice and wheat.

In recent years, the productivity of rice and wheat in southeast Asia has become stagnant. In extensive surveys and research in the Gangetic plains, soilborne pathogens and deficient root-health have been documented as the major constraints on health and productivity of rice-wheat systems (J.M. Duxbury, unpublished). The root-knot nematode (*Meloidogyne graminicola,* Golden and Birchfield, 1965) is widely distributed, and is considered a serious soilborne pathogen reducing the productivity of the rice-wheat system in S.E. Asia (J.M. Duxbury, unpublished; Padgham et al., 2004).

In field tests in Nepal, solarization of soils infested with root-knot nematodes increased rice yields by more than 30% (J.M. Duxbury, unpublished). Application of the nematicide carbofuran to infested fields increased rice yield by 16 - 31% (Padgham et al., 2004). In greenhouse tests, the yield losses caused by this nematode were much higher (31-97%), depending upon the initial inoculum level (Sharma-Poudyal et al., 2005). Applications of nematicides or use of solarization are not economically viable options for nematode control in production fields. Generally, growers are not aware of nematode infestations and potential yield losses. Crop rotation, an effective and sustainable nematode management option, may not be feasible in S.E. Asia due to the limited availability of land, seasonal flooding, and the high priority of growers to produce rice. Thus, the development of nematode-resistant cultivars is the most cost-effective and sustainable means for nematode management for both large and small-scale farmers in developing countries. Minimal breeding efforts have been devoted to developing resistant rice cultivars to this nematode (Bridge et al., 2005). Currently, little attention is given this topic by the International Rice Research Institute (IRRI, Los Banos, Philippines), and the national research systems in S.E. Asian countries where this nematode is the major issue. Little information is available on the host-parasite relationship of this nematode with wheat, a common rotational crop with rice in many South Asia countries including Nepal.

Different approaches and screening methodologies have been used when searching for sources of resistance against *M. graminicola* in rice. The appropriate screening protocol used to identify nematode resistant breeding lines should enable to readily and reliably evaluate thousands of genotypes of a breeding program (Boerma and Hussey, 1992). Several protocols have been published for identifying resistance against other root-knot nematodes including *M. aranaria, M. incognita, M. javonica* and *M. hapla* in soybean, potato, tomato, pepper, lettuce and other crops (Hussey and Janssen, 2002) but not for *M. graminicola* in rice and wheat. Identification of sources of resistance to *M. graminicola* in rice must be performed under environmental conditions favorable for maximum damage by this nematode (Tandingan et al., 2000).

The objective of this project was to identify effective, reliable and efficient protocols for the evaluation of rice and wheat germplasm for resistance to *M. graminicola*. In several greenhouse tests, optimum testing conditions were determined. The results of this study will enhance the methods in host resistant investigations and other greenhouse studies against *M. graminicola* in rice and wheat. Material could be useful for designing appropriate crop rotations and identification of potential resistant germplasm for breeding programs.

Materials and Methods

Inoculum Levels and Incubation Periods: In experiment 1, two inoculum levels (2 and 10 eggs/cm^3 soil) were

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established as main factor and 45 day and 60 day incubation periods as sub-factor. In experiment 2, direct-seeded rice or transplanted four week-old rice seedlings were exposed to inoculum levels of 0.2, 1, 2, 10 and 20 eggs of *M. graminicola*/cm^3 soil. In both the experiments, the inoculum was placed in a hole next to seeds or seedlings in a 500 cm^3 capacity clay pot.

Inoculum Level and Container Size: In experiment 3, nematode infection and reproduction in different container sizes with increasing inoculum levels on rice and wheat were examined. The use of clay pots (10-cm diameter, 500 cm^3 soil capacity) was compared to that of 150 cm^3 -capacity conical plastic tubes (20.6 cm long; Ray Leach Single Cell Container, Stuewe and Sons Inc., Corvallis, Oregon). Tubes and clay pots were filled with pasteurized coarse sandy loam mineral soil, and three rice or wheat seeds were planted in each tube or pot. Then, each tube or pot was inoculated with 0.5, 1, 2, 4 and 8 eggs of *M. graminicola*/cm^3 soil in a hole along with seeds, covered with a thin layer of peat moss and soil mixture, and incubated on greenhouse benches at 25 ± 3 °C for 60 days.

Seedling Age and Inoculation Method: In experiments 4 with rice, responses of direct seeding or transplanting and seedlings of different ages to *M. graminicola* infection and reproduction were studied. Seedlings were grown in wooden boxes filled with pasteurized coarse sandy loam mineral soils, and were transplanted to clay pots of 500 cm^3 soil capacity with the same mineral soil (coarse sandy loam). This experiment was conducted in factorial design with the main factor inoculum level (2 or 10 eggs/ cm^3 soil) and the sub-factor direct seeding and transplanting seedlings of different ages of 1, 2, 3, or 4 weeks. The nematode inoculum in 20 ml of water was placed along with seeds or seedlings into the planting hole.

In experiment 5, different inoculation methods were compared in a split-split-plot design where different inoculum levels of 2 eggs or 10/cm^3 soil constituted the main factor, seeding or transplanting the sub-factor, and inoculation method the sub-sub-factor. The nematode egg incoulum was (a) placed on top of the soil, (b) mixed with the soil surrounding the seed, or (c) placed in holes alongside the seeds or seedlings.

Verification of Screening Protocol: Experiment 6 was conducted to verify the protocol, a total of 11 rice and 9 wheat cultivars from diverse geographic areas were tested against *M. graminicola* in a greenhouse at the NYSAES at Geneva, New York. The cultivars were selected from the collection of rice cultivars developed by IRRI, commercial rice cultivars, or germplasm from Nepal and Bangladesh. The seeds of these cultivars were obtained from the International Wheat and Maize Research (CIMMYT) Regional Offices of the respective countries with the proper permits (PPQ526 permit 63098). The US rice cultivars, 'Bonet 73' (resistant) and 'Labelle' (susceptible) to a Louisiana isolate of *M. graminicola* (Yik and Birchfield, 1979) were obtained from the Small Grain Repository Center, Dale Bumpers, Georgia, US and included for comparisons. Similarly, eight wheat cultivars and promising breeding lines from the CIMMYT Offices in Nepal and Bangladesh were included. The highly virulent isolate of *M. graminicola* from a rice field in Nepal (NP 50) (Pokharel et al., 2007) was used as inoculum because a highly virulent isolate can discriminate genotypes with the highest level of resistance (Hussey and Janssen, 2002). The isolate was maintained in the greenhouse on susceptible rice 'Mansuli' and barnyard grass (*Echinocloa crusgali*) alternately.

The experiments followed a common general maintenance procedure: they were conducted in a randomized complete block design with five replications, and each experiment was conducted twice. The two experiments were combined for analysis. Clay pots of 10-cm diameter (500 cm^3 capacity) or single plastic cones served as replicates. They were filled with pasteurized mineral soil (60 °C for 30 minutes). In initial experiments, clay pots were planted to 10 seeds of rice 'Mansuli' (Nepali) or wheat 'Bhrikuti' inoculated with eggs of *M. graminicola* (NP 50), covered with a thin layer of sterile sand and maintained in a greenhouse at 25 ± 3 °C for 60 days. In experiment 1, one half of the pots were assessed after 45 days of incubation. The pots in all experiments were watered twice daily and fertilized weekly with 30-30-30 NPK fertilizer.

After the incubation period, plants were uprooted, roots were washed free of soil, and scored for infection severity of the nematode-induce root-galling as root galling severity (RGS) on a scale of 1-9 by estimating the proportions of roots galled: 1= no galls (healthy roots), 2 = ≤ 5 % roots galled, 3 = 6-10%, 4 = 11-18%, 5 = 19-25%, 6 = 26-50%, 7 = 51-65%, 8 = 66-75%, and 9 = 76-100% of total roots (Mullin et al., 1991). Nematode eggs were extracted by processing roots for 3 minutes intermittently in a blender (Xtreme series MX1500 half Gallon hi-power, The WEBstaurant Store San Diego, CA) in 1% sodium hypo-chlorite (NaClO) solution of commercial bleach (Barker, 1985). The contents from the blender were transferred into a 150-μm aperture sieve nested on top of a 25-μm aperture sieve to retain nematodes. After 3 minutes of washing with tap water, the eggs were collected in a beaker, the volume was adjusted to 100 ml, and the eggs in a 10 ml aliquot counted under a dissecting microscope. Nematodes were extracted from soil using a modified Baermann tray method.

The reproductive factor, [RF = Pf/Pi; where Pf = total number of eggs and second-stage juveniles (J2) extracted from roots and soil at harvest, and Pi = number of eggs at initiation of the experiment], was calculated for each experimental unit. In each experiment, data were tested for normality of distribution, transformations were done if necessary, and ANOVA was conducted with subsequent means comparison using PROC GLM (SAS

Institute, Cary, NC). Regression analysis was done using PROC REG (SAS Institute, Cary, NC) to determine the relationship of initial inoculum level and plant age with that of RGS and RF factors. The linear, cubic, and quadratic relationship of the independent variable initial inoculum level with that of dependent variables RGS ratings and RF factors were tested in experiments 2 and 3 and in Experiment 5, the relationship of the independent variable plant age at inoculation with that of dependent variables RGS ratings and RF factors was tested. For practical breeding strategies a reaction index (RI) was calculated by a method modified from Mullin et al. (1991), and proposed by Pokharel et al. (2011) for cultivar classification inclusive of both factors, RGS and RF. This index was used in determining the reaction of tested rice and wheat lines to *M. graminicola* by calculating a reaction index (RI). Some modifications were applied because egg masses in this root-knot nematode species are deposited inside the roots and are difficult to obtain (Pokharel et al, 2007). This is known to lead to the absence of correlation between RGS and RF although we surmise that both are critical for describing the resistance reaction. RF-values were converted to a 1-9 scale in relation to RF values on the susceptible check Labelle (rice) or 'Brikuti' (wheat). The reproductive factors (PRF) were converted to the RF scale as follows: 1= 0%, 2 =1-10%, 3=11-20%, 4= 21-30%, 5=31-40%, 6= 41-50%, 7 = 51-60%, 8=61-70% and 9= >70% of the nematode reproduction on the susceptible cultivar. Within each test, observed RGS ratings of the test cultivars were converted to percentages of those of the susceptible check as suggested by Pokharel et al. (2011). The calculated RGS% was converted on the scale of 1-9. The reaction index of the tested materials was computed by the formula by Mullin et al. (1991) RI = RGS rank^2 + RF rank^2. In this scheme, the reaction of a plant to root-knot nematode was classified as: immune (I), RI = <2.0; as highly resistant (HR), RI = 2.1-4.0; as resistant (R), RI = 4.1-18; as moderately resistant (MR), RI = 18.1-50; as Intermediate (IM), RI = 50.1-71; as susceptible (S), RI = 71.5-98; and as highly susceptible (HS), RI ≥ 98.1.

Results

Inoculum Level and Incubation Period: Higher RF values were observed at the lower inoculum level (2 eggs/cm^3 soil) than in the higher inoculum level irrespective of incubation period of 45 or 60 days. At both inoculum levels, higher RF values were observed at 60 days than 45 days; the interaction between the two incubation periods and the two inoculum levels was not significant (P=0.5674) (Table 1). RGS ratings were higher at higher inoculum level of 10 eggs/cm^3 soil than at 2 eggs/cm^3 soil irrespective of incubation period; the interaction of inoculum level × incubation period on the RGS ratings was significant (P = 0.001).

Table 1. Effect of incubation time and inoculum densities on root-galling severity (RGS) and reproductive factor (RF) of *Meloidogyne graminicola* on rice 'Mansuli' [a]

Days after Inoculation	Inoculum Level[b]	RGS[c] ratings	RF values [d]
45	2	6.8ab	366.6 b
45	10	7.3 a	46.1 d
60	2	6.2 b	493.4 a
60	10	7.9 a	115.4 c
Standard deviation		1.54	1042.1

[a] The means with the same letter are not significantly different by LSD test at P = 0.05.
[b] Number of eggs/cm^3 soil.
[c] Root galling severity rating (1-9 scale).
[d] Reproductive factor (RF = Pf/Pi) where Pf = eggs and J2 at harvest; Pi = initial inoculum (eggs).

The relationship of initial inoculum level from 1-20 eggs/cm^3 soil in a pot (500 ml) of *M. graminicola* in rice was best described by cubic relationship with that of log RGS (f(x)= -0.0086 x^3 + 0.2768 x^2 + 2.4073 x + 1.0056, R^2 = 0.8707, P = 0.0001; Fig. 1A) and RF factor (f(x)= 0.7258 x^3 + 22.03 x^2 + 150.19 x + 78.081, R^2 = 0.1933, P= 0.0004; Fig. 1B) irrespective of the inoculation timing of the rice seed and seedling (4 weeks of age) inoculation. ANOVA was used to determine the optimum level of initial inoculum on RGS rating and RF value. RGS ratings in both seed inoculation and seedling inoculation treatments were similar. RF values in both seed and seedling inoculation treatments were the highest at 2 eggs/cm^3 soil (data not shown). The RF values in seedling inoculation at 2 eggs/cm^3 soil were higher than other treatments that were not different from each other (data not shown). In seed inoculation treatments, the RF values at 2 eggs/cm^3 soil was different from 10 eggs/cm^3 soil but not with that of 1 egg/cm^3 soil; remaining treatments were not different from each other (data not shown).

Initial Inoculum level and Container Size: In experiment 3, RGS ratings caused by *M. graminicola* were affected by

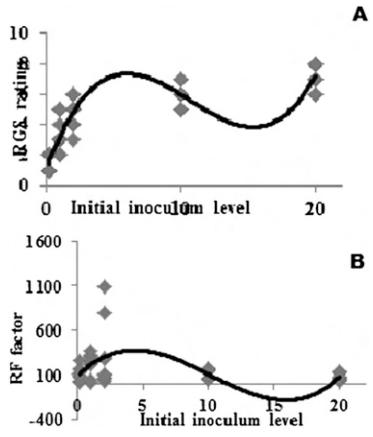

Fig. 1. Relationship of initial inoculum level with that of (A) lnRGS, f(x) = 0.0086 x^3 – 0.2768 x^2 + 2.4073 x -1.0056, R^2 = 0.870, P = 0.0001 and (B) lnRF, f(x) = 0.7758 x^3 – 22.03 x^2 + 150.190 x + 78.08, R^2 = 0.1933, P = 0.0045 of *M. graminicola* in rice inoculated during seeding and as 4-week old seedlings.

container (tube or pot) and inoculum levels (0.5, 1, 2, 4, 8 eggs/cm^3 soil) but not by the crops (rice and wheat; Table 2). The interaction terms container × inoculum level, container × crop, crop × inoculum level were significant but not the interaction of inoculum level × container × crop. Similarly, RF values were affected by container (tube or pot), inoculum level (0.5, 1, 2, 4, 8 eggs/cm^3 soil), and the crop (rice and wheat; Table 2). Similarly, all interaction terms except container × crop, crop × inoculum level were significant for RF (Table 2). In both rice and wheat, RF values increased from 0.5 eggs/cm^3 soil to 2 eggs/cm^3 soil then declined in tubes whereas in pot the RF values increased until it reached to 4 eggs per cm^3 soil then declined thereafter (data not shown).

In rice, the relationship of the independent variable inoculum level of *M. graminicola* with that of dependent variables log of RGS ratings was best described by cubic relationship in pots (data not shown), in tubes (Fig. 2A), and in both pots and tubes together (data not shown) whereas the RF factor can be best described by a cubic relationship in pots (data not shown), and in both pots and tubes (data not shown) but by a quardratic in tubes only (Fig. 2B). The relationship of initial inoculum level with that of lnRGS rating in wheat grown in both pots and tubes (data not shown) but could be best described by a cubic relationship. Similarly, a linear relation in wheat grown in pots (data not shown), and cubic relationship of initial inoculum level with that of LnRGS in tubes only (Fig. 3A) were observed. The relationship with that of lnRF factor was cubic in pots (data not shown), in tubes (Fig. 3B) and the combination of both (data not shown).

Seedling Age and Inoculation Method: The role of plant age combined with two inoculum levels was examined.

TABLE 2. ANOVA table of effect of crop, container, inoculum level and their interaction on lnRGS and lnRF values of *M. graminicola* in rice.

Source	DF	Mean Square	F Value	Pr > F
Dependent Variable: Root galling severity rating				
Inoculum level (I)	4	66.4	56.46	<.0001
Crop (Cr)	1	0.4	0.31	0.5815
Container(Ct)	1	73.7	62.94	<.0001
I × Cr × Ct	4	7.4	6.24	0.0002
I × Cr	4	25.7	21.86	<.0001
I × Ct	4	25.7	21.86	<.0001
Cr × Ct	1	0.2	0.14	0.7131
Dependent Variable: Reproductive factor				
Inoculum Level (I)	4	5641.2	9.24	.0001
Crop (Cr)	1	25154	41.19	<.0001
Container (Ct)	1	3410.5	5.59	0.0205
I × Cr × Ct	4	1304.0	2.14	0.084
I × Cr	4	4261.2	6.98	<.0001
I × Ct	4	607.6	0.99	0.4153
Cr × Ct	1	5126.5	8.4	0.0049

DF is degree of freedom,
F is variance of the group means.
Pr. is the probability ($P = 0.05$).

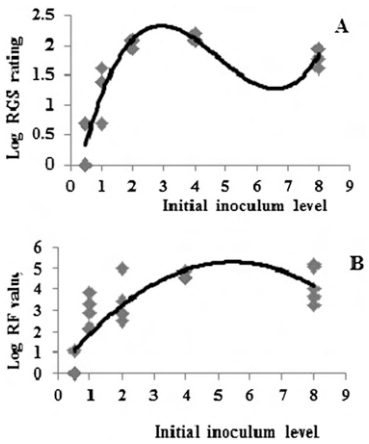

FIG. 2. Relationship of independent variable inoculum level of *M. graminicola* with that of dependent variables (A) lnRGS ratings, f(x) = 0.0429 x^3 – 0.6118 x^2 +2.474 x - 0.7432, R^2 = 0.8965, P = 0.0001 and (B) lnRF factor (f(x) = - 0.1722 x^2 + 1.8786 x = 0.1626, R^2 = 0.7256, and P = 0.0002) in rice grown in tube in greenhouse.

ANOVA analysis exhibited a lack of significant difference on RGS ratings when seedlings of different ages were inoculated as compared to the seeds inoculated at planting. Higher RGS ratings were observed on plants inoculated with 10 eggs/cm^3 soil as compared to 2 eggs/cm^3 soil density (P = 0.0001) and the interaction between seedling age × inoculum level (P= 0.0230) was also significant (data not shown). Generally, in rice, seed inoculation at planting resulted in severe infection and development of visibly larger (data not shown) and higher number of "hook-like" root terminal galls in rice (Fig. 4A) but not in wheat (Fig. 4B) and seedling inoculation in rice (Fig. 4C) where smaller galls without hook-like structure appeared throughout the roots and roots continued to grow. Significant effects of initial inoculum level (P < 0.0001) and age × inoculum level

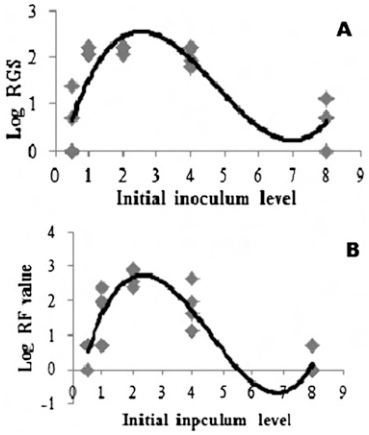

FIG. 3. Relationship of initial inoculum level with that of (A) lnRGS rating, f(x) = 0.0528 x^3 – 0. 7531 x^2 - 2.7931 – 0.5464, R^2 = 0.7405, P = 0.0002 and (B) lnRF factor, f(x) = 0.0762 x^3 + 1.0489 x^2 - 3.6672 x -1.0726, R^2 = 0.7978, P = 0.0001 in wheat grown in tube in greenhouse.

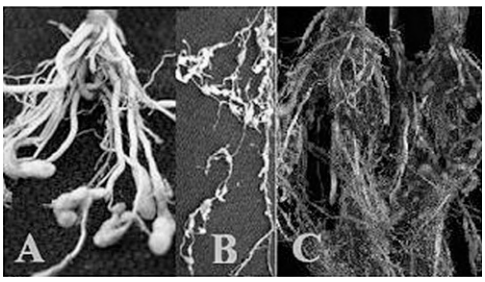

FIG. 4. Symptom of infection by *M. graminicola* inoculated (A) to rice at seeding (B) on wheat at seeding, and (C) to rice seedlings.

($P = 0.0030$) were also observed on the reproduction (RF) of *M. graminicola*.

Higher RF values were observed at the lower inoculum level of 2 eggs/cm^3 soil. This was not true with higher level of inoculum of 10 eggs/cm^3 soil (data not shown). There was no relationship of increasing age of seedling at inoculation with that of RGS (Fig. 5A) ($P = 0.5391$) but a weaker relationship ($f(x) = -0.1333\ x^3 + 0.5286\ x^2 - 0.281\ x + 7.6971$, $R^2 = 0.3191$; $P = 0.0040$) of increasing inoculum level with that of lnRF value soil when 2-weeks or older seedlings were inoculated compared to seeded plants or 1-week old transplants was observed (Fig. 5B).

Experiments evaluating the role of inoculation method compared two inoculum levels, seed versus seedling inoculation, and their interaction terms. The ANOVA analysis models for both RGS and RF values were highly significant ($P < 0.0001$). In RGS rating, the effects of seedling compared to seed inoculation and inoculation methods were significant but not the initial inoculum level (Table 3). In RF, seedling compared to seed inoculation, inoculation method, and initial inoculum level were significantly different (Table 3). The interaction terms initial inoculum level × seedling vs seed inoculation × inoculation method, seedling vs seed inoculation × inoculation method were significant but not inoculation level × seedling vs seed inoculation for both RGS rating and RF value (Table 3).

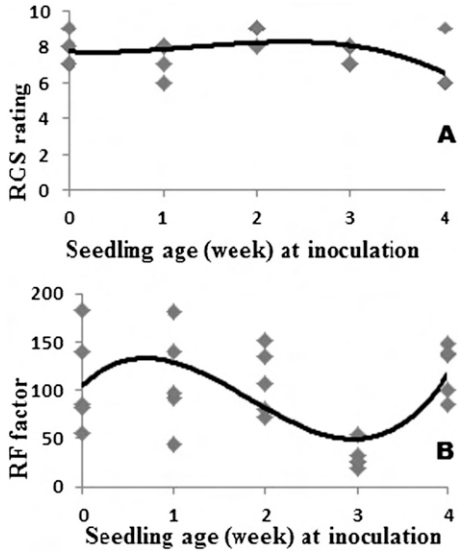

FIG. 5. Relationship of seedling age (0-4 weeks) when inoculated with 2 eggs/cm3 soil in rice by *Meloidogyne graminicola* with (A) RGS, $f(x) = -0.1333\ x^3 + 0.5286\ x^2 - 0.281\ x + 7.6971$, $R^2 = 0.3191$, $P = 0.5391$ and (B) RF factor, $f(x) = -14.295\ x^3 + 78.526\ x^2 + 88.45\ x + 104.67$, $R^2 = 0.3502$, P = 0.400.

TABLE 3. ANOVA table of effect of inoculation method, seed versus seedling inoculation, and, inoculum level and their interaction on lnRGS and lnRF values of *M. graminicola* in rice.

Source	DF	Mean Square	F Value	Pr > F
Dependent Variable: Root gall severity rating				
Inoculum level (IL)	1	0.00338306	0.28	0.5983
Seed vs seedling (SS)	1	0.05503997	4.58	0.0375
Inoculation method (IM)	2	0.04736431	3.94	0.0261
IL × SS	1	0.00055883	0.05	0.8302
SS × IM	2	0.07377127	6.13	0.0042
IL × SS × IM	4	0.08746477	7.27	0.0001
Dependent Variable: Reproductive factor				
Inoculum level (IL)	1	0.70487597	12.17	0.0011
Seed vs seedling (SS)	1	3.80625585	65.71	<.0001
Inoculation method (IM)	2	0.20850383	3.60	0.0350
IL × SS	1	0.07804169	1.35	0.2515
SS × IM	2	0.39498089	6.82	0.0025
IL × SS × IM	4	0.40816259	7.05	0.0002

DF is degree of freedom,
F is the variance of the group means.
Pr-is the probability ($P = 0.05$).

A higher RF was observed with higher inoculum level but not the RGS ratings. Seed inoculation treatment had higher RGS but lower RF values as compared to seedling inoculation. Similarly, three different inoculation methods (a) placed on top of the soil, (b) mixed with the soil surrounding the seed, or (c) placed in holes alongside the seeds or seedlings were compared. The RGS ratings in the methods of placing inoculum on top of the soil (a) were not different from each other, but both were different from (c) placed in holes alongside the seeds or seedlings. But these both were different compared to the method of mixing inoculum along with soil (b). There was no difference in RF values among the above 3 methods (data not shown).

Validation of the Protocol for Screening Rice and Wheat Cultivars: In rice, the RF values ranged from 35 to 430; 'Bonnet73' (a previously reported resistant cultivar) had the maximum RF value whereas 'S. Masino' (Nepalese land race) had the lowest RF value. The RGS ratings that ranged from 2.5 to 8.5 did not correlate with RF values. The highest RGS rating of 8.5 was observed with 'Labelle' (previously reported susceptible cultivar) and the lowest RGS ratings were observed with 3 cultivars (Table 4). The final resistant score ranged from 45 to 162 where 'IR 8' (IRRI cultivar) and 'Baram Kartika' (Nepalese land race) had an index of moderately resistant (Table 4). The RF in wheat ranged from 20-306 where BL 1813 had the highest RF, and BL 1887 had the lowest RF. The RGS ranged from

TABLE 4. Reproduction factors (RF) of *Meloidogyne graminicola* isolate NP 50 and root-galling severity (RGS) rating, on some selected rice cultivars, and resistance index (RI) of these cultivars to the nematode.

	Reproductive factor (RF)		Root gall severity rating (RGS)		Resistant index	
Cultivars	Measured[a]	Rank[c]	Actual[d]	Rank[f]	Score[g]	RI[h]
Ramani	428.0	9	7.8	9	162	HS
Futuje	176.0	9	3.8	7	130	HS
Mansala	409.0	9	4.5	7	130	HS
IR	35.5	3	2.5	6	45	MR
S. Masino	35.0	3	4.3	7	58	IM
Baram Kartika	45.0	3	4.0	6	45	MR
IR 38	94.0	6	2.5	6	72	IM
POBRRO 10	71.0	5	2.5	6	61	IM
BH 1442	104	6	3.5	6	72	IM
Bonnet 73	430.0	9	4.5	7	130	HS
Labelle	226.0	9	8.5	9	162	HS
SD	221.3		2.19			

[a] Reproductive factor (RF = Pf/pi) where Pf = Final eggs+J2; Pi = initial eggs inoculated.
[b] The RF rank converted to a severity scale which was calculated as 1 = (0 % reproduction) to 9 (> 70% reproduction) as compared susceptible check. Rank was calculated by Percentage of RF as compared to that of susceptible check, which was considered 100%.
[c] Root-galling severity determined on a scale of 1 (no galls observed) to 9 = (> 80% of roots galled).
[d] Rank converted to a scale of 1-9 and the rank was calculated based on rank calculated by Percentage of RF as compared to that of susceptible check, which was considered 100%.
[e] Score = RF rank^2 + RGS rank^2.
[f] Resistant Index: Immune (I) ≤ 2.0, Highly resistant (HR) ≤ 4.0, Resistant (R) ≤ 18, Intermediate (IM) ≤ 71, susceptible (S) ≤ 98 and highly susceptible (HS) ≥ 99.

TABLE 5. Reproduction factors (RF) of *Meloidogyne graminicola* isolate NP 50 and root-galling severity (RGS) rating, on commercial wheat cultivars, and resistance index (RI) of these cultivars to the nematode.

	Reproductive factor (RF)		Root gall severity rating (RGS		Resistant index	
Cultivars	Actual[a]	Rank[b]	Actual[c]	Rank[d]	Score[e]	RI[f]
Annapurna 3	23.0	6	8.3	9	117	HS
Annapurna 4	22.0	6	8.3	9	117	HS
BL 1813	306.0	9	7.3	9	162	HS
Brikuti	48.0	9	7.6	9	162	HS
BL 1022	25.0	7	6.2	9	130	HS
BL 1887	20.0	6	5.3	9	117	HS
Annapurna 2	22.0	6	7.5	9	117	HS
SD	76.14		55.66			

[a] Reproductive factor (RF = Pf/pi) where Pf = final eggs + J2; Pi = initial eggs inoculated.
[b] The RF rank converted to a severity scale which was calculated as 1 = (0 % reproduction) to 9 (> 70% reproduction) as compared susceptible check. Rank was calculated by Percentage of RF as compared to that of susceptible check, which was considered 100%.
[c] Root-galling severity determined on a scale of 1 (no galls observed) to 9 (> 80% of roots galled).
[d] Rank converted to a scale of 1-9 and the rank was calculated based on rank calculated by Percentage of RF as compared to that of susceptible check, which was considered 100%.
[e] Score = RF rank^2 + RGS rank^2.
[f] Resistant Index (RI): Immune (I) ≤ 2.0, highly resistant (HR) ≤ 4.0, resistant (R) ≤ 18, intermediate (IM) ≤ 71, susceptible (S) ≤98 and highly susceptible (HS) ≥ 99.

5.3 to 8.3 where 'Annapurna 3' and 'Annapurna 4' had the highest RGS, and 'BL 1887' had the lowest RGS ratings (Table 5). The resistance index ranged from 117 to 162 where all the cultivars had a highly susceptible reaction index (Table 5).

DISCUSSION

The inoculation of rice or wheat planted in 10-cm pots (500 cm^3 soil) with 4 eggs of *M. graminicola*/cm^3 of soil at planting followed by incubation for 60 days in the greenhouse at 25 ± 3 ° C was an effective protocol for screening rice and wheat germplam lines for resistance to *M. graminicola*. Several factors such as incubation period length and conditions during incubation (temperature, light, and soil moisture) were already known to influence the infection and reproduction of *M. graminicola*. In this current study, it was determined that initial inoculum, incubation period, crop, seedling age, container size, inoculation method, and several interacting factors influence the infection and reproduction of *M. graminicola* in rice and wheat. Previously, variable incubation periods of 35 to 90 days had been used when studying *M. graminicola* in rice (Rao and Israel, 1971; Roy, 1977; Yik and Birchfield, 1979; Prasad et al., 1986; Swain and Prasad, 1991; Soriano et al., 2000). This variability made the comparison of results and adaptation of the protocol difficult. Higher RF values and RGS ratings observed in the current study after an incubation period of 60 days compared to 45 days at 25 ± 3 °C indicated that longer incubation was required for *M. graminicola* in rice than for other root-knot nematodes in other crops where incubation periods of 40-45 days after inoculation at temperatures of 25-30 °C had been proposed (Hussey and Janssen, 2002). For *M. graminicola* in rice and wheat, 60 days incubation seemed essential for obtaining consistent results. We did not compare incubation periods longer than 60 days because of potential of restricting root development in the pots and the potential to get multiple generations. Higher infection and reproduction of this nematode with longer incubation time (60 days) may be due to the maturation of larger numbers of females after 45 days of inoculation, producing more eggs and juveniles and emergence of a new generation with longer incubation time. Presence of multiple generations also complicates the analysis procedure.

The inoculum level played a vital role in infection and reproduction of the nematode, and an optimum level of inoculum for the maximum reproduction was critical for the evaluation procedure. A reduction of the reproduction rate of *Meloidogyne* spp. with increasing initial nematode inoculum density was observed in several crops (Di Vito et al., 2004). In the current study, a lower reproduction (RF) rate at lower inoculum level (up to 4 eggs/cm^3 soil), but a higher infection (RGS) rating with higher inoculum levels up to 20 eggs/cm^3 soil was observed. This concept was confirmed in

additional greenhouse tests. The reproduction (RF) of this nematode increased with the increasing inoculum levels from 0.2 to 2 eggs/cm^3 soil, and declined at 10 eggs/cm^3 soil in an experiment when initial inoculum level of 0.2, 1, 2, 10 and 20 eggs/cm^3 soil was tested in 500 cm^3 clay pots. The RF value increased up to 2 eggs/ cm^3 soil in clay pots and up to 4 eggs/ cm^3 soil in smaller plastic tubes in the next experiment. The decrease of the RF values with the increasing inoculum level perhaps was due to limiting resources as a result of competition for nutrition and space among the developing nematodes within the root system. Similar results were reported by Pandey and Haseeb (1997) and Park et al. (1999), while studying pathogenicity of *Meloidogyne* spp. on several plant species. The reproduction of *M. hapla* decreased exponentially with increasing inoculum density from 0.1 to 20 egg/cm^3 soil on three medicinal plants (Park et al., 2005). The increasing RGS rating with increasing inoculum was similar to that of *M. javanica* in potato (Vovlas et al., 2005), and other *Meloidogyne* species in other host plants (Park et al., 2005) where root galling was proportional to the initial nematode population density. The increased RGS ratings with increasing inoculum density may be due to availability of infection sites on roots. Thus caution should be exercised when selecting inoculum density because too high levels could cause extreme injury that masks the identification of potentially useful genetic material (Fassuliotis, 1985).

The maximum reproduction of nematodes at 4 eggs/cm^3 soil observed in these studies was similar to the levels found by Sharma-Poudyal et al. (2005) who reported that the optimum build-up of *M. graminicola* was at an inoculum density of 5 J2/g soil. Sharma-Poudyal used large plastic pots containing 5 kg of soil, whereas in the current study smaller clay pots (10-cm diameter) containing 500 cm^3 of soil were used. In rice, the optimum inoculum level (4 eggs per/cm^3 soil) for the maximum reproduction rate of *M. graminicola* was different from other *Meloidogyne* species on other crops. Reproduction rates of *M. javanica* on potato at an inoculum density of 1 egg + J2/cm^3 soil were similar to those observed for *M. chitwoodi*, and higher than those for *M. fallax* or *M. hapla* on several potato cultivars (Van der Beek et al., 1998). Maximum reproduction rate of *M. incognita* race 1 in spinach was at 0.25 eggs/cm^3 soil (Di Vito et al., 2004). Lack of relationship with the RGS and RF values observed by Pokharel et al. (2005) further complicated the use of only one parameter when evaluating the results of the screening procedure.

Use of small size plastic tubes could be more efficient and economical than larger size clay pots in greenhouse studies for screening rice and wheat cultivars when optimizing resources. In the study of pots of 500 cm^3 capacity and small plastic tubes of 150 cm^3 with rice and wheat, maximum reproduction was found at 2 eggs/cm^3 soil in the plastic tubes and at 4 eggs/cm^3 soil in clay pots of 500 cm^3 capacity. That perhaps was because the larger numbers of juveniles were able to enter host roots in smaller tubes due to proximity of inoculum to roots, resulting in a higher infection rate. The results were similar to those obtained by Hussey and Janssen (2002) working in other crops. Similarly, Jones et al. (2005) observed higher numbers of J2 and eggs of *M. incognita* race 3 in roots growing in plastic tubes with 90, 250, 500 and 750 cm^3 soil capacity infested with 1000 eggs each in small tubes than those in pots with 1000 cm^3 soil capacity. Thus a lower rate of inoculum was required in smaller containers than in larger containers to obtain maximum reproduction. While selecting such containers, several other factors, especially initial inoculum levels, and their interaction factors should be considered based on the targeted parameter to be used such as RGS or RF.

Previous studies on the biology of the nematode and screening for resistance to *M. graminicola* in rice germinated seeds (Roy, 1973), un-germinated seeds (Yik and Birchfield, 1979; Roy, 1977), and seedlings of different ages were used (Swain and Prasad, 1991; Sharma-Poudyal et al., 2005). Un-germinated seeds are easier to handle and to plant in the field. Uniformity in the use of such materials is important as plant age influences the host efficiency to *M. incognita* infection and reproduction in most but not all the crops tested (Mendoza and Jatala, 1985). Direct-seeded Sesbania inoculated with *Meloidogyne javanica* had significantly less nematode galling and smaller nematode populations on the roots than transplanted Sesbania (Desaeger and Rao, 2000). In the current study, higher reproduction of *M. graminicola* on older seedlings but higher infection (RGS ratings) on younger seedlings or seed inoculated seedlings was observed. Higher levels of infection in an early root age may cause more severe damage to the root system and subsequently to plant health. This early infection may cause lower levels of reproduction of the nematode compared to older seedling inoculation. Similar observations were reported in melons inoculated with *M. incognita* (Ploeg and Phillips, 2001) and on carrots inoculated by *Longidorus africanus* (Huang and Ploeg, 2001). The authors concluded that nematodes are much more damaging when they are able to attack the developing roots immediately after seed germination. Higher RF values observed on older seedlings may be due to the availability of greater root mass at inoculation thus providing more nutrition and infection sites for reproduction of the nematode.

It is believed that due to the slow movement of nematodes in soil, inoculation methods can have a major impact on infection severity and reproduction of the nematode. Generally, eggs are added into 2-3 depressions in the soil around the stem base of young seedlings (Starr et al., 2002) or close to the planted seeds or

seedlings. Eggs can also be applied either by mixing with the soil or by mixing with the irrigation water. But the influence of such factors on *M. graminicola* infection and reproduction was not known. In the current study, the role of inoculum level, seed vs seedling inoculation, different inoculation methods on the infection and reproduction *M. graminicola* were examined.

RGS and nematode reproduction are used as parameters for assessing root-knot nematode resistance in crop plants (Roberts and Thomson, 1986). The lack of correlation between RGS ratings and RF values induced by *M. graminicola* in rice (Pokharel et al, 2005) differed from reactions of other root-knot nematode species where inoculum levels correlated with root-galling severity or yield of several crops except soybean (Birchfield and Harville, 1984; Hussey and Boerma, 1981). This observation suggested that root-galling development and nematode reproduction are under the control of independent genetic factors. Roberts et al. (1998) reported that root-galling is often, but not always, closely correlated with nematode reproduction. That may be because the root-galling was reported to be induced by chemical release of the nematode (Trudgill, 1991), but the nematode reproduction was influenced by the host plants (Giebel, 1982). In previous works on evaluation of rice germplasm for resistance against *M. graminicola,* only the root-galling severity (Roy, 1977; Rao and Israel, 1971; Yik and Birchfield, 1979) or only nematode reproduction (Soriano et al., 2000) were considered. In absence of a close correlation between RGS ratings and nematode reproduction, selection for both traits was suggested in the development of highly resistant cotton varieties against *Meloidogyne* spp. (Luzzi et al., 1987).

While working with beans, Mullin et al. (1991) ranked bean germplasm lines for resistance to root-knot nematode calculating a resistance index (RI) that used both root-galling severity and egg mass production, RI= (root galling severity rating^2 + egg mass production rating^2). When evaluating advanced breeding lines it was useful to obtain quantitative data on egg numbers produced in roots which gave a better indication of resistance than either root-gall or egg-mass determination (Luzzi et al., 1987; Hussey and Janssen, 2002), and estimation of egg masses in *M. graminicola* in rice and wheat is rather difficult as eggs are laid and remain inside the root cortex. Therefore, an evaluation system was needed that equally involved infection (RGS) ratings and nematode reproduction (RF) in the search and development of rice germplasm resistance to *M. graminicola.* Similar approaches will be appropriate in wheat, although employing only one of the factors may not affect the results due to the close correlation found between RGS tings rand RF values in wheat (Pokharel et al., 2005).

In the present study, on the basis of using RGS, 'POBBRO 10', 'IR 38', 'Balamchi' and 'Baram Katika' exhibited RGS values of lower than 3.0, thus, these varieties or germplasms could be considered resistant to *M. graminicola* as proposed by Griffin and Grey (1995). But a number of these cultivars exhibited RF values higher than 3.0 of *M. graminicola.* Many of the tested cultivars can be considered resistant as per Trudgill (1991) because they exhibited RF values that were lower than 10% of the susceptible 'Labelle'. He proposed that germplasm resistance be indexed against known standard susceptible and resistant controls. Roberts et al. (1998) concluded that cultivars or germplasm lines could not be considered resistant unless the calculated reproductive factor (RF = Pf/Pi) of the nematode was < 1. In the present study, none of the cultivars and germplasm lines had RF values < 1, thus they were all considered susceptible according to the definition of Roberts et al., (1998). A modified reaction index (RI) scale as suggested by Pokharel et al., (2011) based on the numbers of eggs produced on roots and on root-galling severity may be a reliable option for the study of *M. graminicola* in rice and wheat.

The reproductive factor (RF) of this nematode was almost six times higher in rice than in wheat but not the gall ratings (RGS). Elkins et al. (1979) emphasized that the difference in root length were important in response to nematode invasion and reproduction. Wheat is known to have longer roots than rice, thus it provides more infection sites. Soomro and Hague (1992) emphasized the greater role of host resistance on the RF values of the nematode than the effect of root length. High variability in the RF values on rice and wheat were observed in different experiments suggesting that other factors were also involved in the reproduction of this nematode in addition to host genotype. Higher RF values obtained by inoculating seedlings compared to inoculating seeds further support the assumption of association of greater nematode numbers and higher root biomass. Similar results of lower reproduction of this nematode on wheat than on rice have been reported from India (Gaur and Sharma, 1999), Pakistan (Soomro and Hauge, 1992) and Bangladesh (Padgham et al., 2004). Pokharel et al. (2005) observed a few isolates of this nematode to produce significantly higher RGS and RF values in selected wheat cultivars than a few rice cultivars, and a few isolates of this nematode had a significant interaction between crop variety × isolate of *M. graminicola.*

In summary these studies indicated that various factors, such as incubation period, inoculation levels, container size, crop, inoculation method, and their interactions, might influence the RGS rating and RF factors. Thus, these factors need to be considered while screening rice or wheat germplasms against this nematode. This information is expected to inprove the screening procedure and development of management strategies through the use of resistant or tolerant

germplasm. The protocol will help to compare and understand the biology of the nematode.

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