A screening technique to evaluate pigeonpea for resistance to *Rotylenchulus reniformis**

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Summary

Rotylenchulus reniformis is one of the most important nematode pests of pigeonpea. A simple greenhouse technique has been developed to aid evaluation of pigeonpea genotypes for resistance to *R. reniformis*. In greenhouse pot experiments, eggsacs of *R. reniformis* in pigeonpea (cv. ICPL 87) roots were counted by eye and with the aid of a stereoscopic microscope at 15, 30 and 45 days after seedling emergence in soils infested with various numbers of vermiform *R. reniformis*. Seedlings were rated for the number of eggsacs per root system on a one (no eggsacs) to nine (more than 50 eggsacs) scale. Eggsac ratings were more uniform when roots were evaluated at 30 – 45 days than at 15 days and an inoculum of 15 to 30 individuals/cm³ soil also helped reduce variability. Eggsacs were not easily visible without the aid of a stereoscopic microscope. Of the 14 stains tested, exposure of nematode-infected roots to 0.25% trypan blue for three min was effective in staining the eggsacs blue without staining the roots. Using the stain, the assessment of infestation by *R. reniformis* was equally accurate with or without the aid of a stereoscopic microscope. Exposure of eggsacs to trypan blue enhanced the emergence of juveniles from the eggsacs.

Key words: *Cajanus cajan,* host plant resistance, pigeonpea, reniform nematode, *Rotylenchulus reniformis*

Introduction

The reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, is one of the important nematode pests of pigeonpea (*Cajanus cajan* (L.) Millsp.) in Fiji, India, Jamaica and Trinidad (Sharma & McDonald, 1990). Infestation by this nematode results in poor crop growth and heavily infected plants have yellowish leaves and reduced biomass (Sharma & Nene, 1988). The nematode has a very wide host range, rendering crop rotation an ineffective method of control, and the use of nematicides is uneconomical. Therefore, growing nematode-resistant cultivars of pigeonpea is the most desirable management practice.

Nematode attack does not produce any characteristic above ground symptoms useful in distinguishing susceptible from resistant genotypes. Numbers of nematode females, eggsacs and eggs on a plant are good indicators of the disease stress imposed, and efforts have been made to use such data to screen pigeonpea genotypes for resistance (Chavda, Patel & Patel, 1988; Patel, Chavda, Patel & Patel, 1987; Thakar & Yadav, 1987). Current screening procedures involve inoculation of vermiform stages of *R. reniformis* onto the roots of host seedlings and counting under a stereoscopic microscope the numbers of females and/or eggsacs that develop on the roots. This is usually done after staining the root systems in hot

acid fuchsin lactophenol. This procedure is slow, and impractical for screening large numbers of genotypes in a breeding programme.

The objective of this study was to develop a simple, and reliable greenhouse screening procedure based on the number of eggsacs on the roots. We evaluated 14 stains (dyes) for their efficacy to facilitate counting of eggsacs on the roots without the aid of a microscope.

Materials and Methods

Nematode inoculum

Rotylenchulus reniformis was collected in December 1986 from a pigeonpea field on an Alfisol (red soil) at the research farm (17°N 78°E 545 m) of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Andhra Pradesh, India. The nematode was reared on susceptible pigeonpea cultivars (ICPL 87 and ICP 2376) in 20 cm diameter plastic pots containing autoclaved river bed sand plus farm yard manure plus Alfisol (2:1:1, by volume) in a greenhouse maintained at approximately 30 °C. The plants were harvested after three months and the infested pot soil was mixed with autoclaved river bed sand and Alfisol (1:1, by volume). This soil mix was used to fill thirty more pots. Five seeds of pigeonpea cultivar (ICPL 87) were sown in each pot to increase the nematode inoculum further.

The nematode-infested soil thus produced was bulked, thoroughly mixed to distribute the nematode population uniformly, and divided into eight parts. The first part was maintained as a control, and the other seven parts were mixed with different quantities of autoclaved sand and Alfisol (1:1) to obtain a total of eight infestation levels. The soil samples were incubated at 28 °C for one week to facilitate emergence of juveniles from the eggs. Population densities of *R. reniformis* at each infestation level were estimated in 100 cm^3 soil samples.

Juveniles, males and immature slender females of *R. reniformis* were extracted from the 100 cm^3 soil samples by suspending them in water and pouring the suspensions through nested sieves (850 and 38 μm pore sizes). The residues from the 38 μm pore sieves were placed on double layers of Kimwipes ® tissue papers supported on wire mesh in 9 cm diameter Petri dishes containing sufficient water to cover the residue. The Petri dishes were incubated at 25 ± 2 °C for 48 h to allow the nematodes to pass into the water; the nematodes were then counted. After counting, soils containing inoculum densities of 0.4, 0.9, 1.3, 6.0, 15, 20, 25 and 30 individuals per cm^3 soil were used to fill 12.5 cm diameter plastic pots. Holes in the bottoms of these pots were closed with cotton wool plugs before adding 1000 cm^3 soil to each pot.

Screening procedure

Five seeds of pigeonpea cv. ICPL 87 were sown in each of the pots containing nematode-infested soils. There were six pots for each of the eight infestation levels. At 15, 30 and 45 days after seedling emergence, the roots in two pots from each infestation level were carefully washed free of soil and the eggsacs were counted under a stereoscopic microscope. Each root system was separately rated on a 1 to 9 index where 1 = no eggsacs, 2 = 1 – 5, 3 = 6 – 10, 4 = 11 – 15, 5 = 16 – 20, 6 = 21 – 30, 7 = 31 – 40, 8 = 41 – 50 and 9 = more than 50 eggsacs. The date were statistically analysed for variance and means and standard errors of the means were determined. Coefficients of variation were calculated, and t-tests were used to compare the means.

Influence of staining on the visibility of R. reniformis eggsacs

Fourteen stains (see Table 3) were evaluated. Infected 30 day old seedlings (cv. ICPL 87) were gently removed from the pots and their roots were dipped for 10 min in one of 14 stains.

The roots were then carefully washed with tap water to remove excess stain before spreading them out in water in Petri dishes. The colours of eggsacs and of roots were recorded. Numbers of eggsacs per root system were counted with the unaided eye and with the aid of a stereoscopic microscope. An index of differentiation based on the contrast of eggsacs to root colour was used to evaluate the usefulness of each stain on a 1 (excellent) to 5 (poor) scale. Efficiency of a stain was calculated as a percentage of eggsacs counted with the unaided eye relative to the number of eggsacs counted with the help of a stereoscopic microscope.

Stains with differentiation indices of 1 to 3 were investigated further. Infected roots were exposed for 3, 5 and 10 min to three concentrations (0.25%, 0.5% or 1.0%) of stain, except for Giemsa's stain (where 10.0%, 5.0% and 2.5% were used) and for Coomassie brilliant blue (where 0.5%, 0.25% and 0.15% were used). The roots were rated for eggsacs on 1 to 9 scale by eye as well as with the help of a stereoscopic microscope.

Effects of staining on emergence of juveniles of R. reniformis *from eggsacs*

Stained eggsacs from the lowest and highest concentrations of staining treatments were collected and incubated at $25 \pm 2\,°C$ in water in 2.5 cm diameter Petri dishes. Numbers of emerged juveniles were counted after 2, 4 and 12 days; the emerged juveniles were removed after each counting and fresh water was added to the Petri dishes. After 12 days, the numbers of unhatched eggs and juveniles in the eggsacs were counted. Percentage emergence was calculated and the data were subjected to analysis of variance after arc-sin transformation of percentages.

Drenching of pigeonpea rhizosphere with trypan blue

Nematode infested soil with 15 cm diameter pots containing 30 day old seedlings of cv. ICPL 87 were irrigated with 50 ml of 1.0%, 0.75%, 0.5% or 0.4% trypan blue. Pots irrigated with 50 ml of tap water were maintained as controls. Seedlings were removed gently from the pots 4, 6 and 24 h after adding the stain to the rhizosphere. After gently washing the roots in tap water, the numbers of eggsacs on the roots of five plants for each treatment were counted directly or with the aid of a stereoscopic microscope. Efficiency of a treatment was calculated as previously described. The data were statistically analysed using analysis of variance.

Screening of pigeonpea genotypes

Twenty pigeonpea genotypes obtained from the Genetic Resources Unit of ICRISAT were evaluated for their reaction to *R. reniformis* using the technique of staining roots with trypan blue, and rating procedure described above.

Results

Infestation levels of R. reniformis *and eggsac counts*

Eggsac production differed with the level of soil infestation and the age of seedling. Eggsac counts were lower except at the three lowest infestation levels ($P = 0.05$) 15 days after seedling emergence than at 30 and 45 days (Table 1). Infestation levels of 15 or more *R. reniformis* per cm^3 soil resulted in more ($P = 0.05$) eggsacs per root system than when 6.0 or fewer nematodes per cm^3 were used. Infestation levels of 15 and more individuals per root system gave uniform eggsac indices while lower infestation levels resulted in variable indices (Table 2). Eggsac indices of 15 day old seedlings ranged between 2.0 and 8.3 and the coefficients of variation were high. Eggsac indices of 30 and 45 day old seedlings were less variable, particularly with inocula of 15 to 30 *R. reniformis* individuals per cm^3 soil. Many immature females without eggsacs were observed when roots of 15 day old seedlings were examined.

Table 1. *Numbers of eggsacs of* R. reniformis *on pigeonpea* (*ICPL 87*) *roots 15, 30 and 45 days after seedling emergence in nematode infested soils*

R. reniformis infestation level/cm³ soil	No. of eggsacs after 15 days	No. of eggsacs after 30 days	No. of eggsacs after 45 days
0.4	6.4 ± 6.0^c+	13.2 ± 5.7^d+#	29.5 ± 7.4^cd#
0.9	6.8 ± 6.0^c+	21.8 ± 7.1^cd+	31.5 ± 6.4^cd+
1.3	2.5 ± 6.2^c+	10.2 ± 7.1^d+#	22.3 ± 6.4^d#
6.0	10.3 ± 6.4^c+	35.6 ± 6.2^c#	41.9 ± 6.4^c#
15.0	52.6 ± 8.1^b+	137.5 ± 10.4^a#	137.6 ± 6.8^a#
20.0	37.1 ± 8.5^b+	79.0 ± 9.0^b#	130.4 ± 9.7^a#
25.0	49.0 ± 9.7^b+	89.5 ± 10.4^b#	85.0 ± 9.0^b#
30.0	76.5 ± 6.6^a+	90.6 ± 7.7^b#	130.9 ± 8.1^a#

Figures followed by same symbol in the rows are not significantly different ($P = 0.05$). Figures followed by same letter in columns are not significantly different ($P = 0.05$).

Table 2. *Eggsac indices of pigeonpea* (*ICPL 87*) *roots 15, 30 and 45 days after seedling emergence in soils infested with eight levels of* R. reniformis

R. reniformis infestation level/cm³ soil	Time of evaluation (days after seedling emergence)	Average eggsac index ± S.E.	Coefficient of variation
30	15	8.3 ± 0.33	15.5
25	15	6.6 ± 1.13	45.5
20	15	6.7 ± 0.62	28.1
15	15	7.6 ± 0.60	25.0
6	15	3.2 ± 0.49	61.9
1.3	15	2.0 ± 0.17	35.7
0.9	15	2.8 ± 0.32	48.7
0.4	15	2.8 ± 0.15	23.3
30	30	8.9 ± 0.09	3.4
25	30	8.9 ± 0.13	4.0
20	30	8.5 ± 0.34	9.8
15	30	9.0 ± 0.00	0.0
6	30	6.8 ± 0.37	22.1
1.3	30	3.3 ± 0.37	55.7
0.9	30	5.2 ± 0.44	30.4
0.4	30	4.0 ± 0.32	36.3
30	45	9.0 ± 0.00	0.0
25	45	8.9 ± 0.14	4.3
20	45	8.8 ± 0.41	14.1
15	45	9.0 ± 0.00	0.0
6	45	7.6 ± 0.33	17.2
1.3	45	5.1 ± 0.46	36.2
0.9	45	6.5 ± 0.33	20.2
0.4	45	6.4 ± 0.26	14.0

Eggsac index: 1 = no eggsacs, 2 = 1–5, 3 = 6–10, 4 = 11–15, 5 = 16–20, 6 = 21–30, 7 = 31–40, 8 = 41–50 and 9 = more than 50 eggsacs.

Table 3. *Efficacy of different stains in differentiating* Rotylenchulus reniformis *eggsacs from pigeonpea roots*

Stain	Source	Concentration used for staining (%)	Colour of the roots		Colour of the egg sacs		Index of differentiation‡
Aniline blue*	BDH, UK	1.0	+ +†	Pink	+ +	Purple	3
Azur II eosine*	Loba-Chemie, India	1.0	+ + +	Blue	+ + +	Purple	3
Brilliant blue R*	BDH, India	1.0	+ +	Violet	+ + +	Violet	3
Brilliant green	BDH, India	1.0	+ +	Green	+ + +	Green	3
Coomassie brilliant blue	Bio-Rad Lab, USA	1.0	−		+	Violet	3
Cotton blue	Loba-Chemie Indoaustranal Co. Ltd., India	1.0	+	Blue	+	Blue	5
Eosine	BDH, India	1.0	+	Blue	−		5
Giemsa's stain	Nice New India Chemical, India	10.0	+	Purple	+ +	Purple	5
Haematoxylin*	Loba-Chemie, India	1.0	+	Brown	+ +	Brown	3
Methylene blue	BDH, India	1.0	+ +	Blue	+ + +	Blue	3
Nigrosin	E. Merck, Germany	1.0	−		−		5
Orange G	BDH, India	3.0	−		−		5
Phloxine B	Allied Chemicals, USA	0.015	+	Pink	−		3
Trypan blue	Matheson, Coleman & Bell Manufacturing Chemists, USA	1.0	−		+ + +	Blue	1

Roots were stained for 10 min.
* These were initially dissolved in 10 ml of ethyl alcohol before making up the volume with water.
† + = Light colour, + + = moderate colour, + + + = deep colour, − = no colour.
‡ Index of differentiation (ability of a stain to facilitate visual counting of eggsacs on the roots: 1 = excellent and 5 = poor).

Influence of staining on the visibility of R. reniformis *eggsacs*

Ten stains coloured the eggsacs (Table 3). Azur II eosine, brilliant blue R, brilliant green, methylene blue and trypan blue resulted in marked staining of the eggsacs. Differentiation of eggsacs on the roots was excellent with the use of trypan blue and counting of eggsacs with the unaided eye was simple (Table 4). Although useful in differentiating the eggsacs from root tissues, the other stains were not as good as trypan blue at allowing reliable counts of eggsacs to be made with unaided eye (Table 5). Correlation coefficients of eggsac indices from unaided eye and microscope observations were positive and significant ($P = 0.05$) with 0.25% trypan blue after 3, 5 and 10 min staining of infected roots.

Emergence of R. reniformis *juveniles from stained eggsacs*

Staining of eggsacs significantly ($P < 0.01$) increased the emergence of juveniles from the eggsacs. The two concentrations of stains tested (1.0% and 0.25%) had similar effects on juvenile emergence from eggsacs. Exposure of eggsacs for 10 min significantly ($P = 0.01$) increased the mean emergence (66.9%) of juveniles compared to exposure for 5 min (50.8%) and 3 (44.1%) min. Mean emergence in haematoxylin (64.8%), azur II eosin (61.1%) and brilliant blue R (64.7%) was significantly grater ($P = 0.05$) than that in other stains except Coomassie blue (Table 5). In comparison with emergence in the control (no stain), an increase in emergence of juveniles from eggsacs placed in methylene blue, brilliant green, trypan blue and aniline blue was observed only at an exposure time of 10 min.

Table 4. *Comparison of three concentrations of nine stains for their efficiency to stain* R. reniformis *eggsacs*

Stain dilution* (%)	Time of exposure of roots to the stain (min)	Methylene blue	Giemsa's stain	Trypan blue	Haema-toxylin	Coomassie brilliant blue	Azur II eosin	Brilliant blue R	Brilliant blue	Aniline blue
0.25	10	31.3	46.25	84.1	25.1	31.4	56.1	63·9	40.9	31.9
0.25	5	13.4	48.8	83.4	65.2	50.5	42.5	13.5	46.6	53.0
0.25	3	38.2	14.8	79.0	31.4	19.9	25.1	31.2	37.3	11.3
0.5	10	25.3	34.8	74.0	62.0	38.1	38.7	28.6	33.3	26.4
0.5	5	22.6	34.1	79.8	37.0	40.6	37.7	21.4	29.0	19.4
0.5	3	12.6	35.2	80.6	28.0	18.9	36.0	19.3	21.5	11.7
1.0	10	26.4	43.8	84.8	38.8	55.6	28.8	35.4	30.7	30.6
1.0	5	24.8	27.2	85.0	32.2	34.2	33.1	26.3	32.7	32.7
1.0	3	18.7	39.3	78.3	22.1	38.2	25.2	16.7	42.5	21.8

L.S.D. $(P = 0.05) = 17.72$.

* Stain dilutions were 10.0%, 5·0% and 2.5% for Giemsa's stain and 0.5%, 0.25%, and 0.15% for Coomassie brilliant blue.

Efficiency of a stain = the number of eggsacs counted with unaided eye expressed as a percentage of the number of eggsacs counted with aid of a stereoscopic microscope.

Table 5. *Effect of nine selected stains on emergence of* R. reniformis *juveniles from eggsacs*

	Exposure time of eggsacs in different stains (in min)			
	10	5	3	Mean
Methylene blue	74.8	41.9	27.8	48.2
Giemsa's stain	53.4	53.2	27.0	44.5
Trypan blue	74.3	24.2	34.6	44.4
Haematoxylin	83.8	51.8	58.9	64.8
Coomassie brilliant blue	66.0	65.9	50.1	60.7
Azur II eosin	73.5	64.8	45.0	61.1
Brilliant green	64.3	45.7	54.9	55.0
Brilliant blue R	76.3	64.4	53.3	64.7
Aniline blue	64.6	50.3	51.1	55.4
Control	38.1	38.1	38.1	38.1
L.S.D. $(P = 0.05)$			19.92	5.36
Mean	66.9	50.0	44.1	
			12.55	

N.B. For Giemsa's stain 10% and 2.5%, for Coomassie brilliant blue 0.5% and 0.15%, and for other stains 1.0% and 0.25% concentrations were used. Emergence of juveniles in the two tested concentrations did not differ $(P = 0.05)$. Average emergence presented in the table.

Drenching of rhizosphere soil with trypan blue

Drenching soil with 1.0%, 0.75%, 0.5% and 0.4% trypan blue for 4, 6 and 24 h stained the eggsacs and facilitated counting (Table 6). Ratings of eggsacs on roots by the unaided eye and using a stereoscopic microscope did not differ significantly $(P = 0.05)$. Rating of *R. reniformis* eggsacs on the roots of control plants (no stain treatment) was 3 with the unaided eye

Table 6. *Efficiency of drenching* R. reniformis *infested rhizosphere soil with trypan blue on eggsac ratings on pigeonpea* (*ICPL 87*) *roots*

Trypan blue (%)	Efficiency[*] of drenching rhizosphere soil for		
	4 h	6 h	24 h
1.0	$72.6 \pm 4.57^{+*a}$	$73.1 \pm 4.08^{*a}$	$61.7 \pm 3.23^{+a}$
0.75	$63.0 \pm 3.73^{+a}$	$70.2 \pm 4.57^{+ab}$	$64.4 \pm 3.45^{+a}$
0.5	$53.7 \pm 3.73^{+b}$	$68.9 \pm 3.73^{*ab}$	$60.4 \pm 5.27^{+*a}$
0.4	$41.8 \pm 3.73^{+c}$	$59.1 \pm 3.45^{*b}$	$45.9 \pm 4.08^{+b}$
0.0	$20.7 \pm 3.73^{+d}$	$20.7 \pm 3.73^{+c}$	$20.7 \pm 3.73^{+c}$

[*] Number of eggsacs counts with unaided eye divided by eggsacs counts with aid of a stereoscopic microscope × 100.

Mean ± S.E. in columns followed by the same letter are not significantly ($P = 0.05$) different and mean ± S.E. in rows followed by the same symbol are not significantly different ($P = 0.05$).

and 7 when the roots were evaluated under a stereoscopic microscope. Use of 1.0% and 0.75% trypan blue for 4 and 6 h gave good contrast between eggsacs and root tissues. Occasionally, root tips were faintly stained but this did not affect the contrast between eggsac and root tissue.

Reaction of pigeonpea genotypes

Pigeonpea genotypes ICP 28, 1207, 2627, 7182, 7191, 7194, 7222, 7739, 8520, 11920, 11981, 12974, 13326, 13525 and 14281 were rated 9, ICP 2624, 7647 and 7862 were rated 8 and ICP 7119 and 8518 were rated 7.

Discussion

Pigeonpea genotypes can be evaluated for resistance to *R. reniformis* by counts of eggsacs on infected roots 30 to 45 days after seedling emergence but examination of 30 day old seedlings is faster and more precise due to the smaller root mass. The life cycle of *R. reniformis* is short and eggsacs appear on the roots of 15 day old seedlings; however, assessment after 15 days is not reliable with large variations in eggsac counts and many females without eggsacs. Infestation levels of 6 and fewer *R. reniformis* per cm^3 soil result in variable and low ratings. The use of trypan blue for staining eggsacs is very useful because it does not involve heating the roots in the stain. In addition, viability of eggs is not adversely affected by the stain. This method is faster than screening procedures wherein roots are screened with the aid of a stereoscopic microscope (Birchfield, 1963; Birchfield & Brister, 1969; Birchfield, Williams, Hartwig & Brister, 1971; Chavda *et al.*, 1988; Nayak, Routray & Das, 1987; Patel, Chavda & Patel, 1989; Patel *et al.*, 1988).

Soil infestation levels of *R. reniformis*, age of the seedlings at the time of screening and observations to be recorded for resistance evaluations (counts of females eggsacs or eggs) have not been standardised. Soil infestation levels between 0.2 and 6.0 *R. reniformis* per g or cm^3 of soil have been used and roots screened for *R. reniformis* female or eggsac or egg numbers 12 –25 days after nematode inoculations (Chavda *et al.*, 1988; Patel *et al.*, 1987; Thakar & Yadav, 1987). We suggest that sowing genotypes in soil infested with 15 or more individuals of *R. reniformis* per cm^3 soil and evaluation of the root systems of 30 day old seedlings grown at 25 – 30 °C after staining in 0.25% trypan blue for 3 to 5 min will be helpful in evaluating pigeonpea for resistance to this nematode.

Acknowledgements

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