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## A Screening Procedure for Evaluating Cotton for *Rotylenchulus reniformis* Resistance in Controlled Conditions

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### Summary

*Rotylenchulus reniformis* is one of the most important nematode pests of cotton. Currently, no cotton cultivar resistant to this pest is available. In the framework of a breeding program aiming at producing cotton genotype resistant to this nematode, a *R. reniformis* resistance evaluation protocol based on egg inoculation, extraction and counting, has been established. Two environment conditions (growth chamber and greenhouse), four sieves (75, 50, 20 and 10  $\mu\text{m}$ ), three doses of inoculum (1,500; 3,000 and 6,000 eggs), and five durations of infestation (20, 30, 40, 50 and 60 days after inoculation) have been tested. The growth chamber programmed to provide 12 h of light, 55%-60% relative humidity and 30-26°C day-night air temperatures revealed to be adequate. The NaOCl (sodium hypochloride)-blender-sieving-centrifugation-flotation method, using 75- $\mu\text{m}$  and 20- $\mu\text{m}$  sieves, kaolin powder and MgSO<sub>4</sub> (magnesium sulfate) solution (specific gravity 1.18) proved to be suitable for effective *R. reniformis* egg extraction (from roots) and counting. Inoculation of 6,000 eggs per seedling and 60 days duration of infestation seemed to be sufficient dose and period for a reliable resistance evaluation. The protocol developed has been tested on known susceptible and resistant cotton genotypes: *G. hirsutum* L (main cultivated species through the world, susceptible), *G. thurberi* Tod. (wild cotton species, moderately susceptible) *G. longicalyx* Hutch. and Lee (wild cotton species, very resistant). The results obtained were in accordance with the response expected from the genotypes

tested, proving the reliability of the evaluation procedure developed.

### Résumé

**Mise au point d'une méthode d'évaluation de la résistance du cotonnier à *Rotylenchulus reniformis* en conditions contrôlées**

*Rotylenchulus reniformis* est l'un des plus importants nématodes parasites du cotonnier. Actuellement, aucun cultivar de cotonnier résistant à ce ravageur n'est disponible. Dans le cadre d'un programme de sélection visant à produire des génotypes de cotonnier résistants à ce nématode, un protocole d'évaluation de la résistance à *R. reniformis* basé sur l'inoculation, l'extraction et le comptage des œufs, a été développé. Deux conditions environnementales (chambre de croissance et serre), quatre tamis (de mailles 75, 50, 20 et 10  $\mu\text{m}$ ), trois doses d'inoculum (1500, 3000 et 6000 œufs), et cinq durées d'infestation (20, 30, 40, 50 et 60 jours après l'inoculation) ont été testés. La chambre de croissance programmée pour fournir 12 h de lumière, 55% à 60% d'humidité relative et 30/26°C de température jour/nuit s'est révélée être adéquate au bon développement du nématode. L'utilisation d'un mixer, de tamis de mailles 75  $\mu\text{m}$  et 20  $\mu\text{m}$ , et de la technique de flottaison-centrifugation avec de la poudre de kaolin et une solution de sulfate de magnésium (MgSO<sub>4</sub>) de densité 1,18 s'est avérée efficace pour l'extraction (à partir de racines) et le comptage des œufs de *R. reniformis*. L'inoculation de 6000 œufs par plante et une durée d'infestation de 60 jours semblaient être la dose et la durée suffisante pour

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une évaluation fiable de la résistance. Le protocole mis au point a été testé sur des espèces sensibles et résistantes de cotonnier: *G. hirsutum* L. (principale espèce de cotonnier cultivée à travers le monde, sensible), *G. thurberi* Tod. (espèce de cotonnier sauvage, moyennement sensible) *G.*

*longicalyx* Hutch. et Lee (espèce de cotonnier sauvage, très résistant). Les résultats obtenus étaient conformes à la réponse attendue de la part des génotypes testés, ce qui prouve la fiabilité de la procédure d'évaluation mise au point.

## Introduction

The reniform nematode (*Rotylenchulus reniformis* Linford and Oliveira) is becoming one of the most damaging nematode species of cotton and currently no resistant cotton variety is available (1, 4, 5, 10). The common management practices to manage this nematode in cotton include crop rotation and nematicide applications (4, 11). However, genetic resistance is, because of environmental and economical reasons, the most desirable control method (1, 3).

For a breeding program aiming at developing cotton varieties resistant to this nematode, a reliable screening procedure to evaluate in controlled conditions the resistance of the plants is important. On its host, *R. reniformis* has a semi-endoparasitic habit and only females parasitize plant roots. The immature female imbeds her head into root tissue and establishes a permanent-feeding; the posterior portion remains on the root surface, swells and is surrounded by a gelatinous matrix (eggsac) in which the mature female deposits eggs. Host susceptibility to *R. reniformis* is correlated with the degree of development of the females and with the number of eggs they produced (12). Healthy females and large eggsacs were observed on susceptible plants whereas degenerated females and small eggsacs were common in roots of resistant plants (1, 12). Therefore, the number of *R. reniformis* eggs on a plant can be a good indicator for the assessment of host reaction to this nematode (7, 8, 10). Consequently, a screening procedure should involve inoculation of *R. reniformis* vermiform stages and/or eggs onto plantlet roots and counting the number of eggs developed on these roots. The efficiency of such a procedure depends firstly upon an effective nematode eggs extraction from roots and on an easy and reliable counting. Several basic techniques for nematode egg extractions have been developed from which the NaOCl (sodium hypochloride) -Blender-Sieving method (12) and the NaOCl-Blender-Sieving-Centrifugation-Flotation method (2) can be used for *R. reniformis* egg extraction and count in an evaluation of plant resistance. But, as the efficacy of nematode extractions for many commonly used techniques

ranges from about 10% to 50% (9), it is important to ensure the efficiency of the method chosen, especially when sieving is involved in the procedure (major egg losses can occur during the sieving process if sieves are not appropriate). Moreover, because many other factors (soil composition, inoculum dose, infestation duration, environmental conditions, etc.) can influence the efficiency of the global resistance screening procedure, it is essential to determine the suitable conditions for an effective and reliable screening technique.

This paper deals with experiments carried out to establish a simple and reliable screening technique based in inoculation, extraction, and counting of nematode eggs in the framework of a breeding program aiming at producing cotton resistant genotypes to *R. reniformis*.

## Materials and methods

### Determination of appropriate sieves for *R. reniformis* egg extraction

To determine the appropriate sieves for *R. reniformis* egg extraction, four sieves (75, 50, 20 and 10  $\mu$ m) were tested. A suspension of vermiform stages and eggs of *R. reniformis* was poured through the sieves nested in descending order. A stream of tap water was used to facilitate the sieving process. The residues from each sieve were collected for counting in a petri dish under a stereomicroscope (Wild, Heerbrugg, Switzerland, M5-65480).

### Comparison of NaOCl-Blender-Sieving and NaOCl-Blender-Sieving-Centrifugation-Flotation methods

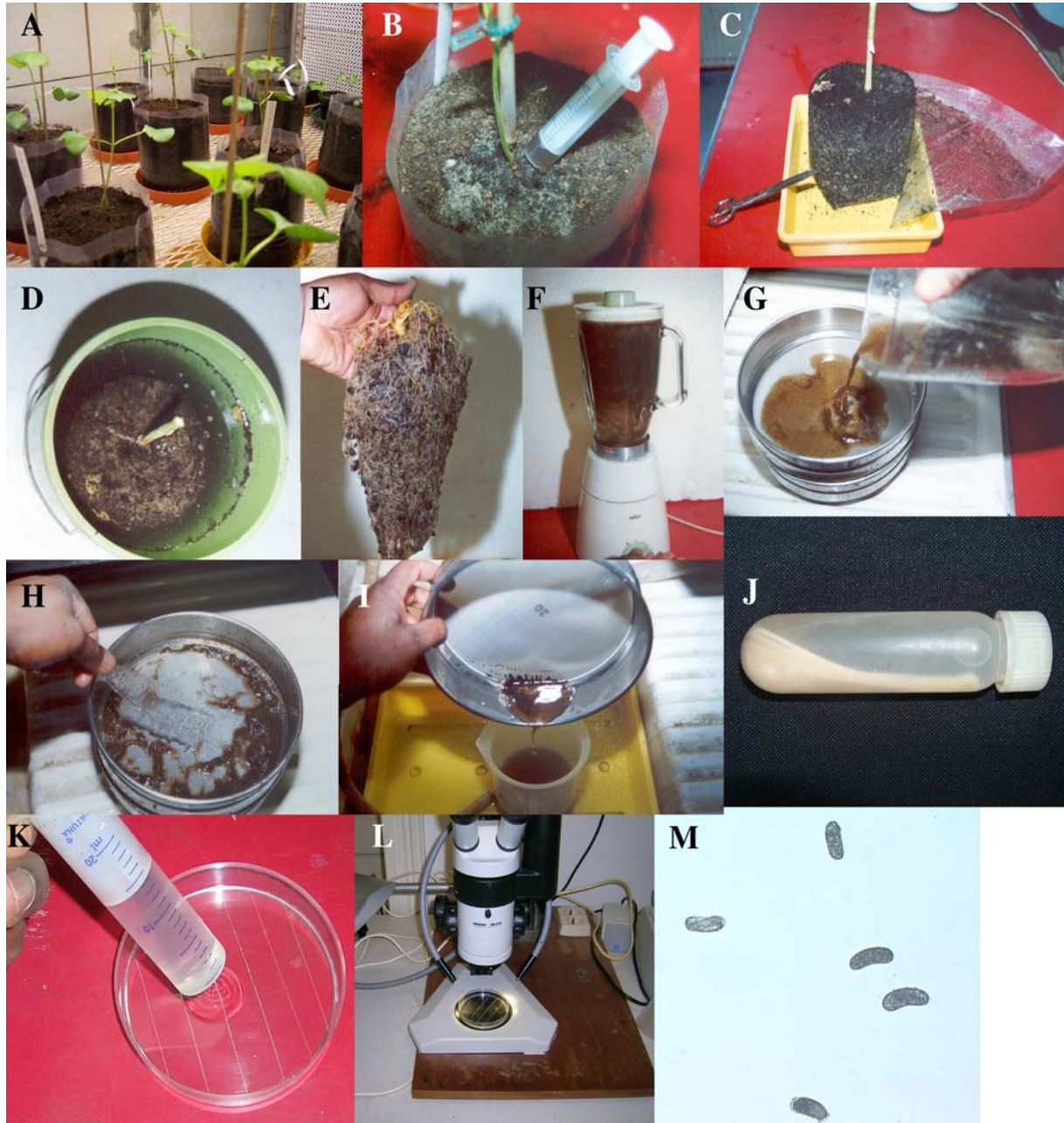
#### NaOCl-Blender-Sieving method:

Roots of *G. hirsutum* infected by *R. reniformis*, were gently washed to remove soil particles. They were then cut into  $\pm$  1-cm pieces and mixed in 0.25% NaOCl solution with a blender (Braun AG, Frankfurt/M, Germany) for 30 seconds to free the eggs from the eggsacs and to disperse them. The mixture was poured through a 75- $\mu$ m sieve, nested over a 20- $\mu$ m sieve. The sieves were placed under a stream of cold water to remove the residual NaOCl and to separate eggs from the root debris.

The residue on the 20- $\mu$ m sieve was gently washed and collected in 1,000 ml water into a beaker. The suspension was agitated while two 20-ml aliquots were taken and eggs were counted.

*NaOCl-Blender-Sieving-Centrifugation-Flotation method:*

The rest of the 1,000 ml suspension was used to test the NaOCl-Blender-Sieving-Centrifugation-Flotation method. The suspension was agitated



**Figure 1:** Main steps of the screening procedure to evaluate cotton for resistance to *R. reniformis*: A) Cotton seedlings planted in 5 litres pots filled with a 3:2:1 (v:v:v) mixture of compost, sand and peat; B) Inoculation with 6,000 *R. reniformis* eggs by injection of the appropriate nematode egg suspension; C) Roots with soil, after 60 days of infestation; D) Soaking of the roots with the soil in a bucket of water; E) Harvest of the roots; F) Blinding of the roots in 0.25% NaOCl solution; G-H) Sieving of the blending with 75 and 20- $\mu$ m mesh sieves; I) Collection of residue retained by the 20- $\mu$ m mesh sieve; J) A centrifuge tube after centrifugation; K) After centrifugal flotation, putting of a 20-ml egg suspension in a petri dish (especially divided) for counting; L) Counting of eggs under a stereomicroscope; M) Some *R. reniformis* eggs seen with the stereomicroscope.

while two 20-ml aliquots were taken in 50-ml centrifuge tubes containing, per tube, 4 g of kaolin powder. The volumes were adjusted with water and were rapidly mixed in a circular motion. The tubes were centrifuged 5 min at 3,000 rpm. The supernatant water was removed and replaced with a MgSO<sub>4</sub> (magnesium sulfate) solution (specific gravity 1.18, or 500 g of MgSO<sub>4</sub> made up to 1 L with water) into which the pellet is thoroughly dissolved. The tubes were centrifuged at 3,000 rpm for 6 min. The supernatant MgSO<sub>4</sub> solution was sieved through a 20- $\mu$ m sieve. The eggs retained by the sieve were washed, collected in 20-mL water and counted.

### Comparison of *R. reniformis* development in greenhouse and in growth chamber

Greenhouse and growth chamber environmental conditions were compared to choose the one which allows the best development of *R. reniformis*. Six 30-days *G. hirsutum* seedlings, planted in 20-cm-diameter plastic pots containing sterile mixture of 3:2:1 (v:v:v) compost, sand and peat, were inoculated each with 5,000 *R. reniformis* eggs. The inoculum preparation consisted in a suspension in water of *R. reniformis* eggs extracted and counted from infested cotton roots according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method as presented above. For inoculation, the planting medium in each pot was infested with 5000 *R. reniformis* eggs by injecting the appropriate egg suspension 2 to 5-cm deep at four points located 2 cm apart from the stem. Three of the infested plants were placed in greenhouse and the three others in a growth chamber. The growth chamber was programmed to provide 12-hr light per day, 55%-60% relative humidity and 30-26°C day-night air temperatures. No effective control was possible on the greenhouse conditions where light, temperature and relative humidity were mostly influenced by outside conditions and were very variable. In the period (summer) corresponding to the experiment in the greenhouse the relative humidity was 30-40%, and the temperature varied from 30°C to 55°C the day and from 28°C to 35°C the night. Sixty days after inoculation, the number of eggs on each plant was determined by extracting and counting them according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method as presented above.

### Determination of the adequate period for egg extraction

To determine the adequate duration of the test before egg extraction, five periods (20, 30, 40, 50 and 60 days after inoculation) were tested in a

growth chamber programmed for 12-hr light, 55%-60% relative humidity and 30-26°C day-night air temperatures. Ten susceptible cotton (*G. hirsutum* cv C2) seedlings of 30 days, planted in 20-cm-diameter plastic pots containing sterile mixture of 3:2:1 (v:v:v) compost, sand and peat, were inoculated with about 5,000 eggs of *R. reniformis*. After each tested period, 2 plants were used for egg extraction and counting according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method as presented above.

### Determination of the inoculum dose

Three doses of inoculum (1,500, 3,000 and 6,000 eggs per plant) were tested in growth chamber programmed for 12-hr light, 55%-60% relative humidity and 30°C-26°C day-night air temperatures. Inoculum preparation consisted in a suspension in water of *R. reniformis* eggs extracted from infested cotton roots according to the NaOCl-Blender-Sieving as presented above. For each dose, three cotton (*G. hirsutum* cv C2) seedlings of 30 days, planted in 20-cm-diameter plastic pots containing sterile mixture of 3:2:1 (v:v:v) compost, sand and peat, were inoculated. Sixty days after inoculation, roots of each plant were weighed and eggs produced on each plant were extracted and counted according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method.

### Check of the reliability of the screening protocol established

A screening procedure was established from the results of the experiments carried out above. The reliability of this procedure was checked using susceptible and resistant cotton genotypes. These genotypes were *G. hirsutum* L. (main cultivated cotton species), *G. longicalyx* Hutch. and Lee (wild diploid cotton species), *G. thurberi* Tod (wild diploid cotton species) respectively susceptible, resistant and moderately susceptible to *R. reniformis* (4, 6, 10, 12). Three seedlings of each genotype were tested. The general protocol for this screening was as follows (Figure 1):

Three 30-days seedlings of each genotypes, planted in 20-cm-diameter plastic pots containing sterile mixture of 3:2:1 (v:v:v) compost, sand and peat, were inoculated with 6000 *R. reniformis* eggs. Sixty days after inoculation, the soil was removed by soaking the roots in water and entire root systems were gently harvested and weighed. Eggs were then extracted and counted according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method as presented above. For each plant, the number of eggs per gram roots was determined.

These numbers were used to assess the relative resistance of each plant compared to the susceptible control *G. hirsutum* by calculating the percentage of eggs per gram root on each plant considering the 100% level for the susceptible control. The scale of relative resistance we used contains the following classes: 0% = immune, 1-10% = highly resistant, 11-25% = resistant, 26-40% = moderately resistant, 41-60% = low susceptible, 61-100% = susceptible as check, and above 100% = very susceptible (12).

## Results and discussion

### Determination of the appropriate sieves for *R. reniformis* egg extraction:

All the tested sieves, allowed passage of vermiform stages through them (Table 1), demonstrating that vermiform stages of *R. reniformis* could be lost during sieving when using these sieves. Concerning eggs, the 75- $\mu$ m sieve let all the eggs pass through it. The 50- $\mu$ m sieve kept some eggs (31 eggs) but let the majority of them (446 eggs) pass through it. The 20- $\mu$ m kept all the eggs (446 eggs) coming from the 50- $\mu$ m sieve. These results show that the sieves used here, have not the same efficiency regarding *R. reniformis* egg collection. The sieves with larger apertures (50 and 75) that let the eggs pass through them, are not good to collect eggs; their aperture seemed be larger than the width of most of *R. reniformis* eggs. The most suitable sieves for a *R. reniformis* egg extraction process, are the 75- $\mu$ m and the 20- $\mu$ m sieves. Both the 75- $\mu$ m and the 20- $\mu$ m sieves can be used efficiently to separate *R. reniformis* eggs from plant debris. Indeed, the 75- $\mu$ m sieve will retain only the root debris while the 20- $\mu$ m sieve will collect the totality of the eggs.

**Table 1**

Number (N.) of vermiform *R. reniformis* (Rr) and eggs retained by the 75- $\mu$ m, 50- $\mu$ m, 20- $\mu$ m and 10- $\mu$ m mesh sieves.

	Size of sieves			
	75- $\mu$ m mesh	50- $\mu$ m mesh	20- $\mu$ m mesh	10- $\mu$ m mesh
N. of vermiform Rr retained	28	39	33	1
N. of Rr eggs retained	0	31	446	0

### Counting of eggs collected with the NaOCl-Blender-Sieving method and with the NaOCl-Blender-Sieving-Centrifugation-Flotation method:

Egg counting from the NaOCl-Blender-Sieving extraction method was not easy. Sieves alone were not efficient in separating all the root debris from

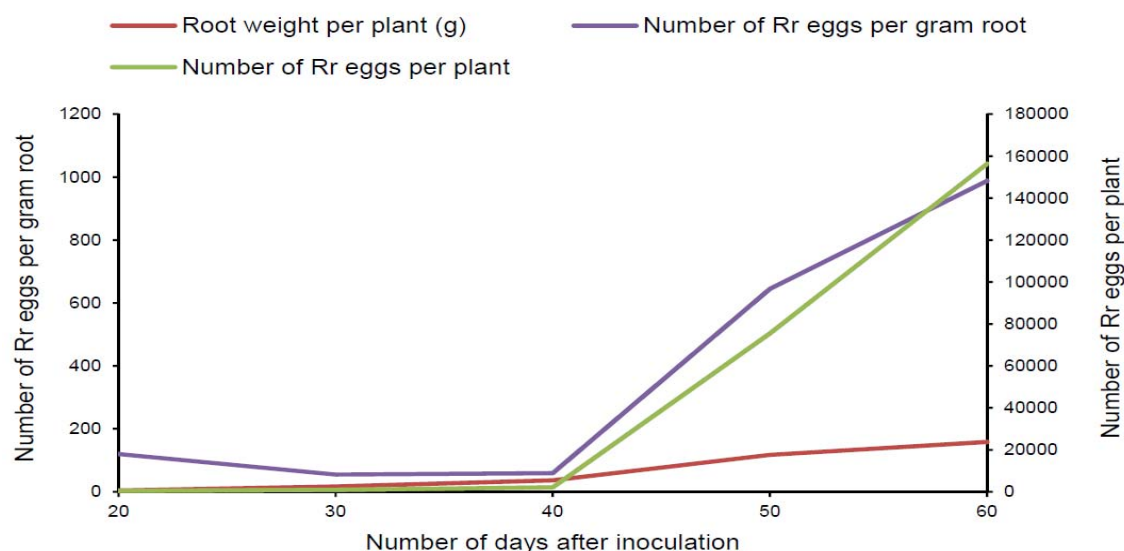
the nematode eggs. The egg suspension contained a lot of residual root debris that can be confused with eggs, rendering the counting difficult. On the contrary, the suspension coming from the NaOCl-Blender-Sieving-Centrifugation-Flotation method was very clear and eggs were readily distinguishable (Figure 1: K and M). The kaolin powder used in this method, bound to roots debris in pellet while nematode eggs floated in the MgSO<sub>4</sub> solution because of its greater specific gravity; the specific gravity of the MgSO<sub>4</sub> solution used was 1.18 while most nematodes have a specific gravity ranging from 1.06 to 1.10 (2). The eggs collected from the supernatant MgSO<sub>4</sub> solution were free of root debris. In the egg suspension obtained by this method, eggs were counted more easily. The NaOCl-Blender-Sieving-Centrifugation-Flotation method is more reliable for the counting of *R. reniformis* eggs.

### Comparison of *R. reniformis* development in greenhouse and in growth chamber:

All the plants grown in growth chamber, gave a greater number of eggs per gram root than the ones grown in greenhouse. The mean number of eggs produced per gram root on plants grown in greenhouse (230 eggs) was twenty times inferior to the mean number of eggs counted per gram root on plants grown in growth chamber (4770 eggs). These results indicate that the nematode reproduced better in growth chamber conditions than in greenhouse. This relative low development of *R. reniformis* in greenhouse could be explained by the high instability of temperature in summer at Gembloux, with temperature in greenhouse going sometimes up to 40°C; while *R. reniformis* has optimal temperatures for movement and reproduction between 27°C and 32°C (5). For a reliable screening procedure aiming at evaluating plant for resistance to *R. reniformis*, the results obtained here suggest that assays must be carried out in conditions avoiding extreme temperature levels.

### Determination of the adequate period for egg extraction:

At 20, 30 and 40 days after inoculation, the number of eggs on the plant were on average inferior to 150 eggs per gram root (Figure 2). The highest production of eggs occurred at 50 and 60 days with 645 and 988 eggs per gram root, respectively. Fifty or sixty days after inoculation can be good periods for the evaluation of the resistance of cotton plants to *R. reniformis*, since these periods allowed the greatest egg productions.



**Figure 2:** Number of *R. reniformis* (Rr) eggs collected on *G. hirsutum* (cv C2) 20, 30, 40, 50 and 60 days after inoculation of 5,000 eggs.

#### Inoculum dose:

Three doses of inoculum were examined: 1,500; 3,000 and 6,000 eggs per plant (Table 2). The level of egg production was a function of the initial dose. The higher the initial dose the higher the number of eggs produced on the plant. The dose of 6,000 eggs gave the largest number of eggs produced (287,042 eggs per plant on an average) while the dose of 3,000 and 1,500 eggs gave respectively 90,841 and 54,142 eggs per plant.

The number of eggs produced with the dose 6,000 represented three times the numbers of eggs obtained with the dose 3,000 and 5 times the number of eggs obtained with the dose of 1,500. As

**Table 2**  
Number of *R. reniformis* (Rr) eggs collected on *G. hirsutum* sixty days after inoculation of 1,500, 3,000 and 6,000 eggs.

Inoculum dose	Number of plants	Mean number of Rr eggs per plant
1,500 eggs	3	54,142
3,000 eggs	3	90,841
6,000 eggs	3	287,042

the objective of the resistance evaluation procedure is to test the reaction of plants to *R. reniformis*, the dose 6,000 which gave the largest number of eggs per gram root seems to be the more suitable dose for this assessment.

Check of the reliability of the screening protocol established: The reaction of each tested genotype to *R. reniformis* inoculation was known before the test. *G. hirsutum* is susceptible, *G. thurberi* is low susceptible, *G. longicalyx* is very resistant (4, 6, 10, 12). Our aim was to assess the reliability of the evaluation procedure developed. *G. hirsutum* presented the highest number of eggs per gram root (14,690 eggs.g<sup>-1</sup>). *G. thurberi* comes to the second position with 8,570 eggs.g<sup>-1</sup> (Table 3). The number of eggs per gram root of *G. longicalyx* was very low (186 eggs.g<sup>-1</sup>). Compared to the susceptible *G. hirsutum*, the wild species *G. thurberi* was lowly susceptible (58% eggs.g<sup>-1</sup>) whereas *G. longicalyx* (1.27% eggs.g<sup>-1</sup>) was very resistant. These results are in accordance with the response expected and prove the reliability of the evaluation procedure developed.

**Table 3**  
Evaluation of the resistance to *R. reniformis* of three cotton genotypes using the procedure developed.

Genotypes	Number of plants	Eggs produced per gram roots	Percentage of egg productions per gram root	Host status
<i>G. hirsutum</i> (C2)	3	14,690	100%	S <sup>a</sup>
<i>G. thurberi</i>	3	8,570	58%	LS <sup>b</sup>
<i>G. longicalyx</i>	3	186	1.27 %	HR <sup>c</sup>

<sup>a</sup> S: susceptible; <sup>b</sup> LS: low susceptible; <sup>c</sup> HR: highly resistant

## Conclusion

The results obtained from the experiments reported in this manuscript and the main conclusions drawn allow us to propose the following protocol for the evaluation of the resistance of cotton plants to *R. reniformis*:

1. **Inoculum preparation:** Inoculum is prepared from the nematode reared on susceptible *G. hirsutum* cultivars. The inoculum preparation consists in a suspension in water of *R. reniformis* eggs extracted and counted from the infested cotton roots according to the NaOCl-Blender-Sieving-Centrifugation-Flotation method.

2. **Inoculation:** Inoculate 30-days seedlings planted in 20-cm-diameter pots with 6000 *R. reniformis* eggs.

3. **Evaluation of the resistance:** Sixty days after inoculation, evaluate the plants for *R. reniformis* resistance according to the following steps (Figure 1):

(i) Collection and weighing of the roots;

For easily collection of roots, a cultivation substrate with too much compost or peat must be avoided. A heavy soil that strongly stuck to the roots can cause a lot of *R. reniformis* eggsacs losses during removal of roots from such a soil. In our works we used 3:2:1 (v:v:v) mixture of compost, sand and peat.

(ii) Extraction of eggs from the roots by the NaOCl-Blender-Sieving method;

(iii) Separation of eggs from the residual debris according to the Centrifugation-Flotation method and counting;

(iv) Assessment of host status for each plant by calculating the percentage of eggs per gram root. Use the scale of relative resistance of Yik and Birchfield (12) that contains the following classes: 0% = immune, 1-10% = highly resistant, 11-25% = resistant, 26-40% = moderately resistant, 41-60% = low susceptible, 61-100% = susceptible as check, and above 100% = very susceptible.

## Acknowledgements

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