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Image Analysis of the Growth of *Globodera pallida* and *Meloidogyne incognita* on Transgenic Tomato Roots Expressing Cystatins[1]

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Abstract: An approach based on image analysis that enables rapid collection and analysis of nematode size and shape during growth is reported. This technique has been applied to assess *Meloidogyne incognita* and *Globodera pallida* during their development over 35 and 42 days, respectively, on transgenic tomato roots expressing the wild-type rice cystatin Oc-I or an engineered variant, Oc-IΔD86. Morphometric values were established that subdivided enlarged saccate females from other life stages. Analysis of this data subset indicates that the size of females and the frequency with which they parasitize roots expressing a cystatin are reduced. Results also demonstrate that cystatins can influence the growth of *G. pallida* prior to the adult stage. Similar image analysis procedures should be generally applicable to the study of host status or environmental factors that influence growth rates of plant-parasitic nematodes.

Key words: Globodera pallida, image analysis, *Meloidogyne incognita,* nematode growth, potato cyst nematode, root-knot nematode, transgenic roots.

Females of *Globodera* and *Meloidogyne* spp. attain a sub-spherical shape and a biomass that is much larger than is typical of vermiform tylenchids (1,7). Their rapid growth depends upon feeding from plant cells that are specifically modified by these parasites (14). For *M. javanica,* fecundity and duration of the period prior to ova production are influenced by host suitability (3), which can be experimentally manipulated to bias the sexual fate of *M. incognita* in favor of males (15).

Transgenic plants expressing a serine proteinase inhibitor suppress growth rates and influence the sexual fate of *G. pallida.* They also suppress the fecundity of *M. incognita* (2,8). This may be due to an effect on digestion of dietary protein. Both serine and cysteine proteinase activity occur in females of *G. pallida* (9), and the second of these two classes has been localized in the intestine of the microbivorous nematode *Caenorhabditis elegans* (13). A plant cysteine proteinase inhibitor from rice (oryzacystatin I, Oc-I) and a protein engineered variant of this cystatin (Oc-IΔD86 [16]) kill feeding *C. elegans.* Oc-IΔD86 has a lower K_i than wild-type Oc-I, measured using the *C. elegans* digestive proteinase gcp-1, and a corresponding lower LD50 against *C. elegans.* Studies using tomato roots transformed with *Agrobacterium rhizogenes* have established that Oc-IΔD86, and to a lesser extent Oc-I, suppress the growth rate of *G. pallida* (16).

Urwin et al. (16) measured the size of *G. pallida* over time during growth using values of cross-sectional area provided by image analysis. This method of estimating body size was preferred to one requiring preparation of drawings by use of a camera lucida or a drawing tube as before (8). We investigated the potential of image analysis for allowing a rapid collection of sufficiently large data sets for detailed analysis. The technique may have potential for studies of nematode development and host status that extend beyond our current interest in evaluating transgenic plant lines that suppress nematode growth.

Materials and Methods

Nematode infection: Methods for the transformation of tomato roots with *Agrobacterium rhizogenes* and their subsequent challenge with surface-sterilized *G. pallida* are given in full by Urwin et al. (16). Pro-

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cedures were similar for *M. incognita,* with the exception of surface-sterilization of the nematodes. Eggs of *M. incognita* were recovered from stock colonies grown on tomato using sodium hypochlorite as described by Daykin and Hussey (5) and were set to hatch in water at 28 °C. Second-stage juveniles (J2) recovered at 24 to 48 hours were counted and sterilized for 10 minutes with sequential applications of the following antibiotics: 0.1% streptomycin sulfate, 0.1% penicillin G, 0.1% amphotericin B, and 0.1% cetyltrimethylammonium-bromide (Cetrimide; Sigma Chemical Co., Dorset, UK). The nematodes were pelleted between treatments by brief micro-centrifugation.

Transformed root lines were cultured for 4 weeks after which time a 2-cm section of fresh root was transferred to fresh media (16). After 3 to 4 days, series of growing roots were challenged with surface-sterilized nematodes. The J2 were washed extensively in filter-sterilized tap water, and 35 J2 per 5 µl aliquot of one species was pipetted onto the root approximately 1 cm behind its tip. A 1-cm^2 piece of sterile GFA filter paper (Whatman) was placed over the area and removed 24 hours later. The roots were then placed at 25 °C (*M. incognita*) and 22 °C (*G. pallida*) for various times up to 35 and 42 days post-infection, respectively.

Growth of M. incognita and G. pallida on transformed roots: Infected roots were removed from petri dishes, rinsed in water, and then placed in 1% (w/v) sodium hypochlorite for 2 minutes (4). Roots were plunged into boiling 0.1% aqueous acid fuchsin for 1 minute, rinsed in water, and then cleared in acidified glycerol at 60 °C overnight before nematodes were dissected out of the roots. An aliquot of preparasitic J2 of each species was stained in a similar manner. Second-stage juveniles in a second aliquot were killed by brief exposure to heat at 50°C immediately before measurement. Nematodes were examined under a microscope (Leica, model number DMRB) at ×50 to 200 magnification.

Image analysis: The preparasitic juvenile nematodes and the parasitic stages were analyzed under the microscope on which was mounted a color camera (Kappa CF15 MCC). The camera output was connected to a frame grabber and analysis was controlled through a software package (Quantimet 500c; Leica, UK). Calibration of the size presented by a pixel was achieved using a micrometer slide before experiments. Values were kept as a subset for *G. pallida* parasitic juveniles in which vermiform male development was evident. Males were not present for *M. incognita* in the experimental conditions that prevailed. They were obtained for this population using procedures given by Triantaphyllou (15). The aerial parts of tomato plants were removed at soil level at 8 days after planting in soil containing this nematode. The animals were recovered 21 days later and stained as described above. Image analysis was carried out for parasites showing vermiform male development. Images of representative nematodes were transferred to an optical disk for later comparison and photography. Data were analyzed by selecting parameters from the range available within the software menus. The captured measurements for each nematode were transferred to a cascaded window of a spreadsheet (Excel 5, Microsoft Corporation) using a macro routine. Data sets were saved as worksheets and transferred subsequently to a statistical computer package (Statistical package for the Social Sciences, SPSS, Chicago, IL, USA).

Results

Acid fuchsin staining did not alter the length (L) or cross-sectional area (A) of the J2 of either nematode species relative to values of unstained, heat-killed individuals. It did reduce both the roundness value (R) and the variance attached to it ($P < 0.001$ for both species, t-test and Levene's test for equality of variances) (Table 1). The shape factor R, which describes the outline of the nematode, gives a minimum

TABLE 1. Image analysis for cross-sectional area, length, and roundness values of living and stained preparasitic second-stage juveniles of *Meloidogyne incognita* and *Globodera pallida*.

Species	Preparation	Area (μm^2)	Roundness (range)	Length (range) (μm)
M. incognita	Heat-relaxed	5,966 ± 91	10.18 ± 0.47 (8.1–15.3)	395 ± 5.9 (312–436)
	Stained	5.910 ± 129	9.4 ± 0.15 (7.9–10.8)	397 ± 2.7 (375–422)
G. pallida	Heat-relaxed	9,365 ± 140	15.29 ± 1.08 (8.5–25.9)	469 ± 5.9 (411–528)
	Stained	9,467 ± 156	8.43 ± 0.19 (7.1–10.5)	465 ± 5.6 (427–511)

Mean values ± SEM (n = 20 in all cases). Comparison between preparations establishes that means for both species are statistically significant for roundness ($P < 0.001$ in both cases) but not length.

value of unity for a circle and is calculated from the ratio of perimeter squared to area. All subsequent analysis was based on stained individuals to ensure comparability of data.

The change in R with development was examined further to distinguish fusiform stages that have a cylindrical form from saccate females that are spheroids. A basis for subdivision of animals on R was obtained from values for parasitic juveniles in which vermiform male development was evident (Table 2). Using the same approach, body length (L) was selected as the second filter. Therefore, the parasites were categorized into three shapes according to their growth: (i) Fusiform: R no less and L no greater than the respective means plus two sample standard deviations for a developing male within its cast cuticles, (ii) Saccate: R below and L within the upper limit described for fusiform individuals, (iii) Enlarged saccate: R as described for saccate but with L above the threshold set for fusiform individuals.

Fig. 1 provides a three-dimensional plot of L against R and cross-sectional area (A) for individuals of *M. incognita* recovered from transformed roots at 14 to 42 days post-infection plus the 20 stained preparasitic J2 used in Table 1. The data show an initial change in R with only a small increase in A and no change in L. R falls to approximately 2.0 before A and L begin to increase appreciably. The increase of L at $R < 2.0$ is not accompanied by a change in R and is therefore indicative of an increase in the diameter of the sub-spherical female.

The critical filter values in Table 2 for *M. incognita* were applied to the SPSS data file to sub-categorize established parasites into two subsets for control or oryzacystatin-expressing roots for 28 and 35 days combined. This is a sufficient period for animals in favorable conditions to reach the enlarged saccate female stage. These individuals were segregated into three groups of fusiform, saccate, and enlarged saccate individuals. Only four of the 233 nematodes present on the roots at 35 and 42 days (two from each treatment) were excluded from this analysis as having unusual values for the two selection criteria (i.e., L above its filter threshold and R not below its value). The roots expressing the

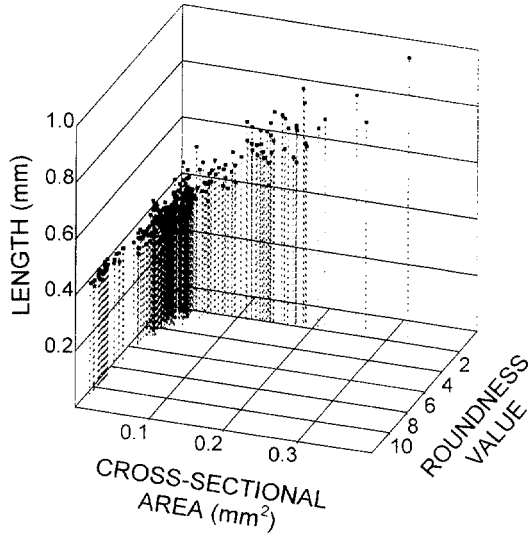

FIG. 1. The relationship between length, roundness value, and cross-sectional area for 261 *M. incognita* individuals growing over 14–35 days at 25 °C on roots of tomato that had been transformed by *A. rhizogenes* harboring pBIN19 lacking a cystatin insert. The figure includes 20 preparasitic second-stage juveniles.

TABLE 2. Image analysis of stained *Meloidogyne incognita* and *Globodera pallida* juveniles recovered from plants in which development of the vermiform male is evident within the fusiform parasite.

Species	Number of measurements	Roundness (filter values)	Length (filter values) (μm)
M. incognita	15	3.93 ± 0.112 (3.06)	350 ± 9.32 (422)
G. pallida	8	2.90 ± 0.152 (2.04)	398 ± 12.6 (469)

Values for *R* and L are means ± SEM. Filter values used to sub-divide data in Table 3 are means for L plus two sample standard deviations and m eans for *R* minus two sample standard deviations.

wild-type rice cystatin Oc-I show both a reduction in the proportion and the cross-sectional area of the nematodes that are placed in the enlarged saccate category (Table 3).

Fig. 2 provides the same relationship as Fig. 1 but for *G. pallida*. Again a fall in *R* occurs before an appreciable increase in L and A. In this case, critical filter values given in Table 2 for *G. pallida* were used to sub-divide the combined data set at 35 and 42 days post-injection in three size categories. Of the 125 individuals in the study, 8, 4, and 6 individuals from the control, Oc-I, and Oc-IΔD86 data sets, respectively, were excluded from analysis. The L and *R* values of these 18 individuals did not place them in one of the three categories used in this work. No attempt was made to distinguish males still within juvenile cuticles from other juveniles still present in the roots at 35 or 42 days (Table 3). The data for the engineered oryzacystatin showed a reduction in the mean cross-sectional area of the females on these roots relative to controls ($P < 0.05$), but the reduction in number was not statistically significant. Furthermore, the effect on body size for roots expressing Oc-IΔD86 was not limited to enlarged saccate females. Juveniles were smaller on these roots than on controls ($P < 0.05$; Table 3). This suggests the transgene influences the growth of the nematode before the enlarged saccate female stage.

DISCUSSION

The image analysis equipment is clearly capable of measuring animals accurately since the mean lengths of J2 in Table 1 are in agreement with previously published values for the species (10). In addition, the measurements are accurate in that the SEM of values in Table 1 are <2.5% of their respective mean values. The rapidity of the approach allows a hundred stained individuals to be measured within a day. The data are readily transferred during collection to a spreadsheet, and the whole data set can be exported to other applications such as the statistical package used in this work. These features facilitate analysis of relatively large data sets and so contribute to the certainty of analysis. In total, 562 and 444 individuals of *M. incognita* and *G. pallida,* respectively, were measured before infection and up to 35 and 42 days post-infection, respectively, in this work.

Furthermore, the sub-division of animals according to the extent of their growth was based on objective, morphometric criteria using filter values within the SPSS analysis. Three categories of nematodes were recognized according to their roundness and length. A fourth category could be devised to define infective, vermiform juveniles as a subset by applying the approach followed in Table 2 to the data in Table 1. This was not done for the data presented in Table 3 because no such individuals were detected for *M. incognita* and only five were recorded for *G. pallida*. The first category recognized in this work was fusiform. It describes nematodes of either sex, of invasive or established parasite stages, and it includes all stages of male development within cast juvenile cuticles and, for females, J2, J3, and J4 *Meloidogyne* and both J2 and J3 *Globodera*. The second category includes those individuals in which the body has swollen toward a subspherical shape with the loss of a cylindri-

TABLE 3. Image analysis of stained *Meloidogyne incognita* and *Globodera pallida* recovered from both wild-type plants and transgenic plants that express a cystatin.

Nematode	Anti-nematode gene expressed	Fusiform juveniles and males[a]		Saccate females		Enlarged saccate females	
		No.	Mean ± SEM	No.	Mean ± SEM	No.	Mean ± SEM
M. incognita	Control	19	0.0144 x ± 0.0011	41	0.0249 x ± 0.0014	61	0.122 x ± 0.0081
	Cystatin (Oc-I)	8	0.0132 x ± 0.0013	63*	0.0244 x ± 0.0010	37*	0.0852 y ± 0.0080
G. pallida	Control	19	0.0422 a ± 0.0062	2	0.0678 a ± 0.0069	11	0.146 a ± 0.014
	Cystatin (Oc-I)	21	0.0356 a ± 0.0036	6	0.0621 a ± 0.010	12	0.135 a ± 0.0096
	Engineered cystatin (Oc-IΔD86)	25	0.0236 b ± 0.0023	5	0.0494 a ± 0.0045	6	0.086 b ± 0.0094

The cross-sectional area (mm^2) and number of individuals of *Meloidogyne incognita* on hairy tomato roots at 28 and 35 days post-infection (combined data) plus similar data for *Globodera pallida* at 35 and 42 days post-infection. The nematodes were categorized into three groups on the basis of roundness values and length (see text). Means in columns for a species share the same letter (a,b; x,y) if not significantly different ($P < 0.05$; SNK or *t*-test as appropriate).

χ^2 test was used to compare the frequency of each stage for a treatment relative to its corresponding control (* indicates statistical significance, $P < 0.05$).

[a] The incidence of males was not recorded separately from juveniles in these experiments.

FIG. 2. The relationship between length, roundness value, and cross-sectional area for 149 *G. pallida* individuals growing over 7–42 days at 22 °C on roots of tomato that had been transformed by *A. rhizogenes* harboring pBIN19 lacking a cystatin insert. The figure includes 20 preparasitic second-stage juveniles.

cal outline. All are females because males do not achieve a spherical shape and remain cylindrical in outline throughout their development. The final category, enlarged saccate, describes females in which their length is greater than that of early parasitic states. The increase in length without a loss of spherical shape (low R value) indicates that they are sub-spheres that are increasing in size. Therefore, they are certainly adults for *Meloidogyne* but could be adult for J4 females of *Globodera*. They indicate established nematodes with a feeding cell complex able to support development toward a gravid condition.

Animals showed a rapid change in shape on establishment as parasites, with roundness values falling for stained individuals from just less than 10 toward the value of <2 that occurs for enlarged saccate females. No males were observed in the experiments with *M. incognita,* but a large number of animals were recorded as saccate females. This term is applied on the basis that the change in R that distinguishes fusiform from saccate forms had occurred but the increase in length (and width) of the sub-spherical female had yet

to be substantial. All the analysis in this work is centered on growth and not development stages because the approach used could not distinguish stages sharing a fusiform shape. Presumably, unenlarged saccate females have failed to establish a feeding position that supported rapid growth within the study period. Many of the saccate females in this work probably correspond to individuals termed stagnant by Grundler et al. (6).

The sub-division of animals into three subsets revealed that the frequency of enlarged saccate females of *M. incognita* was suppressed when development occurred on Oc-I-expressing roots. This is an additional effect to that on mean size for all individuals at one time point, as reported for *Globodera* by Urwin et al. (16). This imaging approach also clearly established that Oc-IΔD86 suppressed growth of the fusiform stages of *G. pallida* (Table 3). The overall effect on growth is considerable. The difference in cross-sectional area indicates that females of *M. incognita* on roots expressing Oc-I were approximately 60% of the volume of the controls. The corresponding value for *G. pallida* on roots expressing Oc-IΔD86 is approximately 45%. In addition, the number of enlarged saccate females was reduced in both cases. This reduction was only statistically significant for the larger data set involving *M. incognita*. We are currently investigating the factors that allow a few animals to achieve some growth as enlarged saccate females on these plants.

Categorizing a population into subgroups each with a mean size has value, particularly as size of enlarged saccate individuals is correlated to fecundity in both *Globodera* (7) and *Meloidogyne* (11). Counts for eggs per root system take no account of distinctions between number and fecundity of egg-laying females. Such distinctions can be pertinent to study interactions involving both natural and transgenic host resistance. In this work we show that the cystatin suppresses growth of both fusiform individuals and enlarged saccate individuals. Stage-specific expression of proteinases has been reported for nematodes (12). Currently, we assume that cystatin inhibits the cysteine proteinase of *G. pallida* (9). If this is the mode of action, the proteinase must be expressed before the last feeding stage.

The approaches described in this work have potential for other applications within nematology. For instance, they could be used to determine differential effects of host status or other environmental stresses on growth before and during the final feeding stage of species with saccate females.

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