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Quantification of Globodera rostochiensis and G. pallida in mixed populations using species-specific thermostable proteins

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Abstract

A method has been developed to quantify species ratios in mixed populations. The method is based on the separation of species-specific thermostable proteins by SDS-PAGE. Densitometric analyses of the 17 kD protein of *Globodera pallida* and the 18 kD protein of *G. rostochiensis* revealed a high correlation ($R^2 = 0.93$) with the species ratio in the mixed samples. Within the limits of 10 to 90% of each species, one can estimate with 95% reliability the species composition with 3 to 6% deviation.

Additional keywords: gel electrophoresis, potato cyst nematodes, species composition.

Introduction

Control of potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, is a major problem in the potato growing areas of the Netherlands. Growing resistant potato cultivars is one of the environmentally sound ways to decrease the nematode populations in the soil and to achieve good yields. A reliable screening test to characterize and monitor field infestations of potato cyst nematodes according to species offers possibilities for optimal use of resistant cultivars because resistance directed to either of the species can be used. In case of mixed populations the effects of resistant cultivars on multiplication rates have to be known for both species. At present we are investigating the effect of growing potato cultivars with species-specific resistance on mixed populations of *Globodera pallida* and *G. rostochiensis* under laboratory conditions and therefore need a reliable method to measure differential population changes.

Various methods are available. Methods based on morphology are laborious and not accurate because of variability and overlap of most of the distinguishing characters (Franco, 1978). Alternative methods are based on biochemical approaches such as disk-electrophoresis (Trudgill et al., 1972), 1-dimensional-electrophoresis (Bakker et al., 1988; Von Stegemann et al., 1982), 2-dimensional-electrophoresis (Bakker and Gommers, 1982), isoelectric focusing (Fleming and Marks, 1982; Ohms and Heinicke, 1983), serology (Schots et al., 1987), DNA-probing (Burrows and Perry, 1988) or combinations of these techniques such as immuno-electrophoresis (Wharton et al., 1983). These methods are suitable for distinguishing *G. rostochiensis* from *G. pallida*. With the exception of IEF by Fleming and Marks (1982; Marks and Fleming, 1985) they have hardly been used quantitatively.

We have analysed the quantitative use of a modified 1-dimensional gel electrophoresis

technique based on the separation of heat-stable species specific proteins (Bakker et al., 1988; Schots et al., 1987). For routine ecological research, the method has to be simple, relatively cheap, rapid, sensitive and reproducible. In addition, the method should utilise large samples of cysts because results should reflect accurately the composition of the populations. The work reported here describes the possibilities and drawbacks of this approach.

Materials and methods

Nematodes. Plants cv. Bintje, susceptible to all pathotypes, were inoculated with an egg suspension (5 eggs/g of soil) of *Globodera pallida* (Pa3; Research Institute for Plant Protection). The plants were grown in pots in an artificial soil mixture (30% gravel, 60% silver sand, 10% clay powder) in a greenhouse at 18-22 °C with 16 h daylight for about three months. The newly formed cysts were separated from the soil using a cyst-elutriator (Seinhorst, 1964).

Preparation of protein extracts. To prepare a protein solution about 1000 cysts were incubated in 1.5 ml demineralized water for 24 h. The cysts were crushed and the suspension was purified using a 150 μm sieve. Eggs were collected by centrifugation (5 min, 2850 *g*) at room temperature. The pellet was resuspended in 700 μl 10 mM Tris-buffer pH 7.4. Subsamples of about 140 μl were homogenized for 10 sec in a tissue grinder (2 ml mortar, teflon pestel, Heidolph microhomogenizer, 2200 rpm) at 0 °C. The eggs shells and juvenile cuticles were removed by centrifugation (5 sec, 10 500 *g*). This solution was heated at 95 °C (TCS-Metallblock thermostat) for 5 min and denatured protein was pelleted (15 min, 10 500 g). The supernatants with the species-specific heat-stable proteins were stored at −20 °C. The protein concentrations of the solutions were determined with the Proti analyzer (Bradford-type analysis (Bradford, 1976), modified Coomassie Brilliant Blue G250 reagent, extinction measured at 465 and 595 nm), as recommended by the manufacturer Marius (Utrecht, the Netherlands).

Gel electrophoresis. Electrophoresis samples were prepared from protein stock solutions. For each replicate separate stock solutions of *Globodera rostochiensis* and *G. pallida* were used. The composite samples covered the species ratios from 100 : 0% to 0 : 100% in steps of 10% by adding from both stock solutions the appropriate proportions of protein solution. The amount of protein added to the gel from mixed samples were 5 μg, 8 μg and 10 μg; 1 μg protein from samples with 100% of the individual species were also applied on the gel. Phosphorylase-b (97 400 kD) was used as reference protein.

One dimensional sodium dodecyl sulfate polyacrylamide slab-gel electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (1970), with 4% (w/v) polyacrylamide stacking gel and 15% (w/v) polyacrylamide separation gel. Running conditions: 50 V stacking gel (1.5 h) and 150 V separating gel (6.5 h, 4-10 °C.). The gels were stained in 0.2% Coomassie Brilliant Blue R250 (12 h) in fixative solution (25% methyl alcohol, 10% acetic acid) and destained in the fixative solution (48 h).

Densitometric analysis. Pictures were taken from the gels (Kodak Ektachrome 64) and the colour positives (10.2 × 12.7 cm) were scanned with the LKB Ultrascan XL

Laser Densitometer. The scanning data were analysed using an interactive program (LKB 2400 gelscan XL^{tm} software package version 1.2.) to determine the peak area by integration via Gausian curve fitting.

Results

Extraction of thermostable species-specific proteins. Our approach was based on the isolation procedure of species-specific proteins of Schots et al. (1987). This method however gave low protein yields, inadequate separation of proteins and low reproducibility. Therefore the method was optimized to meet our criteria.

It was established that the egg/larvae suspension should not exceed the equivalent of the contents of 125 cyst per 100 μl to obtain optimal homogenization of eggs and larvae and extraction of proteins. Greater densities resulted in highly viscous solutions with poorly homogenized eggs. Removal of egg shells and debris after homogenisation by centrifugation gave reproducible recovery of the thermostable proteins; however, prolonged centrifugation times resulted in considerable loss of these proteins.

The heating procedure of the protein solution ('au bain Marie') also influenced the reproducibility considerably. Using a heated metal-block greatly improved this step. The heating time could be reduced as well.

Thus, protein solutions with reproducible concentrations of thermostable proteins were obtained (coefficient of variation 9-10%).

Separation. According to Schots et al.(1987) the thermostable species-specific proteins are 21 kD, 20.5 kD and 17 kD in *G. pallida* and 20.8 kD, 20.6 kD and 18 kD in *G. rostochiensis*. We concentrated our work on the 17 kD and 18 kD proteins in *G.pallida* (Pa) and *G.rostochiensis* (Ro) respectively, because these were consistently present (Fig. 1) and their molecular weight difference was relatively greatest. Since these differences are still small, the separation procedure had also to be optimized. SDS-PAGE showed clear band patterns. Optimal resolution was found with 15% (w/v) polyacrylamide (Fig. 1). At percentages of respectively 10% and 20% polyacrylamide the bands became too diffuse or too close for densitometric analysis. Best separation results were obtained with 8 μg of protein per lane. At 5 μg per lane the minor species was below detectable levels at ratios of 90 : 10 and 10 : 90, and at 10 μg overloading occurred at the 90 : 10 and 10 : 90 ratios for the dominant species. Fig. 1 shows these effects at ratio 90 : 10.

Quantification. The general protein stain Coomassie Brilliant Blue was used as a saturated staining technique. The variability of the stained gels is generally supposed to be negligible. However, analysis of the stain intensity of phosphorylase b, the reference protein, showed considerable variation in separate experiments. Five different gels with 5 μg phosphorylase gave peak area measurements of between 0.32 and 1.54 units and when 0.5 μg was applied the values were between 0.5 and 0.1 units, with variation coefficients of 54% and 38% respectively. Freshly made stain solutions minimize this variability.

Fig. 2 shows the results of five replicate gels of a concentration series of the *G. rostochiensis* stock solution (1, 5, 8, 10 μg per lane). For the 18 kD Ro protein as well as the 17 kD Pa protein a linear relationship was found between the amount of protein and measured peak area with $R^2 = 0.90$ to 0.99.

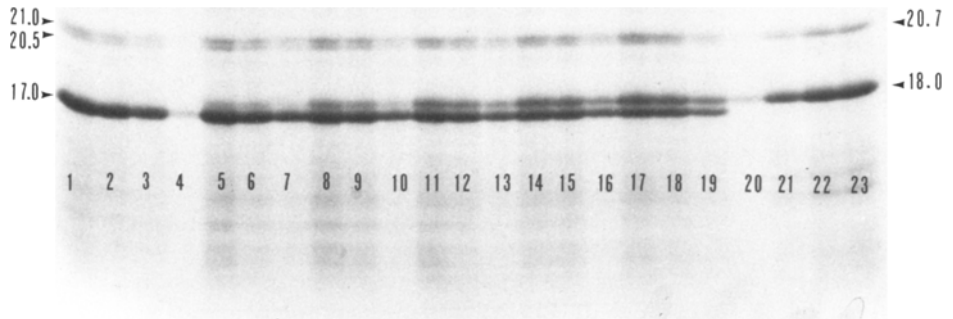

Fig. 1. Protein pattern of thermostable proteins of *Globodera rostochiensis* (20.8 kD, 20.6 kD and 18 kD) and *G. pallida* (21 kD, 20.5 kD and 17 kD) after SDS-PAGE (15% acrylamide, 50V-150V). Lane 1-4: 100% Pa, 10, 8, 5 and 1 μg respectively; lane 5-7: 90% Pa and 10% Ro, 10, 8 and 5 μg resp.; lane 8-10: 80% Pa and 20% Ro, 10, 8 and 5 μg resp.; lane 11-13: 70% Pa and 30% Ro, 10, 8 and 5 μg resp.; lane 14-16: 60% Pa and 40% Ro, 10, 8 and 5 μg resp.; lane 17-19: 50% Pa and 50% Ro, 10, 8 and 5 μg resp.; lane 20-23: 100% Ro, 10, 8, 5 and 1 μg resp.

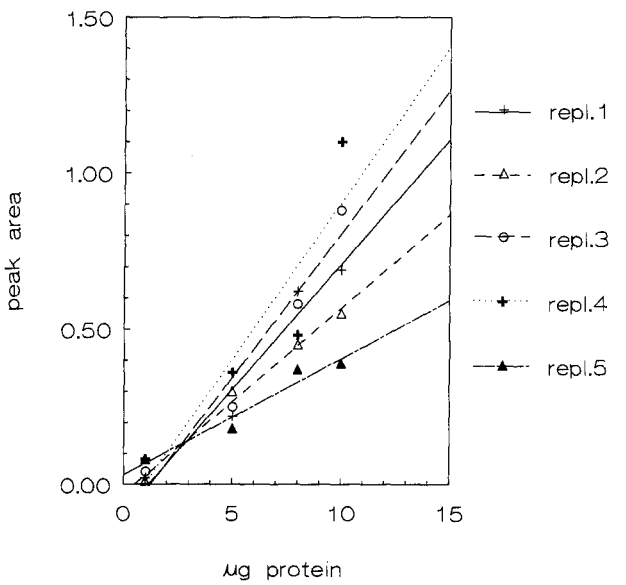

Fig. 2. Standard curves for the 18 kD thermostable species-specific protein of *G. rostochiensis*, determined by regression analysis (R^2 = 0.90 to 0.99), five replicates. Proteins separated in 15% polyacrylamide SDS-PAGE, stained with Coomassie Brilliant Blue R250. Densitometric analysis by LKB-Ultrascan 595 nm.

Between the standard curves considerable variance was observed. At 5 and 8 μg of protein the variation coefficient was smallest (respectively 24% and 27%). The high variability (50%) at the 1 μg values probably resulted from the low amount of protein

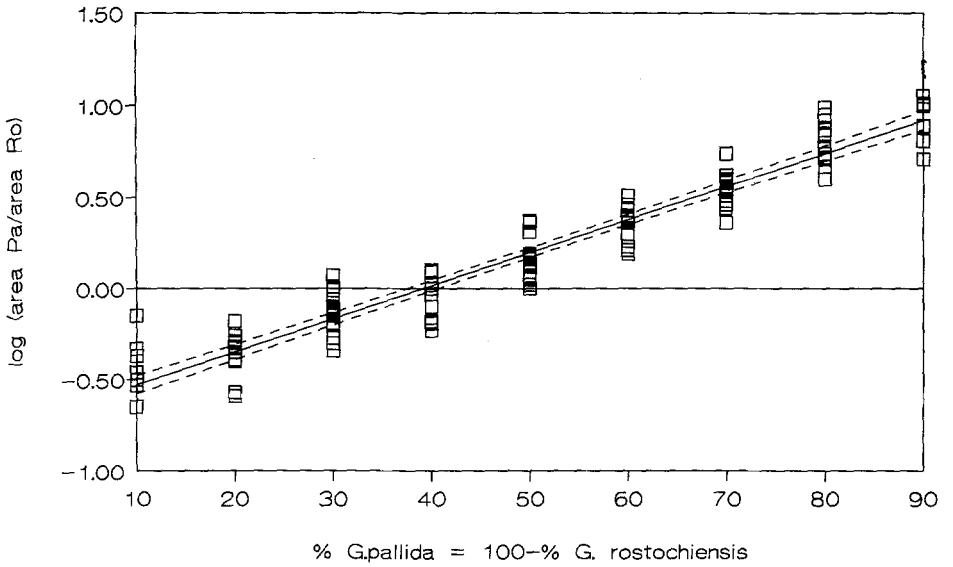

Fig. 3. Calibration curve for assessing the composition of mixed-species samples of the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*. Regression analysis (R^2 = 0.93) and 95% confidence interval for μ (P = 0.05). Proteins separated in 15% polyacrylamide SDS-PAGE, stained with Coomassie Brilliant Blue R250. Densitometric analysis by LKB-Ultrascan 595 nm.

which is close to the detection limits of this method. The detection level for the 18 kD protein of Ro was reached at 0.8 μg of protein stock solution. The 17 kD protein of Pa was still detectable when 0.5 μg of protein stock solution was applied to the gel. This amount is equivalent to 500 eggs, according to the protein content found in the eggs, respectively 1.09 μg per 1000 eggs for Pa and 1.01 μg per 1000 eggs for Ro. Saturation became apparent above 15 μg of protein stock solution.

Five replicate series with nine different Pa − Ro ratios were used for a calibration curve to determine species composition. To circumvent variability between gels due to differences in staining and gel conditions, comparisons between gels were not made. Instead the stain intensities of the bands of the 18 kD of Ro and 17 kD of Pa were compared to each other per lane. The peak area of the Pa protein was divided by the peak area of the Ro protein; the logarithmic value of this quotient was linearly related to the standard ratios of the mixed proteins.

Regression analysis on all data revealed a high correlation (R^2 = 0.93) with the linear relation : y = −0.713 + 0.0182x. A calibration curve was constructed (Fig.3) with 95% confidence intervals (P = 0.05) for mean values. Using the mean of five replicates of peak area quotient measurements species composition can be assessed within a range of 10 to 90% of each species with 3 to 6% deviation. To verify this method protein samples with known ratios, based on larvae counts before protein extraction, were analysed on gels. Table 1 shows the counted percentage of Pa and the calculated percentage of Pa in the samples based on the scanning data and using the calibration curve. All numbers, except one, are acceptable according to the above mentioned criteria.

Table 1. Analysis of composition samples of *Globodera rostochiensis* and *G. pallida* based on counted larvea numbers and estimated by gel electrophoresis and using the calibration curve.

% *G. pallida*		
counted	estimated	difference
24.5	27.7	3.2
24.5	29.5	5.0
28	21.4	−6.6
28	22.5	−5.5
44.7	45.1	0.4
51.6	48.0	−3.6
54	51.4	−2.6
54	50.3	−3.7
73.8	67.6	−6.2
75.1	70.5	−4.6
76	74.0	−2.0
76	71.7	−4.3
86	90.2	4.2
86	87.3	1.3

Discussion

Research on population dynamics of potato cyst nematode species in mixed populations requires quantification of their proportions. Methods based on morphological characters are not suitable because of the overlap in size and shape of most of these characters. Biochemical methods based on intrinsic characters such as thermostable proteins used in this study, depend on presence or absence of a character allowing unambiguous species determination. A routine nematological research and advisory tool for monitoring cyst populations will certainly be based on such an approach (Marks and Flemming, 1985; Schots, 1988). The value of species specific DNA probes, as used for potato cyst nematodes (Burrows and Perry, 1988) and soybean cyst nematodes (Besal et al., 1988), has not yet been developed for routine application but also seems promising.

Because cyst contents vary we used stock solutions of thermostable proteins to prepare composite samples. These samples were made on the basis of protein concentrations, thereby assuming that the thermostable proteins are present in both species in equal amounts. This assumption is realistic in view of the presumed homology of the species specific thermostable proteins (Schots, 1988) and the concentration values of the protein solutions we found (1.01 μg/1000 eggs for *G. rostochiensis* and 1.09 μg/1000 eggs for *G. pallida*). The stain capacity however, differs for Pa and Ro (detection level for Ro is 0.8 μg and for Pa 0.5 μg), therefore the intersection of the calibration curve is not found at the expected ratio 50 : 50 but at the ratio 40 : 60.

Proportions of both species in the composition samples were quantified by SDS-PAGE instead of IEF (Marks and Fleming, 1985). The method of Marks and Fleming (1985) allows analysis of up to 30 cysts per sample. These numbers however are too low to reflect the actual composition of populations. Assuming a Poisson distribution one needs to analyse at least 25 to 30 cysts to establish the presence of 10% of either

of the species in a population with 95% probability. In this approach, aggregation in the field is not taken into account (Fox and Atkinson, 1986). We therefore use samples of at least 500 cysts to determine species ratios. Furthermore, IEF gives narrow protein bands which are difficult to quantify.

Variation found in assessing standard curves (Fig. 2) was circumvented by taking peak area quotients of the proteins per lane. Because ratios are measured instead of absolute amounts the actual population densities of *Globodera pallida* and *G. rostochiensis* have to be derived from counts of the total eggs and larvae contents of a given sample.

The technique presented here estimates proportions of *Globodera pallida* and *G. rostochiensis* with 3-6% deviation at the 95% probability level within the range of 10-90% of each species. The method is useful in monitoring mixed populations for research purposes. In monitoring routine field populations for advisory purposes the method may be too laborious. Also more information on protein contents of single species populations under field conditions is needed to generalize this method. Tests based on ELISA (Schots, 1988) or DNA-probes (Burrows and Perry, 1988) may offer in the near future better possibilities.

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Samenvatting

Het kwantificeren van Globodera rostochiensis en G. pallida in mengpopulaties door gebruik te maken van hun soort-specifieke thermostabiele eiwitten

Een methode is ontwikkeld om de samenstelling van soortenmengsels van *Globodera rostochiensis* en *G. pallida* te kwantificeren. Bij deze methode wordt gebruik gemaakt van de soort-specifieke thermostabiele eiwitten die met behulp van SDS-PAGE gescheiden worden. De kleurintensiteit van het 17 kD eiwit van *G. pallida* en het 18 kD eiwit van *G. rostochiensis* is per gel-laan bepaald m.b.v. een densitometer en heeft een lineair verband met de soortsverhouding in de mengsels ($R^2 = 0.93$). Binnen het bereik van 10 tot 90% van elke soort kan men met deze ijklijn met 95% betrouwbaarheid de soortsamenstelling bepalen op 3 tot 6% nauwkeurig.

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