Comparative Responses of *Globodera rostochiensis* and *G. pallida* to Hatching Chemicals

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Abstract: *Globodera rostochiensis* and *G. pallida* responded similarly to hatch stimulation by potato root leachate, but proportionally more second-stage juveniles (J2s) of *G. rostochiensis* hatched than of *G. pallida* in response to picrolonic acid, sodium thiocyanate, alpha-solaneine, and alpha-chaconine. Fractionation of the potato root leachate identified hatching factors with species-selective (active toward both species but stimulating greater hatch of one species than the other), species-specific (active toward only one species), and -neutral (equally active toward both species) activities. In a comparison of two populations of each of the two potato cyst nematode (PCN) species, however, greater similarity in response to the individual hatching factors was observed among populations of different species produced under the same conditions than among different populations of the same PCN species. Smaller numbers of species-specific and species-selective hatching factor stimulants and hatching inhibitors than of hatching factors were resolved. In a study to determine whether the different hatching responses of the two species to the same root leachate were associated with different ratios of species-selective and species-specific hatching factors, *G. rostochiensis* pathotype Ro1 exhibited greater hatch than did *G. pallida* pathotype Pa2/3 in response to leachate from older plants (more than 38 days old), while *G. pallida* exhibited greater hatch in response to leachate from younger plants (less than 38 days old); the response of *G. pallida* pathotype Pa1 with respect to plant age was intermediate between the other two populations. Combined molecular exclusion-ion exchange chromatography of the root leachates from plants of different ages revealed an increase in the proportion of *G. rostochiensis*-specific and -selective hatching factors as the plants aged.

Key words: *Globodera pallida*, *Globodera rostochiensis*, glycoalkalooids, hatching factors, hatching factor stimulants, hatching inhibitors, potato cyst nematode, potato root diffusate, potato root leachate.

There are two species of potato cyst nematode (PCN): the golden potato cyst nematode, *Globodera rostochiensis* (Woll.), and the white potato cyst nematode, *Globodera pallida* Stone. Large-scale hatch of second-stage juveniles (J2) from eggs within cysts of both PCN species occurs in response to potato root leachate (PRL) (Widdowson and Wiltshire 1958), which contains specific compounds known as hatching factors (HFs). Hatching factors in PRL are known to cause a calcium-dependent permeability change of the inner lipid layer of the eggshell membrane (Clarke and Perry, 1985; Clarke, 1978), which results in the escape of trehalose from the perivitelline fluid; an influx of water follows, which leads to J2 rehydration (Ellenby and Perry, 1976), movement, and, eventually, hatch.

Despite the morphological similarities between the two PCN species, only 30% of their polypeptides (as resolved by 2-dimensional gel electrophoresis) were found to be common (Bakker and Bouwman-Smits, 1988). Since the decision was made to separate PCN into two species (Stone, 1973), there has been much evidence to suggest that many aspects of their respective life-cycles (including J2 hatch) are markedly different. Greet (1974) and Den Nijs and Lock (1992) reported that *G. pallida* has an intrinsically lower spontaneous hatch (in the absence of HFs) in vitro than *G. rostochiensis*: a lower *G. pallida* spontaneous hatch in the absence of the host plant may be a major factor contributing to the slower decline rate of this species during crop rotation in the field (Whitehead, pers. comm.). There is also abundant evidence in the literature to suggest that potato-induced hatch in vitro is more rapid from cysts of *G. rostochiensis* than those of *G. pallida* (Evans, 1983; Marshall, 1984; McKenna and Winslow, 1972; Parrott and Berry, 1976; Robinson et al., 1987; Salazar and Ritter, 1993; Whitehead, 1992). Furthermore, several studies have shown that the optimal in vitro hatching temperature for *G. pallida* is lower than that for *G. rostochiensis* (Franco, 1979; Parrott and Berry, 1976; Robinson et al. 1987). Devine et al. (1996) demonstrated the presence of at least 10 HFs active toward *G. rostochiensis* in PRL. Analysis of purified HFs showed that they are closely related structures, with the same molecular weight but different mass spectrometry profiles (Devine and Jones, 1999). In addition to HFs, Byrne et al (1998) have shown that PRL also contains at least two other classes of hatching compound active toward *G. rostochiensis*: hatching inhibitors (HIs) and hatching factor-stimulants (HFS). Hatching inhibitor compounds inhibit HF-induced hatch (this inhibition was shown to be reversible and not associated with the death of unhatched J2 in the eggs) (Byrne et al., 1998). Hatching factor-stimulant compounds, on the other hand, are hatch-neutral host chemicals that can nevertheless stimulate the amount of HF-induced hatch when eggs are incubated in HF and HS mixtures (Byrne et al., 1998).

The aim of the research described here was to investigate whether *G. rostochiensis* and *G. pallida* exhibited qualitative or quantitative differences in vitro response to the same hatching chemicals, i.e., are indi-
vidual hatching chemicals PCN species-specific (affecting only one PCN species) or species-selective (affecting one species more than the other)? Among the HF s studied were artificial HFs (such as sodium metavanada-
date) and natural HFs present in potato root leachate (such as alpha-solanine and alpha-chaconine).

Materials and Methods

Nematodes: Four PCN populations (two each of G. rostochiensis (pathotype Ro1) and G. pallida (pathotype Pa2/3)) were used in the initial PRL fractionation experiments. These populations came from two different sites (site H in the Netherlands and site I in Northern Ireland), with one population of each species from each site. The four PCN populations were denoted by the codes Ro1 (H), Pa2/3 (H), Ro1 (I), and Pa2/3 (I). In all other experiments, unless stated to the contrary, only the populations from site I were assayed; one population of G. pallida pathotype Pal from this site also was included in the experiment investigating the effect of host-plant age on PCN hatch and in an inter-population study of response to picrolonic acid. All cysts used were from single-generation populations, i.e., all cysts from each population were produced in the same year and were 2 years old. Only cysts that passed through a 500-µm aperture sieve and were retained on a 300-µm aperture sieve were used (Twomey et al., 1995). Prior to use in the assays, cysts were soaked on filter paper discs saturated with distilled water in petri dishes at 22 °C for 7 days.

General assay procedure: All assays described here used a modified version of the microtitre plate assay technique described by Twomey et al. (1995) in which the cysts were suspended in a mesh thimble in a test chemical aliquot in a well. The changes made in modifying the standard bioassay unit of Twomey et al. (1995) were as follows: (i) elimination of the plate containing the thimbles, with cysts resting directly on the bottom of the well, and (ii) reduction in test sample volume (from 340 µl to 70 µl per well). These modifications were shown to have no significant effect on the hatch response of the two species (Byrne, 1997) and had the advantages of reducing both the bench space/growth room area required for assays and the amount of sample required for bioassay. The numbers of J2 that hatched were counted weekly over the incubation period. The unit of hatch (unless otherwise specified) was the percentage of J2 that hatched from all cysts over ve cysts over a 10-concentration logarithmic dilution series, with a stock HF solution concentration of 10 mg/ml. A PRL preparation (pre-treated with Amberlite XAD-4 exchange resin to remove hatch-inhibitory inorganic nutrient salts) (Twomey et al., 1995) also was assayed at 10 concentrations in a logarithmic dilution series (with a stock PRL concentration of 1 mg/ml). J2 hatch was counted each week for 3 weeks using three replicates per treatment.

Hatching chemical fractionation and assay: Six pre-sprouted tubers of potato cv. Kerr’s Pink were planted in pre-washed gravel in a PRL collection box (Twomey et al., 1995) that measured 430 × 350 × 120 cm. Potato ‘root and haulm’ leachate (Byrne et al., 1998) was collected each day for 5 days (starting 2 weeks after shoot emergence) as follows. Each day, 2 liters of distilled water was sprayed onto the potato haulms (applied as 1 liter at the start and end of each day), allowed to drip down into the rooting medium, and drained from the gravel the following morning. A 1-ml aliquot of a highly concentrated PRL preparation (concentrated 800-fold by rotary evaporation at 30 °C) was applied to a Sephadex G-10 column (2 cm × 43 cm) and eluted at a rate of 1 ml/min. Column preparation, calibration, and fractionation parameters were as described by Devine et al. (1996). The distribution coefficients (Kd) of the compounds eluted from the column were determined as follows:

\[ K_d = \frac{(V_e - V_o)}{(V_t - V_o)} \]

where \( V_e \) = elution volume of test material, \( V_o \) = void volume, and \( V_t \) = total bed volume of the column.

The first 50 (HF activity) or 40 (HS and HI activity) fractions eluting from the Sephadex G-10 after the void volume were assayed for HF (using all four PCN populations), HI, and HS activities (using PCN populations Ro1 (H) and Pa2/3 (H)), according to the procedure of Byrne et al. (1998).

The hatching activity of each fraction was assayed at two concentrations: the original concentration and a 1:10 dilution. A PRL preparation (at a concentration that previously had been shown to induce maximal hatch of all four populations) was used as the positive control, and the column running buffer was used as the negative control. The percentage hatch induced by each fraction was then expressed as a percentage of hatch achieved by the same PCN population in the positive control. In this way, variation in hatching potential of eggs between the populations was minimized. Active fractions (those deemed to contain HFs) were those that induced hatch greater than the mean value obtained from the negative controls plus 95% confidence limits. The presence/absence of HI and HS compounds in each fraction was determined by assaying each fraction in both the presence and absence of a PRL “spike” following the method of Byrne et al.
The presence of HI or HS activity in a fraction was demonstrated by a significant ($P < 0.05$) increase (HS) or decrease (HI) in observed hatching activity (in the “fraction + spike” mixture) relative to the mean hatching activity expected if the effects of the individual activities “fraction alone” and “spike alone” samples were simply additive (Byrne et al., 1998).

**Presentation of the PRL hatch activity:** An experiment was designed to develop a single term that would accurately summarize all the hatch data generated from the assay of an individual PRL preparation over several concentrations. For each PRL sample, a Hatch Activity (HA) value was calculated in a two-step procedure:

1. For each dilution of a preparation within a replicated dosage-response study, a hatch rating (HR) value was calculated:

   $$HR = \frac{(% \text{ hatch} \text{ fraction} + \text{ spike}) \times [\log_{10} \text{ dilution of PRL} + 1]}{[\log_{10} \text{ dilution of PRL} + 1]}$$

   (The addition of 1 to the PRL dilution was necessary as $\log_{10}$ of the most concentrated preparation (1:1) would otherwise give a value of zero (i.e., $\log_{10} (1) = 0$).)

2. The HA value for each replicate of the PRL being assayed was then calculated:

   $$HA = \Sigma HR / \text{No. of PRL dilutions}$$

This method also allowed the calculation of HA values for the negative buffer controls by assuming that the % hatch values in the HR formula are the same for all

**Activity = % hatch (fraction + spike) − [% hatch (fraction) + % hatch (spike)]**

The assay was designed to eliminate environmental factors that might favor the hatch of one PCN species over the other (e.g., by employing an incubation duration of 3 weeks and an incubation temperature of 22 °C). After the incubation period, the optimal hatch responses of *G. rostochiensis* and *G. pallida* to PRL were
not different (Fig. 1), whereas alpha-solanine and alpha-chaconine stimulated a greater ($P < 0.05$) hatch response in *G. rostochiensis* than in *G. pallida*. The hatch response of the latter species to these two potato-specific glycoalkaloids present in PRL (Byrne, 1997) was not significantly greater than that in water. The general trend was that *G. rostochiensis* responded more actively to natural and artificial HFs (resulting in higher hatch) than did *G. pallida* (two-way parametric ANOVA, PCN species Main Effect, $F(1,32) = 7.74; P < 0.01$) despite the fact that the two species responded similarly to PRL. Apart from the glycoalkaloids, this difference between the two species was significant only for picrolonic acid and sodium thiocyanate. The greater hatch of *G. rostochiensis* than *G. pallida* in picrolonic acid ($P < 0.05$) is in agreement with the findings of Greet (1974).

When the picrolonic acid study was expanded to include the Pal pathotype of *G. pallida* from the same site (I) as the two other pathotypes, differences ($P < 0.05$) in the percentage hatch induced by picrolonic acid were obtained between each of the three pathotypes: Ro1 (72.3%), Pa2/3 (0.3%), and Pa1 (25.7%). The Pa2/3 response to this artificial HF was below the water hatch (1.0%) of this pathotype. Broadly, the results presented in Figure 1 suggest that the *G. pallida* hatch response is intrinsically more conservative than that of *G. rostochiensis* (i.e., fewer compounds were capable of initiating significant *G. pallida* hatch).

**Resolution of HFs from PRL:** Fractionation of HFs on Sephadex G-10 occurred on the basis of size (molecular weight range 0–700 da) and secondary interactions, primarily ion exchange chromatography (Devine et al., 1996). Comparison of the hatch profiles obtained at the two concentrations (undiluted and 1:10 concentration) indicated that lower hatch occurred at the original concentration of the most active fractions ("supraoptimal inhibition"). Hence, only the 1:10 profiles are presented.

When the fractions from the ‘root and haulm’ leachate were assayed for their ability to induce hatch in each of the four PCN populations, up to 16 peaks of activity were discernible (Fig. 2), each representing at least one HF. Most of the activity was associated with 8 to 10 peaks, an observation similar to that reported earlier (Devine et al., 1996).

Both qualitative and quantitative differences were observed between the responses of the different PCN populations to individual HF peaks. For example, the minor peak HF 12 appeared to be specific for *G. rostochiensis*, with *G. pallida* not responding to this HF (Fig. 2). Further examination, however, revealed that there were more similarities between the profiles of the *G. rostochiensis* and *G. pallida* populations from the same site than between the two populations of the same species from different sites. For example, both populations from site H responded maximally to HF 3, whereas the two populations from site I responded maximally to HF 2 (i.e., individual HFs were population-selective). This relationship was supported (particularly in the case of *G. rostochiensis*) by correlation analysis between percentage hatch of the different populations in response to
the individual fractions eluted from the Sephadex G-10 column. A positive correlation was obtained over the 50 fractions between the hatch of the two populations collected from site I ($r = 0.84$, $n = 50$; $P < 0.01$) and between those from site H ($r = 0.91$, $n = 50$; $P < 0.01$). Although correlations ($n = 50$; $P < 0.01$) also were obtained between the responses of the two *G. rostochiensis* and two *G. pallida* populations, the respective coefficients were lower (0.65 and 0.83, respectively). The similarities in HF responses between populations of the two PCN species from the same site suggest local adaptation, either environmental (via cues from the infected plant responding to abiotic factors) or genetic, to modify hatch response and thereby maximize infectivity (in the presence of the host) and/or persistence (in its absence).

When the two species from site H were assayed for response to HS and HI compounds in the fractions, little similarity was found between the responses (Fig. 3). Ten and twelve zones of HS activity (labeled A to O in Fig. 3) were identified for *G. rostochiensis* and *G. pallida*, respectively. Of these, five (L, I, O, M, and N) were active toward both species; the others were species-specific (e.g., zone H for *G. pallida*, zones D and K for *G. rostochiensis*). Many of the zones exhibiting HS activity for both species also corresponded to zones of HF activity (compare Figs. 2A,C and 3A,B), and this hatch synergy may have been due to HF-HF interactions. True HS activity was clearly evident in fractions in zones L–O (from which HF activity was absent) for both species (with additional species-specific HS activities toward *G. pallida* in zone C and toward *G. rostochiensis* in zone K).

Fewer peaks of HI than HF or HS activity were observed—four for *G. rostochiensis* and three for *G. pallida*. A major zone of HI (G*) was active toward both PCN species.

The finding of PCN species-specificity and species-selectivity toward individual HFs could explain reported differences in the hatch response of *G. pallida* and *G. rostochiensis* to the same leachate. Inter-cultivar (Evans, 1983) and inter-species (Devine and Jones, 2001) differences in hatch induction of the two PCN species could be attributed to varying proportions of HFs selective or specific for individual species. To test this hypothesis, the model system used was the effect of plant age on the induction of PCN hatch. However, before this was tested, a suitable method of hatch expression was devised.

**Expression of PRL hatch activity:** To determine the true hatching activity of a PRL sample, it is necessary to consider PCN hatch over several concentrations. By plotting percentage PCN hatch against PRL dilution, Fenwick (1952) determined the hatching curve to be curvilinear. The dosage-response relationship was linearized by logarithmic transformation of the PRL dilution, and the log of the dilution at which hatch equaled that found in water was termed the Log Activity (LA) value. However, since the downward side of the hatching curve is essentially sigmoidal, the fitting of a straight line to the downward part of the hatching curve is somewhat arbitrary because it requires careful selection of only those data points considered to be on the linear portion of the curve. Furthermore, Fenwick (1952) devised the LA method when it was not known that there were two PCN species. To use LA values to compare the HF responses of the two species, it has to be assumed that (i) the *G. rostochiensis* and *G. pallida* hatching curves are roughly parallel (this cannot be assumed), and (ii) the water hatch for both species is not significantly different (now known to be incorrect; Greet, 1974). Therefore, the use of LA values was deemed unsuitable for studying the effect of plant age on the hatch responses of the two PCN species, and an alternative method (the HA method) was devised. This was similar to the LA method but was based on the integrated area under the curve (total hatch for all dilutions) rather than the slope of the downward part of the curve. Accurate comparison of the HA values of different PRL samples demands that the highest concentrations of all compared leachates be the same (as is the case in LA calculations).

Although the LA method is flawed, it is tried and tested. Hatching curves involving six potato cultivars and four PCN populations gave positive correlations between LA values and HA values for *G. rostochiensis* ($r = 0.94$; $n = 5$; $P < 0.05$) and *G. pallida* ($r = 0.87$; $n = 5$; $P = 0.06$). Hence, this new method of hatch expression was considered suitable for expressing the hatching activity of a particular PRL preparation.
The primary advantages of the HA method were the elimination of the requirements for similar hatch responses in water and parallel hatching curves for the two PCN species. In addition, the arbitrary selection of suitable regression points was eliminated from the calculation.

The effect of plant age on hatching activity of PRL: From Figure 4A, it is clear that the three pathotypes exhibited different patterns of hatch response to leachates as plants aged. This is borne out by the 'plant age × pathotype' interaction ($F = 11.49; \text{df} = 26; P < 0.01$). Further analysis showed significant differences in each of the three pairwise (pathotype × plant age) comparisons ($\text{Ro1} - \text{Pa2/3}: F = 17.13; \text{Ro1} - \text{Pal}: F = 11.04; \text{Pa2/3} - \text{Pal}: F = 3.86; \text{df} = 13, P < 0.01$). Thus, the three pathotypes were shown to respond differently to the 14 different PRL samples, with the greatest similarity (as shown by the smallest $F$ value) occurring between the two $G. pallida$ pathotypes. These differences were not due to differences in percentages of J2s that were capable of hatch, as maximum % hatch values in excess of 90% were obtained for all pathotypes.

More $G. pallida$ (Pa2/3) J2s hatched than did those of $G. pallida$ (Pa1) and $G. rostochiensis$ (Ro1) in response to early-produced leachates; HA values towards Pa2/3 of leachates from 4 to 7-day and 8 to 10-day-old host plants were greater ($P < 0.05$) than those of both Pal and Ro1. A hatching peak for both Ro1 and Pa2/3 occurred in response to the leachate produced by 8 to 10-day-old host plants. The first Pa1 hatching peak was slightly later than that of both Ro1 and Pa2/3, occurring in response to PRL from 11 to 14-day-old host plants. A number of later hatching peaks and troughs were evident for all three pathotypes. The time course profiles of Pa2/3 and Pa1 were very similar from 15 to 17 days onward, with a broad peak between days 25 and 35 followed by a gradual decline to 46 to 49 days, and then a steady increase to 53 to 56 days. Although the hatching response of $G. rostochiensis$ to leachates from plants up to day 35 was less than that of $G. pallida$, more $G. rostochiensis$ than $G. pallida$ hatched from 36 to 45 days. All three pathotypes exhibited a steady increase in hatch from 46 to 49 days to 53 to 56 days. Overall, the Pa1 hatch response appeared to be intermediate between that of Ro1 and Pa2/3, supporting the earlier observation of the different responses of the pathotypes to picrolonic acid.

As was evident from the peak/trough hatching pattern in Figure 4A, HF production by the host plant was not steady over the 56-day period but seemed to occur in distinct phases. The phenomenon of the hatching activity of host leachates toward a cyst nematode varying with the stage of plant growth has also been demonstrated with *Heterodera goettingiana* (Perry et al., 1980).

The observation that the two PCN species responded differently to the same leachate (e.g., $G. rostochiensis$ hatch increased with plant age from 32 to 38 days whereas that of both $G. pallida$ pathotypes declined over this period) suggests that differences in hatch dynamics between the two species may be due to differential response to individual HFs in the leachate. To investigate this hypothesis, the HFs in leachates exhibiting differential $G. rostochiensis$/G. pallida response were resolved by low pressure liquid chromatography.

Two leachates, to each of which the PCN species showed different responses (4 to 7 days: $G. pallida$ hatch > $G. rostochiensis$ hatch; 39 to 42 days: $G. rostochiensis$ hatch > $G. pallida$ hatch), were chosen for resolution of hatching activity. In leachate from the young plants, most of the HFs were species-specific, with $G. pallida$-
specific HF(s) eluting from Sephadex G-10 ahead of G. rostochiensis-specific HFs (Fig. 4B). The leachate from the older plants contained increased amounts of G. rostochiensis-selective HFs (zones 1 to 4 in Fig. 4C). However, the fractionation of PRL from 4 to 7-day-old plants had revealed G. pallida-selective HFs in this same region (Fig. 4B). There are two possible explanations. The first is that, although the G. pallida-selective HFs in PRL from young plants (zones 1 to 4 in Fig. 4B) eluted in the same region as the G. rostochiensis-selective HFs (zones 1 to 4 in Fig. 4C), these were different sets of compounds. The second possibility is that these HFs (in zones 1 to 4 in Figs. 4B and 4C) were, in fact, the same compounds and that they became selectively active toward one of the two PCN species in the presence of a second co-eluting species-selective HS or HF compound. For example, HS zones D and F (Fig. 3A), which eluted in this area, were selective toward G. rostochiensis. It may be that young potato plants do not produce these G. rostochiensis-specific hatching compounds (explaining the poor hatch response of this species to HFs in zones 1 to 4 in Fig. 4B). The involvement of hatching chemicals in addition to HFs is suggested by Figure 4C, where G. rostochiensis hatched in higher numbers than G. pallida to individual fractions, whereas the difference in hatching response of the two PCN species to the corresponding leachates was much smaller (Fig. 4A).

Recognition that the two PCN species respond differently to natural and artificial HFs opens up several new avenues for fundamental and applied research. The availability of species-selective HFs will facilitate studies on the mode of hatch induction (e.g., receptor-ligand interactions). It is also important to recognize that PRL is a dynamic product, changing in composition as the plant ages. Figure 4 reveals marked differences in the hatching responses of the two PCN species to leachates from plants at different stages of growth. In studies by other authors, PRL typically has been collected from 6 to 7-week-old potato plants, whereas hatching in soil is maximal in the first 3 to 4 weeks after planting (Brodie, 1998; Marshall, 1998). The use of leachates from older plants may well be inappropriate when attempting to mimic in-soil behavior with in vitro studies.

From a practical point of view, the induction of ‘suicide hatch’ of PCN by direct application of HFs to infested soil in the absence of the host plant has potential as an environmentally protective PCN control strategy (Devine and Jones, 2000). The use of pure HF preparations such as picrolonic acid (Whitehead, 1977) and sodium metavanadate (Whitehead, 1992) could be ineffective if the effect is PCN species-selective (as with picrolonic acid). Mixed-HF preparations, as in PRL, would minimize such problems as well as reduce the risk of selection of nonresponsive PCN populations.

**Literature Cited**


