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Estimation of the viability of *Heterodera schachtii* field populations by measuring ATP, ADP and AMP contents of eggs and juveniles using HPLC

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Abstract

An HPLC method has been developed to determine adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) in eggs and juveniles of *Heterodera schachtii* in cysts extracted from soils. ATP levels in eggs and juveniles were highly correlated with the numbers of hatched juveniles in root diffusate ($r = +0.92$) in series of soil samples originating from field trials testing different doses of fumigant nematicides. The measured ATP concentrations in the cysts explained 59% of the sugar-yield reduction on these fields, which was similar to the value based on the hatching test. After fumigation, carried out in a laboratory experiment, ATP and the adenylate energy charge, $(AEC = ATP + 0.5 * ADP)/(ATP + ADP + AMP)$, decreased greatly within a few days. In a series of greenhouse trials in which *H. schachtii* had been parasitized severely by *Verticillium clamydosporium* and other antagonists, there were significant correlations between the numbers of hatched juveniles and the ATP content of the cysts ($r = +0.85$), and between the percentages of hatched juveniles and the AEC ($r = +0.75$). The HPLC method is faster than conventional methods and can be used to establish the viability of *H. schachtii* field populations affected by soil fumigation or egg pathogens.

Introduction

Systems of integrated pest management involving cyst-forming nematodes require estimates of the populations of viable eggs and juveniles. The viability of *Heterodera schachtii* and *H. trifolii f. sp. betae* can be influenced by the application of fumigants and the activity of egg pathogens such as *Verticillium chlamydosporium* and *Nematophthora gynophyla* [Maas and Heijbroek, 1982; Crump and Kerry, 1987].

For experimental purposes, a hatching method using oil-seed rape diffusate as a hatching agent is commonly used to determine the viability of beet-cyst nematode populations [Steele *et al.*, 1982]. However, this method is time consuming and laborious. Atkinson and Ballantyne [1977a] developed a rapid method to determine field populations of *Globodera* spp. measuring the ATP content of cysts using bioluminescence [Strehler, 1974]. This enabled the effect of fumigants [Storey and Atkinson, 1979; Storey, 1982] or resistant potato cultivars [Storey and Mark, 1983] on pathotypes of *Globodera* spp. to be assessed.

High Pressure Liquid Chromatography (HPLC) has the advantage that the equipment is widely available and, in contrast to the method using bioluminescence, it can be used to measure directly and simultaneously ATP, ADP and AMP. This allows the calculation of the adenylate energy charge, $AEC = (ATP + 0.5 * ADP)/(ATP + ADP + AMP)$, [Viarengo *et al.*, 1986]. The AEC can provide additional information about the viability of the cyst contents [Atkinson and Ballantyne, 1977b].

We developed an HPLC method for direct detection of ATP, ADP and AMP in eggs and juveniles of *H. schachtii* recovered from cysts that have been extracted from field soils. The accuracy of this method in detecting the viability of field populations and the efficacy of nematocidal fumigants is compared with that of conventional hatching and counting methods.

Materials and methods

Sampling
Soil samples consisting of 20 cores were taken from each plot before cropping. Subsamples for the determination of eggs and juveniles for the hatching test and for the HPLC analysis consisted of 100 ml of soil.

Analysis of cysts and their contents
Cysts were extracted by the centrifugation method, using a saturated solution of magnesium sulphate [Coolen, 1979]. After centrifugation, cysts were collected on a cheese cloth filter and separated from debris. After identification, the cysts were crushed in about 10 ml water using a Braun Potter S homogenizer, and the numbers of eggs and juveniles were counted to determine the initial densities.

Hatching tests
The population of viable eggs and juveniles of *H. schachtii* was determined by placing all filled cysts in Jasper's and Kort's hatching tubes as described by Hoestra [1978]. At least 20 cysts per sample were needed for accurate determination of viability. Cysts were incubated at 25 °C in a closed box with polythene foam drenched in water to avoid drying out. After pretreatment with Streptomycine sulphate, oilseed rape diffusate was added. Every week the hatched juveniles were counted and the diffusate was renewed. After three weeks, cysts were crushed and the remaining egg content was determined [Maas and Heijbroek, 1982].

HPLC analysis
Apparatus. An HPLC apparatus with solvent delivery system (SP 8800, Spectra Physics) and UV/VIS detector (Spectra 200, Spectra Physics) was used with a Spherisorb 5 ODS-2 column (l = 250 mm, i.d. = 4.6 mm).

Standards
ATP: adenosine-5′-triphosphate.3 H_2O, di-sodium salt (Boehringer, Mannheim)
ADP: adenosine-5′-diphosphate, di-sodium salt (Boehringer, Mannheim)
AMP: adenosine-5′-monophosphate.6 H_2O, di-sodium salt (Boehringer, Mannheim)

Analytical conditions. Mobile phase: The buffer solution was prepared by adding 0.2 M Na_2HPO_4 solution to 0.2 M KH_2PO_4 solution until pH = 6.25. The buffer was filtered through a 0.45 μm membrane filter and air was removed by vacuum in an ultrasonic bath. The mobile phase was permanently degassed with helium.

Flow: 1.5 ml min^{-1}
Wave length: $\lambda = 260$ nm
Injection volume: 200 μl
Retention times: ATP = 4.6 min, ADP = 5.3 min, AMP = 8.1 min

Calibration. Series of standard solutions for calibration were prepared by dissolving ATP, ADP and AMP in buffer with the same composition of the mobile phase. Final concentrations of the standard solutions: 10, 50, 100, 500, 1000 and 2000 μg/l for each adenine nucleotide. Each standard solution was analysed in duplicate and the calibration curves were calculated.

Sample preparation. Cysts were crushed in about 1 ml buffer solution with the same composition of the mobile phase, using a Braun Potter S homogenizer. The content was transferred to a 10 ml tube with buffer solution to a total volume of nearly 3 ml. The buffer with cysts was boiled for a few seconds over a Bunsen burner, and then cooled in an ice bath. The buffer was made up to 3 ml and the solution filtered through a 0.45 μm membrane filter and injected for HPLC analysis.

Recovery. Standard solutions, containing about 0.3 nmol ATP, ADP and AMP per ml, were analysed after the same treatment as described under sample preparation, including a threefold dilution from 1 ml to a final volume of 3 ml. The results were compared with the analyses of diluted standard solutions without preteatment to establish the recoveries for ATP, ADP and AMP.

Field trials with soil fumigation
Trials were initiated on fields where previous soil sample analysis suggested economic damage by *H. schachtii* was likely. They were laid out as randomized blocks with four replicates of each treatment.

Soil fumigation was carried out on two trials in autumn 1990 (Oud-Vossemeer and Halsteren) and on four trials in autumn 1991 (Halsteren, Kruisland, Ouwerkerk and Swifterbant). In the 1990 trial at Halsteren, unfumigated blocks were compared with those receiving 150 l dichloropropene per ha. The aim of the other trials was to investigate the efficacy of reduced rates of cis-dichloropropene purified from the biolog-

ically inactive trans-dichloropropene. All 1991 trials comprised fumigation with 0, 50, 75, 85 and 100 l cis-dichloropropene per ha.

Soil samples for the determination of eggs and juveniles were taken at least one month after soil fumigation and stored at 4 °C. Hatching tests and ATP analyses were carried out within 6 months.

At harvest, root weight and sugar content were determined using standard procedures [De Nie and Van der Poel, 1991] and sugar yield was calculated.

Fumigation experiment for measuring changes of ATP, ADP, AMP and AEC in time

Fifty cylinders (height: 25 cm, diameter: 6.5 cm) were filled with soil, containing 3000 to 5000 eggs and juveniles of *H. schachtii* per 100 ml, and placed in dishes containing water to a depth of 6 cm. In 25 cylinders 100 μl dichloropropene was injected at 18 cm depth; the remaining 25 cylinders were not fumigated. The cylinders were covered with aluminum foil and stored at 15 °C.

After 1, 2, 7, 14 and 28 days soil samples were taken from four fumigated and four unfumigated cylinders. The cysts were extracted and the viability of the eggs and juveniles was determined by hatching tests and ATP, ADP and AMP analysis, from which the AEC was calculated. After 30 days, the remaining cylinders were heated to 80 °C for 4 h and after a further 3 days soil samples were taken for analysis.

Experiments with V. chlamydosporium *and other antagonists of* H. schachtii

Two experiments were carried out in a greenhouse with pots containing soil with different levels of antagonists of *H. schachtii*. Two sugar-beet seeds were sown per pot, one of which was removed after establishment.

In the first experiment soil taken from the trial field in 1991 at Halsteren was used. This contained 2,000 to 20,000 eggs and juveniles of *H. schachtii* per 100 ml of soil, which were infected by the egg parasites *V. chlamydosporium* and *Cylindrocarpon destructans* to such an extent that hatching did not exceed 16% of all eggs present. To create differences in the degree of parasitism, this soil was diluted 2 and 10 times with silver sand and 500 hatched juveniles were added to half of the pots containing each dilution.

In the second experiment 500 hatched juveniles were added to potting compost and silver sand. In one set of treatments chlamydospores of *V. chlamydosporium* were added to create differences in the degree of parasitism.

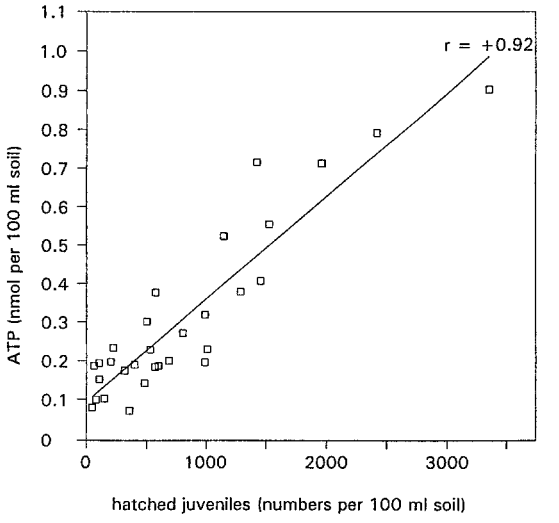

Fig. 1. Correlation between total numbers of hatched juveniles and ATP content of cysts per 100 ml of soil in a series of six field trials.

In both experiments the plants were harvested after 13 weeks; the newly formed cysts in the soil matured for a further five weeks before hatching tests and ATP, ADP and AMP determinations were carried out.

Results and discussion

HPLC analysis

The detection limit for ATP, ADP and AMP is 0.01 nmol ml^{-1} solution, corresponding to 0.03 nmol per 100 ml of soil.

Based on 14 determinations the percentage recoveries (± standard deviations) for ATP, ADP and AMP were 96 ± 6, 92 ± 10 and 100 ± 12, respectively.

Field trials with soil fumigation

Linear regression analysis of the results from six field trials showed a good correlation ($r = +0.92$) between the mean numbers of hatched juveniles for each treatment and the corresponding ATP concentrations in the cysts, expressed per unit volume of soil (Fig. 1).

The lowest measured ATP value was 0.08 nmol per 100 ml of soil, which was considerably above the detection limit. The results showed a mean calculated ATP content in the cysts of about 0.4 pmol per hatched juvenile. This is similar to the ATP contents of viable eggs in cysts of *G. pallida* and *G. rostochiensis* [Atkinson and Ballantyne, 1977a]. However it should be noted that ATP is still present in cysts without viable eggs and juveniles.

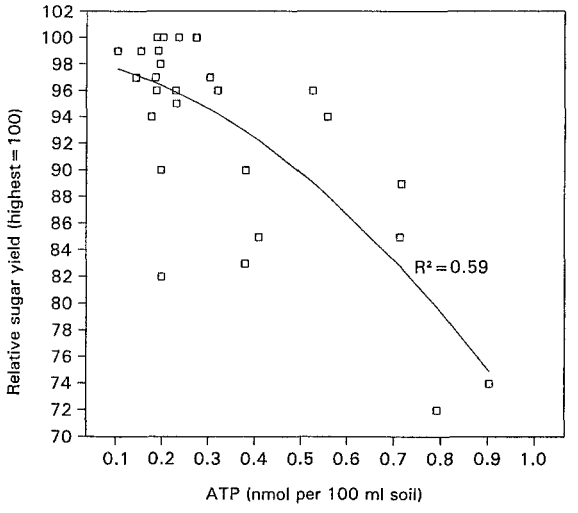

Fig. 2. Correlation between the ATP content and the relative sugar yield in a series of six field trials. The highest sugar yield on each field is set to 100.

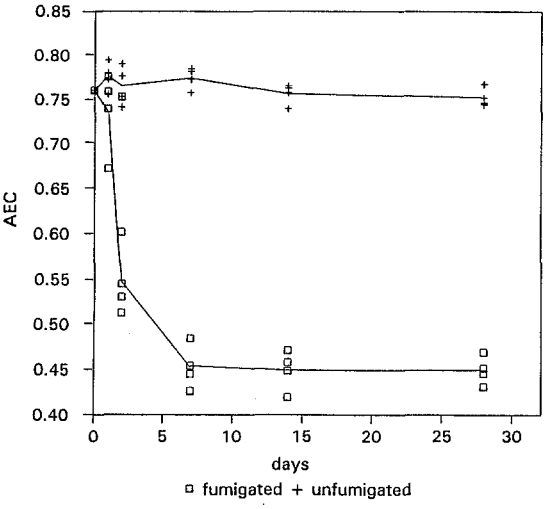

Fig. 4. AEC of cysts from fumigated and unfumigated soils in time. Laboratory trial in cylinders at 15 °C. The individual results of each replicate are shown. The lines connect the means of the four replicates at each time interval.

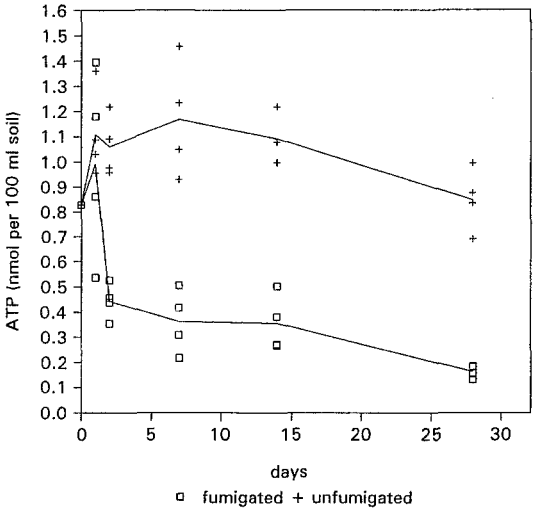

Fig. 3. ATP content of cysts from fumigated and unfumigated soils in time. Laboratory trial in cylinders at 15 °C. The individual results of each replicate are shown. The lines connect the means of the four replicates at each time interval.

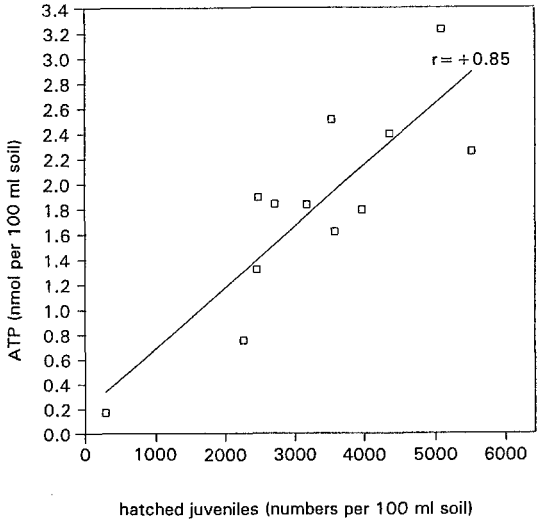

Fig. 5. Correlation between the numbers of hatched juveniles and the ATP content of the cysts in experiments in a greenhouse with pots containing soil with different levels of parasitizing fungi.

In Fig. 2 the ATP contents of cysts from each treatment are plotted against the relative sugar yield, to estimate whether the reduction in sugar yield by *H. schachtii* corresponded to the viability of the population as measured by the ATP method. The treatment producing the highest sugar yield was set to 100 for each trial, on the assumption that fumigation would reduce the population below the tolerance limit. In most fields this was the case.

Statistical evaluation [Payne RW, 1994] showed that 59% of the variation in relative sugar yield in these fields was explained by a model using a quadratic fit (relative sugar yield $= a+b*\mathrm{ATP}+c*\mathrm{ATP}^2$). Negative deviations from the regression line may be due not only to inaccuracies in the determinations but also to reductions of the sugar yield caused by other factors. A same model based on the results of the hatching tests account for a similar amount (61%) of the variation in

relative sugar yield. This suggests that ATP measurement is as reliable as a hatching test in predicting the efficacy of soil fumigation.

Fumigation experiment for measuring changes of ATP, ADP, AMP and AEC in time

After fumigation, the mean ATP content of the cysts decreased quickly to 0.45 nmol per 100 ml of soil within two days, as the ATP content of the untreated cysts remained at about 1.0 nmol per 100 ml of soil. During the next four weeks the ATP content of the treated cysts continued to decrease slowly, to a mean of 0.16 nmol per 100 ml of soil (Fig. 3).

The mean calculated ATP content in the untreated cysts was 0.4 pmol per hatched juvenile, which is similar to the ATP levels per hatched juvenile found in the field trials.

Based on the ATP levels the calculated mortality caused by fumigation is 84%. However the hatching tests showed that the mean hatching percentage declined from 65% in untreated soil to less than 0.5% one week after fumigation and even less than 0.1% four weeks after fumigation. This means that a few weeks after fumigation hardly any juvenile hatched, although ATP was still present. This may be caused by ATP from other sources, such as fungi, or by an insufficient AEC of the eggs and juveniles in the treated cysts.

After fumigation ADP decreased slightly, while AMP increased. This resulted in a rapid decrease of AEC from 0.76 to 0.45 during the first week, after which it remained constant (Fig. 4).

It can be concluded that at an AEC below 0.5 juveniles do not hatch. These results are in agreement with theoretical and emperical considerations that in all organisms under physiologically optimal conditions values of AEC would be between 0.8 to 0.9 and that organisms showing an AEC below 0.50 do not recover when returned to optimal conditions [Atkinson, 1968; Chapman, Fall and Atkinson, 1971]. Therefore, calculation of the AEC can provide information about the efficay of treatments.

The results show that the effects of fumigation can be established by ATP measurement a few days after treatment, as the effect on AEC is obvious after one week.

Killing all eggs and juveniles by heating the soil to a temperature of 80 °C for 4 h reduced ATP further to 0.03 nmol per 100 ml of soil in the fumigated samples and to 0.08 nmol per 100 ml in the unfumigated samples. Heating the samples reduced ADP to 0.11 nmol per 100 ml of soil in the fumigated samples and 0.28 nmol per 100 ml in the unfumigated samples, while AMP was increased to 0.09 and 0.52 nmol per 100 ml of soil, repectively. This resulted in an AEC of 0.37 and 0.25, respectively.

Experiments with V. chlamydosporium *and other antagonists of* H. schachtii

In the experiments with soil containing cysts of *H. schachtii* which were severely parasitized with *V. chlamydosporium* and *C. destructans*, a significant correlation ($r = +0.85$) was found between the numbers of hatched juveniles and the ATP content of the cysts (Fig. 5). The numbers of hatched juveniles were calculated from the total numbers of eggs + juveniles in the cysts used for the HPLC determination and the percentages of hatched juveniles from the hatching test. This was necessary because the numbers of cysts extracted for the hatching test for some treatments deviated considerably from the numbers used for the HPLC determination.

The mean calculated ATP content in the cysts was 0.57 pmol per hatched juvenile. This is somewhat higher than the 0.4 pmol per hatched juvenile, which was found in the field trials and in the fumigation experiment for the unfumigated samples. Higher ATP levels per hatched juvenile may not only be caused by the contribution of ATP from the fungi but also by ATP from eggs and juveniles with an AEC below the critical level of viability. The AEC in the cysts varied from 0.67 to 0.78 and a significant correlation ($r = +0.75$) was shown between the percentage of hatched juveniles and the AEC for the different treatments.

These results show that, even if cyst contents are parasitized by fungi, the ATP, ADP and AMP determination with HPLC can give reliable information about the viability of the eggs in the cysts. The ATP content of the cysts is related to the number of hatched juveniles, as the AEC provided information about the percentage of the eggs and juveniles in the cysts which are able to hatch.

Conclusions

Analysis of the ATP content of filled cysts by HPLC can replace the time consuming and laborious hatching test as a method of determining the viability of *H. schachtii*.

The HPLC method can assess the efficacy of fumigants quickly within a week after application. This means that further measures can be taken if the efficacy of the fumigation is not sufficient (e.g. as a result of incorrect application or rapid degradation of active ingredients).

Additional information concerning mortality can be obtained by calculating the AEC from the ATP, ADP and AMP concentrations. No or few juveniles are likely to hatch when the AEC in the cysts is below 0.5.

The HPLC method can also be used to establish the effect of *V. chlamydosporium* and other antagonists on the viability of *H. schachtii* populations. Even in severely parasitized populations of *H. schachtii* significant correlations between (i) ATP concentrations and the number of hatched juveniles and (ii) AEC and the percentage of hatched juveniles could be detected.

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