Eur J Plant Pathol (2010) 128:459–471
DOI 10.1007/s10658-010-9673-2

Modifications in the potato rhizosphere during infestations of *Globodera rostochiensis* and subsequent effects on the growth of *Rhizoctonia solani*

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Accepted: 5 August 2010 / Published online: 6 September 2010

Abstract Two controlled environment experiments were conducted to explore the hypothesis that invasion and damage caused to potato roots by the potato cyst nematode *Globodera rostochiensis* might result in quantitative or qualitative changes in the release of root exudates to subsequently affect the growth of *Rhizoctonia solani* (AG3) in the potato rhizosphere. The growth of five *R. solani* isolates was compared on media amended either with root exudates from *G. rostochiensis*-infested or uninfested potato (cv. Désirée) plants at different time intervals after the introduction of the nematodes. In Experiment 1, the growth of *R. solani* was higher on medium amended with potato root exudates from *G. rostochiensis*-infested compared to uninfested plants, collected 4, 6, 8 and 12 days after the *G. rostochiensis* treatments were administered. Similarly, in Experiment 2, *R. solani* isolates grew faster on medium amended with potato root exudates from *G. rostochiensis*-infested than uninfested plants. This trend was particularly pronounced at the 12-day collection. At this time, 49% of the *G. rostochiensis* juveniles in roots were found to belong to the juvenile moults J2 and J3, indicating that root exudates were modified during the earlier stages of juvenile invasion. Carbohydrate analysis of root exudates indicated significantly higher levels of sucrose in root exudates from *G. rostochiensis*-infested than uninfested plants, whereas no significant differences were found in total nitrogen content. The results are discussed to help elucidate the mechanism behind the disease complex found between *G. rostochiensis* and *R. solani* in previous field research.

Keywords Black scurf · Disease complexes · Interactions · Potato cyst nematodes · Root exudates · Stem canker

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Introduction

Rhizoctonia solani Kühn anastomosis group 3 (AG3) (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) is a soil-borne fungus of potato (*Solanum tuberosum* L.) on which it causes stem and stolon canker and black scurf. It is frequently found in potato-growing areas worldwide, but infections and significant damage usually occur in areas with wet and cool soil conditions, such as in the UK, where it affects 60–80% of crops each year (Budge et al. 2002).

Initial disease symptoms of *R. solani* occur prior to crop emergence when the developing shoot tips become affected by sunken brown lesions. As the stem grows these lesions often expand and coalesce with one another to completely girdle the affected tissues. This disrupts the movement of water and nutrients throughout the plant and eventually stems may be pruned (Back et al. 2006). The collective name for these symptoms is widely known as stem canker (Read et al. 1989; Simons and Gilligan 1997). In a similar manner, stolons and roots can also be infested and pruned by *R. solani* and the resulting symptoms are known as stolon and root canker, respectively. Stem canker causes patchy and delayed emergence in crops and reduces tuber initiation (Read et al. 1989). Following the senescence or haulm destruction of potato plants, *R. solani* forms sclerotia on the daughter tubers, which appear as black 'tar' like encrustations and are widely known as 'black scurf'. Most reports claim that *Rhizoctonia* disease of potato does not usually reduce total tuber yield (e.g. Simons and Gilligan 1997). It may however substantially degrade tuber quality and marketability, which is the consequence of one or more of the following: (a) hard sclerotia on tuber surfaces (black scurf), (b) greater numbers of non-target size tubers, (c) tuber greening, (d) sessile tubers or 'little potato' and (e) malformed tubers (Scholte 1989; Simons and Gilligan 1997).

The potato cyst nematodes (PCN), *Globodera rostochiensis* (Woll.) and *G. pallida* (Stone) are economically important pests of the potato crop in Europe. In the UK, PCN are recognised as the most important pests faced by potato growers (Haydock and Evans 1998). With the exception of England and Wales (Minnis et al. 2002), *G. rostochiensis* is generally more frequent in Europe than *G. pallida* (EPPO/CABI 1997). Both species of PCN hatch from encysted eggs as second-stage juveniles (J2) in response to multiple hatching factors (HF) produced in host root exudates (Devine et al. 1996) and have an absolute requirement for host roots to feed and reproduce (Back et al. 2006). The damage caused to roots by PCN is initiated when J2 invade the roots and start moving through the root cortex dissolving cell walls. Eventually they become sedentary near the endodermis forming feeding sites called syncytia (Hooker 1981). Roots are substantially damaged and typical water and nutrient (N, P, K) deficiency symptoms (e.g. foliage yellowing and wilting) appear on the plant when large numbers of PCN juveniles feed simultaneously in the roots (Haydock and Evans 1998; Grove et al. 1999).

A review of the scientific literature on disease complexes between plant parasitic nematodes and soilborne fungi have indicated a variety of specific mechanisms involved in the interaction between these microorganisms (Back et al. 2002). Two years of field studies conducted by this research group had consistently shown a strong and positive relationship (r^2= 0.55) between invasion of potato roots by *G. rostochiensis* and infection of stolons by *R. solani* (Back et al. 2006). In view of these findings and because nematode and fungus affected different plant parts, however of close proximity (stolons and roots, respectively), the authors suggested that an indirect mechanism is probably involved in the interaction between *G. rostochiensis* and *R. solani*. It was hypothesised that damage inflicted on potato roots during invasion and subsequent colonisation by *G. rostochiensis* might result in quantitative and/or qualitative changes in the release of root exudates, which could subsequently influence the growth and attraction of *R. solani*.

Root exudates can broadly be described as substances (other than water) that are released from root surfaces (Hale et al. 1971). The most common constituents of root exudates include mucilage (a gelatinous material predominantly comprised of polysaccharides), ectoenzymes, sloughed cells, organic acids, sugars, phenolics, vitamins and amino acids (Hale et al. 1971; Nelson 1990). Many of these exuded substances provide sources of nutrition for microorganisms existing within the subterranean environment (Nelson 1990) including fungal pathogens such as *R. solani* (Reddy 1980). By providing a source of nutrition, root exudates may influence the attraction and growth of *R. solani* (Reddy 1980). In particular, positive relationships have been found between amino acids (aspartic acid, asparagines and histidine) and sugars (glucose and sucrose) and the pathogenicity and in vitro mycelial growth of *R. solani* (Reddy 1980).

Plants infested with sedentary endoparasitic nematodes, such as *G. rostochiensis*, may produce higher quantities of root exudate constituents that are favourable to the growth and pathogenicity of fungal pathogens. For example, Van Gundy et al. (1977) attributed increases in the severity of *R. solani* root rot

of tomato during concomitant infections with root-knot nematodes (*Meloidogyne incognita*) to a shift in the concentrations of carbohydrates and nitrogenous compounds within the root exudates of nematode-infested plants.

During the life cycle of *G. rostochiensis* there are a number of stages where the profile or volume of potato root exudates (PRE) could be modified with possible subsequent effects on *R. solani* behaviour. These include: (a) damage to cortical cells during nematode penetration and invasion, (b) increased release of exudate constituents from roots via the syncytium and (c) damage to the root cortex during emergence of female nematodes. The research described in this communication was designed to test the hypothesis that changes in root exudation occurring during the invasion of potato roots by *G. rostochiensis* increase the severity of *R. solani* infections. To address this hypothesis, controlled environment studies were conducted to: (a) record the growth of five *R. solani* (AG 3) isolates on media amended with PRE from *G. rostochiensis*-infested or uninfested potato plants at different time intervals after the introduction of *G. rostochiensis* and (b) determine the concentrations of carbohydrates and nitrogen in each of the PRE samples collected.

Materials and methods

Experiment 1

Leachate and potato root exudate collection

The initial step of this experiment was to collect PRE from potato plants either uninfested or infested with *G. rostochiensis*. The potato cultivar Désirée (class SE2) was selected for continuity between the current experiment and the previous field experiments (Back et al. 2006). Prior to planting, 60 tubers were physiologically aged in the glasshouse at temperatures between 15–20°C until 3 mm sprouts had developed. Following ageing of tubers, a 'melon ball scoop' was used to remove the eye from the rose end of each tuber, taking care to avoid damage to the sprouts. The resultant potato balls were left to suberise for at least 72 h. The potato balls were then immersed in 1% sodium hyperchlorite (*v/v*) for 5 min and rinsed twice in distilled water before being allowed to air dry.

The potato balls were planted in pots (plastic 'vending' cups, height: 8 cm, bottom diam.: 4 cm). Each pot had a 5 mm diam. hole drilled into the centre of the base and a layer of fine mesh (gauge: 0.5 mm) placed over the hole. This provided drainage for exudates but prevented potting medium from escaping. Fifty pots were partially filled with *c.* 200 g of twice-autoclaved silver sand. By using a sterile stirring rod, a 4 cm deep×2.5 cm wide hole was made into the centre of 40 sand-filled pots to allow a potato ball to be planted. The 10 remaining pots were left unplanted and served as controls for the potting medium. Following planting the pots were transferred to a controlled environment cabinet (Conviron Model S10H, Controlled Environments Ltd, Winnipeg, Manitoba, Canada) with a 15 h photoperiod, day- and night-time temperatures of 18°C and 10°C, respectively and a relative humidity of 50% to represent springtime conditions in the UK. Pots were arranged in 10 randomised blocks with the treatments shown in Table 1. Treatments of *G. rostochiensis* were implemented 3 weeks after planting when the potato plants had fully emerged. The pots were watered every 72 h prior to emergence and every 48 h following emergence by using a syringe to administer 20 ml of sterile distilled water to each pot.

Juveniles as opposed to cysts of *G. rostochiensis* were used in nematode treatments as it was speculated that a pre-determined number of hatched, viable juveniles would produce a more uniform invasion of potato roots in each treatment group. The level of *G. rostochiensis* infection shown in Table 1 was based upon soil densities of 100 eggs g^{-1} soil and the assumption of a 50% hatch rate (Perry et al. 1992). Juveniles of *G. rostochiensis* were induced to hatch from cysts using PRE (Clarke and Hennessy 1984) as outlined below.

Table 1 Treatments used to produce exudates from plants uninfested or infested with *Globodera rostochiensis* (PCN) in the two controlled environment experiments

Treatment	Potato plant	Level of PCN infection (juveniles pot^{-1})	
		Experiment 1	Experiment 2
1	−	0	0
2	+	0	0
3	+	9990	15000

Approximately 10 kg of *G. rostochiensis*-infested soil was collected from the 'Four-Gates Field', Harper Adams University College (Ordnance Survey Grid Reference: SJ 707195) and dried at 25°C for 4–5 days. Nematode densities were determined from three 200 g sub-samples of the soil bulk using standard methods (Shepherd 1986). The cysts were divided into groups of 100, placed into watch glasses containing 2 ml of distilled water, covered with a cover glass and placed in an incubator at 15°C for 7 days. Following incubation, a Pasteur pipette was used to remove the water from each of the watch glasses. The cysts were then removed from the watch glasses and divided into 10 sterile Petri dishes, each containing 14 ml of PRE collected from 3-week-old potato plants (cv. Désirée) using the method of Fenwick (1949). The Petri dishes containing the cysts and PRE were incubated for 5 days at 15°C and examined on a daily basis with a binocular microscope (×30) to monitor their progress. Following incubation, the contents of Petri dishes were passed through two sieves into a 250 ml glass beaker. The upper sieve (gauge: 300 μm) was used to collect the cysts and the lower (gauge: 10 μm) to collect the juveniles. The juveniles in the lower sieve were washed 3 times with distilled water. The lower sieve was then inverted over a glass funnel on a 250 ml volumetric flask and the contents were flushed into the flask via a stream of sterile distilled water. Once all juveniles had been transferred, the flask was made up to 250 ml with sterile distilled water and moved to a magnetic stirrer. Three 2 ml aliquots were removed from the juvenile stock suspension and transferred to DeGrisse counting slides. Each slide was examined under a binocular microscope (×30) to obtain an estimate of viable juveniles ml^{-1} water.

Since the quantity of *G. rostochiensis* juveniles within the stock suspension had been pre-determined, the volume of juvenile suspension required for infection (Table 1) could be calculated. As previously mentioned, potato plants within the controlled environment cabinet had been grown for 3 weeks before nematode infection treatments were undertaken. Prior to infection, a stirring rod was used to make four holes, 3 cm deep in the potting medium around each of the potato plants. Potato plants were infected, by using a pipette to gently apply equal volumes of the calculated quantity of juvenile suspension to each hole. To ensure equal volumes application, an additional quantity of sterile distilled water was added to make the total volume of distilled water applied up to 20 ml in each pot. After all pots had been treated and the holes were closed, pots were returned to their designated position in the controlled environment cabinet. In treatments where no nematode infection was required, 20 ml of sterile distilled water was applied. Following nematode infection the 48 h watering regime was resumed and the pots were given a weekly dose of a 7-7-7 NPK fertiliser, used at the rate of 20 ml of product in 5.5 litres of water (J. Arthur Bowers Multi-Purpose Liquid Plant Food, Garotta & Silvaperl Products, Lincoln, UK).

Leachates and PRE were collected 4, 6, 8, 12 and 18 days after the nematode infection treatments were administered. Prior to PRE/leachate collection, pots were left without water for 48 h. The PRE/leachates were collected with the aid of specialised racks, which comprised of an upper shelf for holding 10 pots, a middle shelf holding short-necked funnels and a lower shelf with 25 ml sample tubes for collecting the exudates. Each of the funnels was lined with Whatman ® No. 1 filter paper (gauge: 11 μm) (Whatman International Ltd., Maidstone, UK). Pots were removed from the controlled environment cabinet in blocks and placed on an assembled rack with correspondingly labelled sampled tubes beneath them. A sterile syringe was used to administer sufficient sterile distilled water to saturate each of the pots (*c.* 40 ml). Following a 5 min interval, a further 25 ml of sterile distilled water was applied to each pot. When each sample tube was full it was removed from the rack and a new tube was placed underneath to collect any excess leachates. When all the PRE/leachates had been collected, the pots were returned to the controlled environment cabinet and the sample tubes were taken back to the laboratory for processing. Each sample tube was shaken by hand for 10 s before being filtered using a syringe attached to a 0.2 μm Millipore ® filter (Millipore Filter Corporation, Bedford, MA, USA) to remove any bacteria. Twenty millilitre samples were removed from each filtered sample and transferred to 25 ml disposable Sterilin ® tubes (Sterilin Ltd., Feltham, UK) and maintained at 4°C.

When the last PRE collection had been made, the plants were removed from their pots, washed, placed in accordingly labelled polythene bags and taken back to the laboratory for processing. The root system of

each plant was removed, weighed and preserved in formal acetic alcohol (FAA) for the determination of *G. rostochiensis* juvenile densities (Hooper 1986) at a later date.

Assessment of radial growth of R. solani

A series of in vitro experiments were conducted to compare the growth rate of *R. solani* isolates on media amended with PRE from *G. rostochiensis*-infested or uninfested potato plants. Since the PRE were collected at different times after *G. rostochiensis* infection treatments, separate experiments were conducted for each PRL collection date. Each experiment included a treatment where *R. solani* isolates were grown on medium amended with leachate from pots without potato plants. This treatment was used to provide a comparison between the potting medium with and without potato plants.

Five *R. solani* (AG 3) isolates from different UK geographical origins were obtained from the Harper Adams University College culture collection: (a) I3 (Shropshire), (b) X44 (Yorkshire), (c) X50 (Scotland), (d) X56 (Cornwall) and (e) X62 (Suffolk). All isolates had been originally isolated from black scurf on potato (cv. Désirée) tubers. Prior to each experiment, the isolates were sub-cultured on to Petri dishes containing potato dextrose agar amended with streptomycin sulphate and incubated at 15°C for 7 days.

Four 1 l Schott ® bottles (Schott UK Ltd, Stafford, UK) containing 800 ml of distilled water and 10 g of Agar No. 2 (Lab M, Amersham, UK) (water agar) were autoclaved for 20 min at 121°C. On cooling (*c.* 45°C), each bottle of medium was amended with 200 ml of either PRE from pots containing potato plants infested with *G. rostochiensis*, or PRE from pots containing uninfested potato plants, or leachate from pots without a potato plant or sterile distilled water. Each 200 ml of PRE or leachate was made from a composite of 10×20 ml samples collected from individual replicate pots. The amended medium from each bottle was poured into 50 sterile Petri dishes (plates) at 20 ml plate^{-1}. When the medium was set, the underside of each agar plate was marked with a central cross. Ten replicate plates of each medium were inoculated with each of the 7-day-old *R. solani* isolates by placing a 6 mm *R. solani* plug in the centre of each plate. The plates were then sealed with Parafilm ® (Sigma Aldrich, Gillingham, UK), arranged into 10 randomised blocks (stacks) and incubated at 15°C. The diameter of the growing colonies was measured along both the vertical and horizontal marked lines of each plate on a daily basis. A mean of the two measurements was calculated in order to obtain a single value of radial growth. The experiment was run until a colony was found to have reached the edge of a plate. This procedure was repeated for each date that PRE and leachates were collected.

Experiment 2

Leachate and potato root exudate collection and assessment of R. solani radial growth

Following the procedure outlined in Experiment 1, a second controlled environment experiment was set up with the treatments (Table 1) replicated in 15 blocks. The potato seed stock (cv. Désirée), controlled environment cabinet settings and *G. rostochiensis*-infested soil were identical to those used in Experiment 1. Similarly, water was supplied as in Experiment 1. Unlike Experiment 1, plants did not receive any nutrient supplements. Leachates and PRE (20 ml) were collected as previously described but were filtered by Whatman ® No. 5 filter paper (gauge: 2.5 μm) in the rack assemblage and were not subsequently filtered by 0.2 μm Millipore ® filters. In addition to taking a 20 ml sample, two 2 ml samples were placed in Eppendorf tubes and stored at −80°C for subsequent carbohydrate and nitrogen analyses.

Leachates and PRE were collected 4, 8 and 12 days after infection with *G. rostochiensis* juveniles. At each PRE/leachate collection date, an in vitro radial growth experiment was conducted using the procedure and *R. solani* isolates detailed in Experiment 1. However, several modifications were introduced with the aim of optimising the experiment. Firstly, the quantity of PRE/leachate used to amend agar was increased to 300 ml (15×20 ml replicates). The agar was accurately dispensed at 10 ml plate^{-1} by using a sterile 100 ml syringe attached to a 30 cm length of plastic tubing. In between treatments, the syringe and tubing was soaked in 1% sodium hyperchlorite (*v/v*) for 3 min and rinsed 3 times with sterile distilled water. The plates were incubated at 10°C and measured every 48 h.

Determination of sugar content within leachates and potato root exudates

The sugar content of the PRE and leachate samples was determined by adapting the enzyme-coupled microplate method of Spackman and Cobb (2002). Two hundred μl of each PRE or leachate sample was added to 3 vertically adjacent wells (strips) of a Falcon® flat-bottomed, 96-well microtitre plate (Fahrenheit, Northampton, UK) in triplicate. On each plate, a block of 15 wells (5×3 well strips) was reserved for standard sugar solutions of glucose, fructose or sucrose at concentrations of 0, 25, 50, 75 or 100 μg ml^{-1}, which were assayed at 10 μl $well^{-1}$. When the plate was loaded with both samples and standards, the absorbance of each well was determined using a Benchmark ® microplate reader (BioRad, Hemel Hempstead, UK), which was programmed to shake the plate for 20 s before reading at 340 nm.

A 30 μl dose of glucose reagent (Bayer, Newbury, UK) was added to the first well of each strip in order to produce an enzymic oxidation of the sample glucose to 6-phosphogluconic acid coupled with a reduction of NAD to NADH (Spackman and Cobb 2002). Subsequently, a 30 μl dose of glucose reagent amended with 0.2 units of phosphoglucose isomerase, type IV from rabbit muscle (Sigma, Poole, UK) was added to the second and third wells of each strip. Spackman and Cobb (2002) stated that phosphoglucose isomerase converts fructose to 6-phosphogluconic acid. The third well of each strip was additionally treated with 10 μl of invertase grade VII from baker's yeast (Sigma, Poole, UK) in 0.1 mol sodium acetate (pH 5), which converts sucrose to 6-phosphogluconic acid (Spackman and Cobb 2002). The plate was incubated at room temperature for 40 min before the absorbance at 340 nm was determined. A repeat reading was taken 60 min after the enzymes were applied. The sugar concentration of each well was quantified by comparing the increase in sample absorbance with the absorbance of standard sugar solutions.

The increase in sample absorbance was calculated using the difference between readings taken at 0 and 40 min for glucose and fructose and the difference between readings taken at 0 and 60 min for sucrose (Spackman and Cobb 2002).

Determination of nitrogen content

The nitrogen content of PRE and leachates was determined using Leco® FP-528 combustion based nitrogen/protein apparatus (Leco Corporation, St Joseph, MI, USA) as per manufacturers' instructions.

Statistical analysis

All statistical analyses were conducted with the aid of Genstat Release 10.1 © 2007 (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Frequency histograms were used to examine the distribution within variates. Because glucose, fructose and sucrose data were skewed, a square root transformation was applied to each data set prior to analysis to normalise their distribution. Analysis of variance was conducted with the explanatory variates, which were collection date and experimental treatment.

Results

Experiment 1

Radial growth of R. solani

The data of mean radial growth of *R. solani* isolates on agar amended with leachates or PRE from *G. rostochiensis*-infested or uninfested plants are presented in Table 2. The mean radial growth of *R. solani* isolates, 72–96 h after inoculation was significantly higher ($P<0.001$) on agar amended with PRE from *G. rostochiensis*-infested plants compared to agar amended with PRE from uninfested plants, when PRE were collected 4 days after the introduction of *G. rostochiensis* juveniles. However, the highest recording of *R. solani* radial growth was found 24–96 h after inoculation on agar amended with leachates collected from pots containing sand alone. Subsequent radial growth experiments using PRE and leachates collected 6, 8 and 12 days after the introduction of *G. rostochiensis* juveniles produced similar results to the 4-day collection. The final radial growth experiment (18-day collection) revealed fewer significant differences

Table 2 The mean radial growth of *Rhizoctonia solani* (AG3) isolates grown on unamended water agar (01-CON), water agar amended with leachate from pots containing sand only (02-LCH), water agar amended with root exudates from potato plants (cv. Désirée) uninfested (03-POT) or infested with *Globodera rostochiensis* (04-NEM). Exudates and leachates were collected 4 (a), 6 (b), 8 (c), 12 (d) and 18 (e) days after *G. rostochiensis* treatments were administered in Experiment 1

	Mean radial growth (mm) of *R. solani* isolates				Statistics		
Time	01-CON[a]	02-LCH[a]	03-POT[a]	04-NEM[a]	SED	LSD	CV %
(a)							
24 h	–	9.04b	7.33a	7.45a	0.313	0.620	19.7
48 h	–	24.96b	19.81a	20.90a	0.585	1.158	13.4
72 h	–	42.29c	33.36a	36.04b	0.698	1.382	9.4
96 h	–	57.84c	46.89a	51.13b	1.158	2.291	11.1
(b)							
24 h	8.31a	9.53b	8.27a	8.51a	0.454	0.896	26.2
48 h	18.37a	21.45b	18.69a	20.07a	0.667	1.317	17.0
72 h	30.41a	35.44c	30.24a	33.06b	0.741	1.463	11.5
96 h	43.66a	52.22d	45.84b	49.16c	0.755	1.491	7.9
(c)							
24 h	9.27a	11.49b	9.85a	11.07b	0.399	0.787	19.1
48 h	20.30a	25.86c	21.44a	23.37b	0.602	1.188	13.2
72 h	31.37a	41.06d	33.58b	36.37c	0.728	1.437	10.2
96 h	43.74a	57.52d	47.62b	51.70c	0.851	1.680	8.5
(d)							
24 h	6.48a	7.73b	6.59a	7.75b	0.357	0.705	25.0
48 h	17.04a	21.25c	17.00a	19.28b	0.586	1.156	15.7
72 h	27.43a	35.54c	28.00a	31.73b	0.715	1.410	11.6
96 h	40.18a	51.94c	41.61a	46.66b	0.849	1.676	9.4
(e)							
24 h	10.10a	11.16b	9.84a	10.55ab	0.372	0.733	17.8
48 h	21.71a	23.72c	20.85a	22.21b	0.520	1.026	11.7
72 h	35.30a	38.83b	34.49a	35.30a	0.605	1.194	8.4
96 h	48.77a	54.48c	50.09ab	51.15b	0.796	1.572	7.8

[a] Within rows means with a common letter are not significantly different according to Fisher's protected LSD test at the $P=0.05$ level

between treatments. In particular, the growth of *R. solani* isolates on agar amended with PRE from *G. rostochiensis*-infested plants compared to agar amended with PRE from uninfested plants was not significantly different 24, 72 and 96 h after the isolates were inoculated onto the agar.

Invasion of potato roots by G. rostochiensis juveniles

The mean (±SE) total number of *G. rostochiensis* juveniles (J2, J3, J4 and adults) recovered from potato roots 18 days after their introduction to 3-week-old plants was 572.5 juveniles g^{-1} root (±57.0; CV= 31.5%). The distribution of the juvenile developmental stages was: 4% J2, 39% J3, 37% J4 and 20% adults. As expected, no juveniles were found in plants uninfested with *G. rostochiensis*.

Experiment 2

Radial growth of R. solani

The first assessment conducted with PRE/leachates collected 4 days after *G. rostochiensis* juveniles were introduced (Fig. 1), revealed that *R. solani* isolates X50 and I3 had a significantly higher growth on agar amended with PRE from *G. rostochiensis*-infested plants compared to all other treatments. In addition, *R. solani* isolates X44 and X62 had a higher growth rate on agar amended with PRE from *G. rostochiensis*-infested plants compared to agar amended with PRE from uninfested plants but were not significantly different from the other treatments.

The second radial growth assessment conducted with PRE/leachates collected 8 days after *G. rostochiensis*

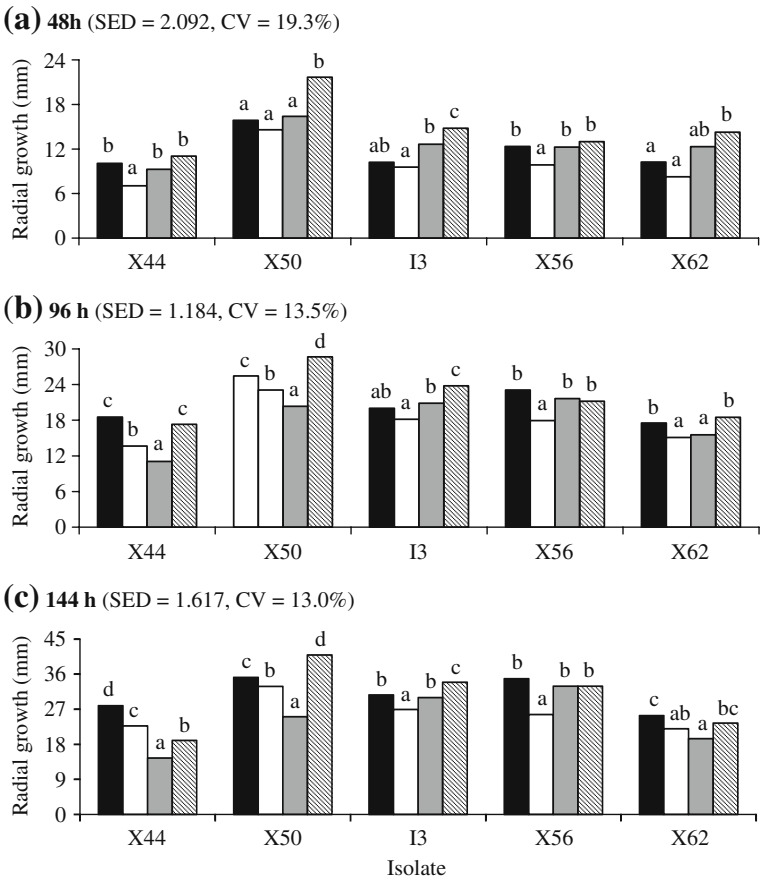

Fig. 1 The radial growth of *Rhizoctonia solani* (AG3) isolates grown on un-amended water agar (■), water agar amended with leachate from pots containing silver sand only (□) water agar amended with exudates from potato plants (cv. Désirée) uninfested (▩) or infested with *Globodera rostochiensis* (▨) at 48 (**a**), 96 (**b**) and 144 (**c**) hours after inoculation with *R. solani* isolates. Exudates and leachates were collected 4 days after *G. rostochiensis* juveniles were introduced. In each subfigure and within each isolate means in columns sharing a common letter are not significantly different according to Fisher's protected LSD test at the P=0.05 level

juveniles were introduced (Fig. 2), showed that the radial growth of all *R. solani* isolates was higher on agar amended with PRE from plants infested with *G. rostochiensis* than on agar amended with PRE from uninfested plants. Furthermore, *R. solani* isolates I3 and X56 had a significantly higher growth on agar amended with PRE from *G. rostochiensis*-infested plants compared to all other treatments.

The final radial growth assessment (12-day collection; Fig. 3) showed that the radial growth of all *R. solani* isolates was higher on agar amended with PRE from *G. rostochiensis*-infested plants than that of all other treatments. This effect was more pronounced at 96 h and 144 h after inoculation with *R. solani* isolates.

Analysis of sugar content in exudates and leachates

Sucrose was found to be significantly higher ($P<$ 0.01) in PRE from *G. rostochiensis*-infested plants (mean of 671 ng ml^{-1}) than in PRE from uninfested potato plants (498 ng ml^{-1}) and in leachates from pots containing silver sand alone (484 ng ml^{-1}). There were no significant differences between harvest dates and therefore the values of the three dates (4, 8 and 12 after *G. rostochiensis* treatments were administered) were pooled and treated as a single sample. Glucose and fructose content did not differ significantly between treatments.

Analysis of nitrogen content in exudates and leachates

There were no significant differences between the total nitrogen content of PRE from *G. rostochiensis*-infested or uninfested plants or the leachates collected from pots containing sand alone (mean values ranged from 0.014% to 0.034%).

Invasion of potato roots by G. rostochiensis juveniles

The mean (±SE) total number (J2-adults) of *G. rostochiensis* juveniles found in potato roots 12 days

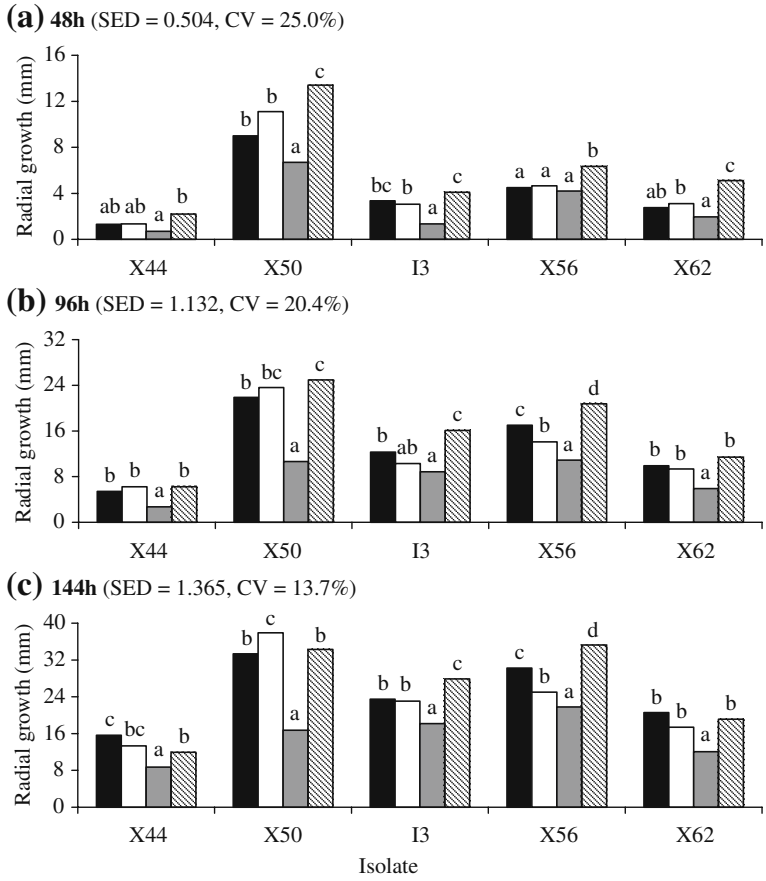

Fig. 2 The radial growth of *Rhizoctonia solani* (AG3) isolates grown on un-amended water agar (■), water agar amended with leachate from pots containing silver sand only (□) water agar amended with exudates from potato plants (cv. Désirée) uninfested (▩) or infested with *Globodera rostochiensis* (▨) at 48 (**a**), 96 (**b**) and 144 (**c**) hours after inoculation with *R. solani* isolates. Exudates and leachates were collected 8 days after *G. rostochiensis* juveniles were introduced. In each subfigure and within each isolate means in columns sharing a common letter are not significantly different according to Fisher's protected LSD test at the P=0.05 level

after their introduction to 3-week-old plants was 880 juveniles g^{-1} root (±91.4; CV=38.9%). Juvenile stages were distributed as follows: J2 20%, J3 29%, J4 25% and adults 26%. Similarly to Experiment 1, no juveniles were found in plants uninfested with *G. rostochiensis*.

Discussion

Plant root exudates are fundamental in stimulating the growth of microorganisms due to the release of organic compounds (Bolton et al. 1993; Grayston et al. 1997). Furthermore, the rate at which root exudates are released can be further increased by root injury (Bolton et al. 1993). On this basis, several workers have hypothesised that root damage caused by plant parasitic nematodes may increase the release of root exudates and ultimately increase the attraction of soil-borne pathogens (e.g. Bergeson et al. 1970; Van Gundy et al. 1977).

The current work has clearly demonstrated an increase in the radial growth of the soil-borne fungus *R. solani* on medium amended with root exudates from *G. rostochiensis*-infested potato plants compared to medium amended with PRE from *G. rostochiensis*-uninfested plants over two consecutive experiments. In Experiment 1, the radial growth of *R. solani* was higher on medium amended with PRE from *G. rostochiensis*-infested plants than on medium amended with PRE from uninfested plants collected 4, 6, 8 and 12 days after the *G. rostochiensis* treatments were administered. However, no significant differences were observed 18 days after the introduction of *G. rostochiensis* juveniles. This may suggest the importance of different stages in the lifecycle of *G. rostochiensis* in relation to the release of PRE. These results indicate that *G. rostochiensis* juveniles modify root exudation during the initial stages of root invasion, such as penetration, migration through the root tissue and initialisation of feeding sites. Since very few adult females were recorded in the juvenile analysis of harvested potato

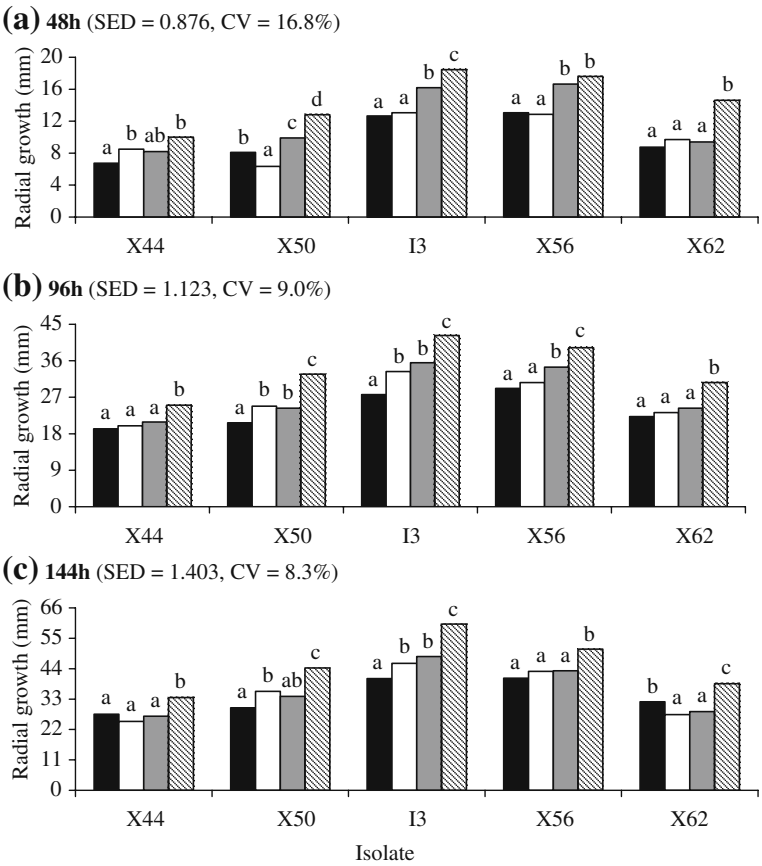

Fig. 3 The radial growth of *Rhizoctonia solani* (AG3) isolates grown on un-amended water agar (■), water agar amended with leachate from pots containing silver sand only (□) water agar amended with exudates from potato plants (cv. Désirée) uninfested (▩) or infested with *Globodera rostochiensis* (▨) at 48 (**a**), 96 (**b**) and 144 (**c**) hours after inoculation with *R. solani* isolates. Exudates and leachates were collected 12 days after *G. rostochiensis* juveniles were introduced. In each subfigure and within each isolate means in columns sharing a common letter are not significantly different according to Fisher's protected LSD test at the $P=0.05$ level

roots, the effects of female emergence on root exudation were not accounted for in this experiment.

In all of the *R. solani* radial growth assays conducted during Experiment 1, the growth of *R. solani* was highest on medium amended with leachates collected from pots containing silver sand alone. This finding may relate to the weekly dose of fertiliser applied to all of the pots to sustain the potato plants during the experiment. Where potato plants were present, the fertiliser is likely to have been utilised by the plant, whereas pots without plants may have retained higher quantities of fertiliser. Since positive interactions have been demonstrated between nitrogen sources and the growth of fungal pathogens (Solomon et al. 2003), it could be hypothesised that the increased growth of *R. solani* observed in the current experiment was due to residual nitrogen. As a result of these findings, fertiliser treatments were not administered in Experiment 2.

The second experiment investigating the radial growth of *R. solani* on media amended with PRE from *G. rostochiensis*-infested or uninfested plants provided a much clearer picture than that in Experiment 1. Each *R. solani* isolate grew faster on medium amended with PRE from *G. rostochiensis*-infested plants than on medium amended with PRE from uninfested plants. This growth trend was particularly pronounced when the medium was amended with PRE collected 12 days after *G. rostochiensis* juveniles were introduced to potato plants. At 12 days after the introduction of juveniles, there was a relatively equal split of the juvenile moults J2, J3 and J4 as well as adults recovered from potato roots. The relatively high proportions of J2 (20%) and J3 (29%) found at this time help support the earlier suggestion that *G. rostochiensis* juveniles modify root exudates during the early stages of invasion. It should also be noted that although adult juveniles were recovered at this date, only 8% were female. This would, therefore, negate the potential effects of female emergence on root exudation.

In view of the modifications used in Experiment 2, it could be reasonably expected that the *R. solani* radial growth assessments might have greater clarity than those recorded in Experiment 1. One such change was the increase in replication (from 10 to 15) and hence in the chances of detecting more significant differences between treatments. Furthermore, the lack of nutrient supplementation in Experiment 2, as opposed to Experiment 1, may have also contributed to the clarity of the results obtained in the former by eliminating the potential impact of the nutrient salts on *R. solani* growth as well as on the sugar and nitrogen content of the exudates. In fact, potassium fertilisers have been found to increase the incidence of *R. solani* in tomatoes (Kassim et al. 1989). Other factors contributing to the clearer results found in Experiment 2 may have been the higher number of *G. rostochiensis* juveniles used to infect potato plants, the higher volumes of exudate used to amend the agar or the reduction in exudate filtering stringency.

The age of plants used in the two experiments for the various assessments ranged from 3.5 to 6.5 weeks. Due to the length of the experiments, the omission of nutrients in Experiment 2 was not considered to be a limiting factor. This speculation was later confirmed, as no nutrient deficiency symptoms were observed for the duration of the experiment. Also, the absence of nutrients from the soil solution would mean limited buffering of the potting medium. How this would affect nematode behaviour is difficult to judge with certainty without performing comparative hatching bioassays in root exudates collected from fertilised and non-fertilised pots. In such case to reduce differences in root development characteristics (i.e., between fertilised and non-fertilised plants), which affect root exudate production and hence the amount of hatching activity (Devine and Jones 2001), exudates should be standardised on the basis of their carbon content. This method was developed by Deliopoulos et al. (2007) and allowed direct quantitative and qualitative comparisons of the hatching activities of different root exudates towards the two PCN species.

Carbohydrate analysis of PRE and leachates collected during Experiment 2 indicated significantly higher levels of sucrose in PRE from plants infested with *G. rostochiensis* than in PRE from uninfested plants. Since the growth and activity of microorganisms is limited by the availability of nutrients in agricultural soils (Nelson 1990), the presence of higher sucrose levels coupled with the observations of increased *R. solani* radial growth may be critical in explaining the disease complex found between *G. rostochiensis* and *R. solani* in the field experiments of Back et al. (2006). The growth of *R. solani* in response to the nutrients within exudates has been observed previously by Reddy (1980) and Ritz (1995). Reddy (1980) found a correlation between the decline of compounds such as sugars (sucrose and glucose) and amino acids within exudates from the hypocotyls of groundnut seedlings of different ages and the in vitro growth of *R. solani*. In addition, Ritz (1995) illustrated that spatial densities of *R. solani* mycelium would correspond to spatial patterns of nutrient distribution. In the present study, there were indications that the relationship between *G. rostochiensis* invasion of potato roots and the infection of potato stolons by *R. solani* may have been the consequence of modified root exudation caused by elevated root damage during the invasion of *G. rostochiensis* juveniles.

No significant differences were found between the total nitrogen content of the PRE/leachate treatments. This result is surprising considering the number of workers who have found nitrogenous molecules in root exudates (e.g. Reddy 1980; Grayston et al. 1997). However, it should be noted that the publications listed above relate to root exudates from plants other than potato. Although considerable work has been undertaken on the constituents of root exudates in relation to the hatching process in PCN, there have been only few reports on the concentration of nitrogenous compounds in PRE (e.g. Ryan and Jones 2004; Deliopoulos et al. 2007). A possible explanation for the nitrogen results could be that the filtering process retained a proportion of nitrogenous macromolecules such as proteins. Further analyses using unfiltered PRE from *G. rostochiensis*-infested and uninfested plants would help clarify this hypothesis.

Van Gundy et al. (1977) investigated a similar hypothesis to that of the current investigation when examining interactions between *Meloidogyne incognita* and *R. solani* root rot disease on tomato. Root exudates collected from *M. incognita*-infested tomatoes had higher total carbohydrate levels than uninfested tomatoes 14 days after nematode introduction, which is in agreement with the present work. The

authors pointed out that this was related to the time at which *R. solani* was attracted to *M. incognita*-infested roots. At the period between 14–21 days following nematode introduction, the carbohydrate content of root exudates from nematode-infested tomato plants was lower (relative to uninfested plants) and was associated with the development of sclerotia by *R. solani*. Increased nitrogenous compounds such as amino acids and proteins were found in exudates from nematode-infested tomato plants 28 days after the introduction of *M. incognita*. This appeared to coincide with the germination of *R. solani* sclerotia and the subsequent invasion of nematode galls. In the present work, no significant differences were found between the nitrogen content of root exudates from potato plants infested or uninfested with *G. rostochiensis* 4–12 days after nematodes were introduced. Later collections of PRE from *G. rostochiensis*-infested plants may have shown a shift in nitrogenous compounds compared to PRE from uninfested plants. However, there are distinct differences in the life-cycles of *G. rostochiensis* and *M. incognita*, which may have been reflected in the results of the current investigations and that of Van Gundy et al. (1977). Most significantly, *M. incognita* and other root-knot nematodes induce the formation of galls on the roots of their host plants, whereas root morphology is generally unaffected in plants infested by cyst nematodes such as *G. rostochiensis* (Endo 1987).

In order to try and elucidate the effects of root exudates from nematode-infested tomato plants on the development of *R. solani* root rot, Van Gundy et al. (1977) used a drip irrigation system to leach out the exudates from *M. incognita*-infested tomatoes that were also co-inoculated with *R. solani*. With this treatment, symptoms of root rot did not develop. However, when exudates collected from *M. incognita*-infested tomato plants were applied to uninfested tomato plants inoculated with *R. solani*, root rot symptoms appeared. Tomato plants inoculated with *R. solani* alone did not develop symptoms of root rot. However, it is not clear from this work whether a treatment was included to test the effect of root exudates collected from uninfested plants on tomato plants inoculated with *R. solani* alone. The inclusion of such treatment would determine whether the root rot was incited by quantitative changes in the exudates (the volume of exudates applied) or qualitative changes in the exudate constituents caused by nematode invasion. It should also be noted that the drip irrigation system used to leach root exudates might have produced unsuitable environmental conditions for *R. solani* root rot to develop. For example, Lootsma and Scholte (1997) have shown that the development of *R. solani* stem canker diseases on potato is reduced in soils with increasing moisture contents.

It should be noted that all of the *R. solani* radial growth studies were undertaken using dilutions of filtered root exudates. A possible alternative to the methods used, would leave the root exudates unfiltered and undertake radial growth experiments on *R. solani* selective media (Castro et al. 1988). This would reduce the potential loss of root exudate constituents during filtering, whilst keeping contamination to the minimum. Another strategy would be to inoculate known volumes of undiluted root exudates, amended with selected antibiotics and fungicides, with *R. solani* and measure the mycelial dry weight after a specific period of time.

In conclusion, the current findings provided clear evidence that the hypothesis that the severity of *R. solani* infections is increased by *G. rostochiensis*-induced changes in root exudation was correct. The outcomes of the present research may be utilised towards determining suitable management strategies for the dual control of *G. rostochiensis* and *R. solani*.

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