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In vitro optimisation of *Meloidogyne* infections on *Arabidopsis thaliana*

Nicola von Mende[1]

Entomology & Nematology Dept., IACR Rothamsted, Harpenden, Herts. AL5 2JQ, UK,
[1]Present address: 25 Pickford Hill, Harpenden, Herts. AL5 5HE, UK
e-mail: loxdale@ukonline.co.uk

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Summary. The growth conditions of *Meloidogyne incognita* on *Arabidopsis thaliana* were optimised by examining the type of medium and agar, agar concentration and volume applied, and pH and sucrose concentration in the medium. The media which supported high rates of invasion and nematode development were 'Gamborg's B5', 'M&S' (0.1x), 'Knop' (0.2x) and 'White's', independent of the addition of vitamins. More nematodes invaded roots of *A. thaliana* and developed in media containing 1.5% sucrose with a pH of 6.0. Loading 8 ml of a 0.8% agar medium into 9 cm i.d. Petri dishes gave the best agar thickness and concentration to support root and nematode growth. There were no differences in gall development when using 'Daichin' agar or purified agar.
Key words: agar, *Arabidopsis,* invasion, pre-galling, root knot nematodes, sucrose concentration.

Sijmons *et al.* (1991) first described the use of *Arabidopsis thaliana* as a model host plant in phytonematology and, since then, it has been used extensively to study cellular and molecular aspects of host-nematode interactions. The culturing conditions described by Sijmons *et al.* (1991) were optimised for the cyst forming nematode *Heterodera schachtii* but the same conditions were used for root-knot nematodes (*Meloidogyne* spp.), such as *M. incognita* and *M. arenaria*. Even though root-knot and cyst-forming nematodes both produce permanent feeding sites linked to the vascular system of the host, the development and behaviour of these two groups of nematodes differ in many respects. For this reason, growing conditions may need to be adjusted for *Meloidogyne* spp. on *Arabidopsis*. Here, the influences of different growth media, sucrose concentration and pH, and the effects of the type of agar, its concentration and the thickness of the layer, are investigated in relation to improved invasion rate, galling and egg production of *M. incognita* on *A. thaliana*.

MATERIALS AND METHODS

Nematode/*Arabidopsis* cultures. The root-knot nematode *M. incognita* Race 1 (NCSU N78) and *A. thaliana* ecotype Landsberg *erecta* were used for all experiments. The nematodes were maintained on aubergine (*Solanum melongena* L. cv. Blackbell) in the glasshouse. Egg masses were extracted in bulk (McClure *et al.*, 1973) or picked and placed on nylon mesh (30 μm) filters in dishes filled with tap water. This allowed second-stage juveniles (J2) to hatch and settle at the bottom of the dish. Eggs were surface sterilised with 0.1% $HgCl_2$ (Sijmons *et al.*, 1991). J2 were sterilised with 0.01% $HgCl_2$ for 2 minutes and washed four times in sterile distilled water, each time being centrifuged at 1000g for 2 minutes (Gravato Nobre, 1996). The pelleted J2 were transferred to 0.25% Agargel (Sigma) and the nematode concentration adjusted to about 10 J2/μl.

Arabidopsis seedlings were grown under monoxenic conditions in Petri dishes (Sterilin), 9 cm i.d., on solidified medium, on a layer of either *ca.* 3 mm or formed from measured volumes (as indicated in the tables). The seeds were surface sterilised (Sijmons *et al.*, 1991) and four seeds were placed in a row on one half of each dish. For easy handling, plates were either placed in general propagator boxes or stacked in specially prepared holders. These were made by cutting drain pipes, with an inner diameter of 9.5 cm, to a length sufficient to hold 20 Petri dishes and with about a third of their circumference removed to allow exposure to light from one side. The pipes

Table 1. Components and final molarities of media tested for *in vitro* culturing of *Meloidogyne incognita* on *Arabidopsis thaliana*.

Media (source)	0.2xKnop Knop, 1860	Sijmons *et al.*, 1991	M&S (Sigma M-5524)	Gamb. B-5 (Sigma G-5768)	Hoagland (Sigma H-2395)	White (Sigma W-0876)
Components	mM					
NH_4^+	–	–	20.0	1.0	1.0	–
K^+	2.4	2.5	20.04	24.7	6.0	1.71
Mg^{2+}	0.16	0.2	1.5	1.02	2.0	1.02
Ca^{2+}	0.0012	1.27	3.0	1.0	4.0	1.22
Na^+	0.0069	0.002	0.1	1.2	–	1.54
NO_3^-	2.0012	2.54	38.8	24.7	100	2.80
SO_4^{2-}	0.16	0.2	1.86	2.28	2.01	4.47
$H_2PO_4^-$	0.4	0.5	1.25	1.09	1.0	,0.14
Cl^-	0.011	0.024	0.19	0.19	–	0.87
NaEDTA	0.008	0.02	0.1	0.1	–	–
	μM					
BO_3^{3-}	9.24	9.0	100.0	0.049	0.046	0.024
Co^{2+}	0.046	0.024	0.19	0.19	–	–
Cu^{2+}	0.09	0.06	0.16	0.16	0.62	–
Fe^{3+}	8.0	20	183	183	9.57	16.0
I^-	–	–	5.0	4.5	–	4.52
Mn^{2+}	4.52	1.8	120	66.2	12.0	33.4
MoO_4^{2-}	0.057	0.06	1.21	1.21	0.11	–
Zn^{2+}	0.44	0.14	53	12.4	1.36	16.5

were glued to a solid base. For germination, seeded plates were kept at 23° C with 16.5 hr light. After germination, holders were tilted to an angle of about 20° to allow roots to grow across the plates for about 10 days. Plates were then inoculated with J2 and left for up to 3 weeks, when observations were made on root growth, nematode behaviour and development, and gall formation using a binocular microscope (Wild) at 60x, 120x and 250x magnification. The data were analysed using ANOVA and Student's t-test.

Effects of sucrose concentration and pH. Four sucrose concentrations (0.5, 1, 1.5, 2%) were tested at pH 6.4, and four pH values (5.5, 6, 6.5, 7.0) were tested with 1% sucrose in two independent experiments. In both cases, Gamborg's B-5 medium (Sigma) (10ml/plate; 0.8% Daichin agar) was used. Four seedlings were placed in a square on each plate and the number of replicates per treatment is shown in Tables 2 and 3. The numbers of root tips, juveniles close to roots and galls per plate were assessed 1, 2 and 3 weeks after inoculation (*ca.* 200 J2/plate).

Effect of different media. The following media, with or without vitamins, were tested: Hoagland's No. 2 basal salt mixture (Sigma, H-2395); White's basal salt mixture (Sigma, W-0876); Murashige and Skoog basal salt mixture using 1x and 0.1x strength (Sigma, M-5524) (M&S); Murashige and Skoog basal medium with Gamborg's vitamins (0.1x strength) (Sigma, M-0404) (M&S+V); Gamborg's B-5 basal salt mixture (Sigma, G-5768); Gamborg's B-5 basal medium with minimal organics (Sigma, G-5893); Knop medium (Knop, 1860) with and without Gamborg's vitamins (Sigma) in 0.2x strength and the medium described by Sijmons *et al.* (1991). The components of each medium and their molarities are listed in Table 1 whilst the numbers of replicates per treatment are indicated in Table 4. An important feature of this test was to identify media that are commercially available and, therefore, allow replication of experiments between laboratories. For all media experiments, 8 ml of medium were used in each plate with 0.8% agar (Sigma) and 1.5% sucrose at pH 6.5.

In addition to root growth, nematode behaviour, development and the frequency of 'pre-galling' was studied. This observation of occasional swelling of plant tissue at the invasion site has been described by Gravato Nobre *et al.* (1995). Root growth was studied with 1 week-old seedlings and measured by following the shape of the piece of grown root using a small piece of wire. A similar technique was described by Hooper (1986) for measuring the lengths of nematodes. Hatching of J2 was studied on media plates without seedlings. Each plate was inoculated with *ca.* 3,000 eggs. Six days later the numbers of hatched J2 were counted as numbers of empty eggshells. Galling was studied 8 days after inoculation of

Table 2. Influence of pH of Gamborgs-B5 medium on root growth, attraction to roots and gall formation of *Meloidogyne incognita* on roots of *Arabidopsis thaliana*.

pH	1 week after inoculation			2 weeks after inoculation			3 wk a.i.
	Root tips/plate	J2 at root/plate	Galls/plate	Root tips/plate	J2 at root/ plate	Galls/plate	Galls/plate
5.5 n=3	50±10.4	33±3.5	7±3.5	61±10.5	42±10.4	23±12.1	92±23.0
6.0 n=4	57±14.2	30±8.2	8±2.1	69±21.3	73±23.9	21±8.4	76 ±16.6
6.5 n=4	57±6.4	43±12.0	13±6.1	84±25.0	98±44.4	31±14.0	104±25.3
7.0 n=3	38±11.6	19±7.8*	4±2.1	50±19.3	97±17.1	5±2.7	32±24.0*

1% sucrose; * = significantly different at $P = 0.05$; 200 J2/plate; ± s.e.

Table 3. Influence of sucrose concentration in Gamborgs-B5 medium on root growth, attraction to roots and gall formation of *Meloidogyne incognita* on roots of *Arabidopsis thaliana*.

Sucrose	1 week after inoculation			2 weeks after inoculation			3 wk a.i.
	Root tips/plate	J2 at root/plate	Galls/plate	Root tips/plate	J2 at root/ plate	Galls/plate	Galls/plate
0.5%	42±11.5	28±8	7±1.2	48±13.6	70±14	11±0.6	54±11.5
1%	49±9.5	31±9.5	10±4.9	52±9.5	42±3.1	15±4.9	80±34.2
1.5%	87±39.5	67±31.8	17±10.1	104±31*	121±59	33±20.7	148±57.4*
2%	56±18.4	33±17.8	7±1.5	65±22.7	69±36.9	12±1.2	90±4.5

pH 6.4; n = 3; * significantly different at $P = 0.05$; 200 J2/plate; ± s.e.

10 day-old seedlings with *ca.* 100 J2/plate. Galling studies were repeated on Gamborg B5 (n = 7) and Knop medium (n = 9) only (0.8% Daichin agar; 1.5% sucrose; pH 6.4), in combination with 'pre-galling' observations. Three weeks after inoculation (*ca.* 3600 eggs/plate) the numbers of galls and pre-galls were counted.

Effects of type of agar. Daichin agar (Brunschwig Chemie BV, Amsterdam), as used by Sijmons *et al.* (1991), is expensive and difficult to purchase in the U.K. To find a replacement, other sources and types of agar were tested. Daichin agar (0.8% w/v) was compared with Agargel (Sigma, A-3301, 0.5% and 0.35%) and purified agar (Sigma, A-7921), 0.8%. All agars were added to 0.2x Knop medium (1.5% sucrose; pH 6.1). The numbers of root tips per plate were counted when seedlings were 1 week old and three weeks later after their inoculation with *ca.* 3600 eggs/plate the numbers of galls were counted. The numbers of replicates per treatment are indicated in Table 4.

Effects of agar concentration. Four concentrations of Daichin agar [0.2, 0.4, 0.6 or 0.8% (w/v)] were tested in M&S 0.1 x medium (1.5% sucrose; pH 6.2). There were three replicates per treatment.

Effects of layer thickness. Nine volumes of medium (0.2x Knop, 0.8% Daichin agar, pH 6.5, 1% sucrose) were tested, from 4 ml to 20 ml/dish in 2 ml intervals. There were three replicates per treatment.

RESULTS

Influence of sucrose concentration and pH. Within the pH range tested, at pH 7.0 attraction of J2 to roots was reduced (Table 2) during the first week only ($P = 0.05$) and gall formation was reduced 2 to 3 fold 3 weeks after inoculation ($P = 0.05$). Similarly, the numbers of root tips developed at pH 5.5 to 6.6 did not differ, but were reduced at pH 7.0; however, these differences were not significant. Generally, attraction to roots as well as gall formation was greatest at a sucrose concentration of 1.5% (Table 3); in the third week, up to three times more galls were found than at other concentrations ($P = 0.05$). This observation correlated well with the greater number of available root tips, which was about twice as large as in other concentrations in the second week ($P = 0.05$).

Influence of medium. Of the 5 media tested, the following four proved to be suitable for culturing *Meloidogyne* on *Arabidopsis*: M&S (0.1x), B-5, White's and Knop (Table 4). The presence of vitamins did not make any difference but, in the case of

Table 4. Influence of different media on root growth, hatching and gall formation of *Meloidogyne incognita* on roots of *Arabidopsis thaliana*.

Media	Root growth cm/48hrs.	n	Hatched J2, n = 2 (3000 eggs/plate)	Galls/plate (100 J2/plate)	n
M&S (0.1x)	0.96±0.410	20	119±20.5	7.5±5.2	4
M&S (1x)	0.926±0.380	19	49±46.7*	0.3±0.58	3
M&S (0.1x)+V	0.90±0.404	14	159±12.7	3.5±3.42	4
Gamb B-5	1.475±0.416	16	108±14.9	9.0±8.68	4
Gamb B-5+V	1.189±0.545	18	124±21.9	12.3±12.1	3
Hoagland	1.70±0.673	19	122±43.8	1.8±1.26	4
White	1.695±0.512	20	177±12.0	4.8±2.06	4
Knop (0.2x)	1.611±0.382	18	141±33.2	6.0±7.21	3
Knop (0.2x)+V	1.455±0.417	20	201±24.8	2.0±1.63	4
Sijmons *et al.*	1.38±0.662	15	158±33.2	6.3±5.77	3

* significantly different at $P = 0.05$, v = vitamins.

Table 5. Influence of type of agar on root growth and gall formation and incidence of *Meloidogyne incognita* on *Arabidopsis thaliana*.

Type of agar	Root tips/ plate ± s.e.	n	Galls/plate ± s.e.	n
Agargel 0.35%	n/a	–	15±14	6
Agargel 0.5%	n/a	–	13±5.8	5
Agar 0.8%	85±31.4	8	31±16.6	14
Daichin agar 0.8%	98±44.1	7	26±19.2	11

0.2x Knop medium, 1.5% sucrose, pH 6.1.

the M&S medium, the strength of the medium did influence hatching and invasion. The 1x M&S medium resulted in poor hatching responses ($P = 0.05$) and little gall formation. Any hatched J2 soon became motionless, rarely entering the agar layer and unhatched J2 appeared to be motionless within the egg. Hatching was not inhibited in any of the other media.

Upon repeating the media test, 31±22 galls were found on 0.2x Knop medium, and 17±12 galls were found on Gamborg B5 medium, with 32% and 37% of pre-galling incidences, respectively. The differences in galling and pre-galling were not significant.

Generally, root growth was poorest on M&S medium (0.9 cm/48 hr; $P = 0.01$), independent of the strength of the medium (Table 4). Best root growth was observed with Hoagland, White's and Knop media, but root growth could not always be correlated with the invasion and development of the nematode as, for example, only a few J2 invaded when using Hoagland medium.

Influence of the type of agar. The purified agar proved to be just as suitable for *in vitro* culturing as Daichin agar (Table 5). It supported similar root growth, nematode invasion and development. In contrast, agargel, at both concentrations, did not support gall formation as effectively. However, these differences were not significant.

Influence of agar concentration and thickness of layer. A 0.8% agar solution and the layer thickness resulting from 8 ml medium gave the best galling results. Less medium did allow J2 invasion and gall formation but thinner layers of agar dried out more quickly (data not shown).

DISCUSSION

The *in vitro* growing conditions for *M. incognita* on the roots of *A. thaliana* have been optimised with respect to root growth, nematode invasion and gall formation. In comparison to the conditions described by Sijmons *et al.* (1991) (modified Knop, pH 6.4, 1% sucrose, 0.8% (w/v) agar), which had been adjusted to culture *H. schachtii,* the pH range and concentration of agar optimal for *M. incognita* were the same, but a higher sucrose concentration (1.5%) improved invasion and gall formation.

In addition to Knop medium, three commercially available media (M&S 0.1x; Gamborg B5; White's) proved to be suitable for *Meloidogyne-Arabidopsis* cultures and had no adverse effects on hatching

and/or invasion (Table 4). This is important when comparative experiments are performed at different locations. In the case of the M&S media, the greater strength of 1x resulted in fewer galls, probably due to its inhibiting effect on hatching of the J2. *Arabidopsis* root growth on M&S medium was not as good as on the other media tested. Gamborg B5 and White's medium has previously been used successfully to culture plant parasitic nematodes *in vitro* (Mountain, 1955; Verdejo *et al.*, 1988). The phenomenon of pre-galling seemed to occur with about one third of all galls induced by *M. incognita,* independent of the medium. It is possible that this proportion changes with the nematode species.

The lower invasion rate at pH 7.0, which is reflected in the number of galls formed, appeared to be linked to the reduced attraction of J2 to the roots at that pH. However, the pH effect on attraction disappears after about two weeks. This could be explained by the way attraction was measured. Juveniles were counted along the whole root, therefore not differentiating attraction to root tips specifically. The greatest degree of galling, found at a sucrose concentration of 1.5%, was strongly correlated with the number of root tips available.

The two agar types tested resulted in similar numbers of galls (Table 5). This suggests that the agar type *per se* does not play as important a role as previously assumed. For economic reasons, the agar concentrations and amounts used per plate were tested. Results indicated that 8 ml of agar at a concentration of 0.8% supported sufficient root growth as well as the development of nematodes.

In conclusion, optimisation of a growth medium for *Meloidogyne* spp. on *Arabidopsis* is based on providing good root growth, the availability of healthy root tips and attraction of the nematode to the root. In the present study, this was achieved with four growth media and at one specific agar concentration, which was important both in supporting the nematode during invasion and allowing good root growth.

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von Mende N. *In vitro* оптимизация заражения мелойдогинами *Arabidopsis thaliana.*

Резюме. Оптимизация условия роста проведена подбором состава питательной среды, агара, pH, и концентрации сахарозы. Среди сред, поддерживающих высокие уровни заражения и развития нематод вне зависимости от концентрации витаминов, отмечены B5 Гамборга, M&S (0.1x), среда Knop (0.2x) и среда Уайта. Наибольшее число нематод проникало в корни и развивалось в среде, содержащей 1.5% сахарозы при pH 6.0. Оптимальным оказался розлив по 8 мл 0.8% агаровой среды на чашку Петри внутренним диаметром 9 см, что обеспечивало наилучший рост корней и нематод. Различий в развитии галлов при использовании очищенного агара или агара Daichin не отмечено.