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Monoxenic culture of the Ufra nematode *Ditylenchus angustus*

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Summary — New techniques for the monoxenic culture of the Ufra nematode *Ditylenchus angustus* utilising seedlings of rice and wheat callus tissue are described. Plant callus tissues differed in their ability to support the reproduction of *Ditylenchus angustus* *D. angustus* multiplied on callus from wheat cv. Copain (79 996 nematodes/callus) and to a lesser extent on that from rice (3710) and lucerne (1060). Only one of 25 rice callus cultures supported nematode reproduction. Within a " host " callus type *D. angustus* reproduced better on faster growing tissue, but overall reproduction was not related to callus size. Monoxenic culture on rice seedlings of rice cv. IR 36 yielded fewer nematodes (21 982) than wheat callus but were more reproducible. *D. angustus* did not reproduce on *Alternaria* spp. or *Botrytis cinerea.*

Résumé — ***Culture monoxénique du nématode* Ditylenchus angustus *agent de l'Ufra du riz*** — De nouvelles techniques utilisant des plantules de riz cv. IR36 et des cals de blé en vue de la culture monoxénique du nématode *Ditylenchus angustus*, agent de l'Ufra du riz, sont décrites. La capacité du tissu du cal à maintenir la reproduction de *D. angustus* varie selon l'espèce de la plante. *D. angustus* se reproduit sur le cal de blé cv. Copain (79 996 nématodes/cal) et, plus faiblement, sur les cals de riz (3710) et de luzerne (1060). Le nématode ne se reproduit que sur une seule des 25 cultures de cals de riz. Dans le cal « hôte », le nématode se multiplie mieux dans les tissus en croissance rapide, mais la valeur de la reproduction totale n'est pas en corrélation avec la taille du cal. Bien que les pousses de riz cv. IR36 cultivées en conditions stériles produisent moins de nématodes (21 982) que le cal de blé, les résultats sont plus constants. *D. angustus* ne se reproduit ni sur *Altenaria* spp., ni sur *Botrytis cinerea.*

Key-words : *Ditylenchus*, culture, callus tissue.

Ditylenchus angustus, the causal agent of " Ufra " in deepwater and lowland rice, has not previously been cultured *in vitro*. Fungal feeding nematodes described as *D. angustus* have been cultured on several genera of fungi (Vuong, 1969) but experience suggests that these nematodes could have been *Aphelenchoides* spp. which commonly occur with *D. angustus* in the field and are difficult to separate when observed under low magnification (max. × 50). Conventional glasshouse culture techniques, using susceptible plants in pots or microtanks, utilise considerable space and require careful management of factors which affect invasion and multiplication i.e. plant age, water temperature and depth and atmospheric relative humidity. Monoxenic, plant tissue culture techniques have been widely used for culturing plant parasitic nematodes (Krusberg & Babineau, 1979) and have recognised benefits of reliability, species purity and efficient use of space. The objective of this work was to develop a reliable monoxenic culture technique for *D. angustus.*

Materials and methods

A field population of *D. angustus* originally from rice cv. Chet Som (local variety) in Hau Giang province, Vietnam was maintained using conventional techniques on rice cv. NC 492 in thermostatically controlled water tanks at 30 °C in a glasshouse. The culture was not pure but reflected the field sample, having a concomitant infection of an undescribed species of *Aphelenchoides.* Tillers of NC 492 were harvested above the top meristematic node, cut into 1 cm lengths, sliced longitudinally and teased in tap water. Nematodes were collected on a 5 µm mesh sieve after cleaning the extract through a 313.5 µm mesh. Nematodes were further cleaned by allowing them to migrate through a cellulose sponge filter into water or an antibiotic-antimycotic solution (1 % v/v) (Sigma Chemical Co.). To facilitate recognition of *D. angustus* and in particular its separation from the *Aphelenchoides* n. sp., carbon dioxide gas was bubbled through the suspension to inactivate the nematodes. Inactive worms were pipetted into a covered channel of a glass slide (Fig. 1) which permitted observation at × 500 magnification. All juveniles and species other than *D. angustus* were removed from the channel and discarded using a fine aperture Pasteur pipette (0.1 mm diam.). Remaining *D. angustus* were pipetted into an excavated glass block where they regained activity within 5 min. Several hundred adult *D. angustus* were identified in this manner and transferred to sterile distilled water. *D. angustus* were surface sterilised in a solution of malachite green (0.1 % w/v). Nematodes were transferred singly to a drop of sterilant on a sterile cavity slide and treated in batches of ten. After 15 min exposure they were transferred directly to sterile distilled water.

Axenic *D. angustus* were cultured using a new technique which utilised seedlings of rice cv. IR36 grown in sterile conditions on a nutrient medium in sealed Petri

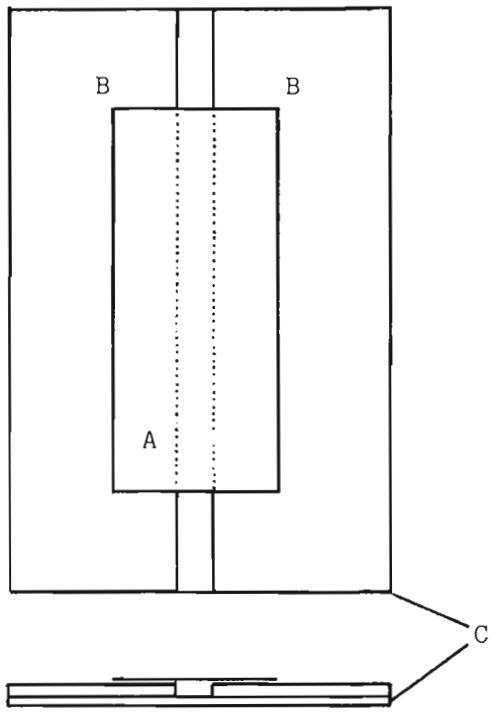

Fig. 1. Observation channel for the identification of *Ditylenchus angustus*. Glass slides (B, 76 × 23 × 0.8 mm) fixed to glass base (C, 76 × 50 × 1 mm) to create a groove (50 × 4 × 0.8 mm) covered by a fixed cover slip (A, No. 0,50 × 22 mm). All bonds made using a glass adhesive.

Fig. 2. Monoxenic culture of *Ditylenchus angustus* on seedling of rice cv. IR36. Note foliar symptoms.

dishes (Fig. 2). The cultures were set up in the following manner. Hulled rice seed, surface sterilised in mercuric chloride (0.1 % w/v) for 30 min and rinsed five times in sterile distilled water, were placed on Gamborg's B5 (1968) basal medium supplemented with sucrose (2 % w/v) and solidified with agar (1 %) in 9 cm diam. plastic Petri dishes. The cultures were maintained in a controlled environment maintained at 23-27 °C with a 12 h photoperiod. Thirty days after sowing, seedlings were inoculated with twenty, predominantly adult female, *D. angustus* pipetted in 5 µl of SDW, onto a leaf base adjacent to a newly emerging leaf. Petri-dishes were sealed with elastic PVC tape immediately after inoculation and returned to the same controlled environment.

The reproduction of *D. angustus* on the following tissues was assessed and compared with that obtained using the above seedling technique : 1) callus tissues, of *i) Oryza sativa* cvs NC492 and IR36 induced from mature embryos and cv. Speaker induced from immature inflorescences, *ii) Triticum aestivum* cv. Copain from immature embryos, *iii) Triticum monococcum* from immature embryos, *iv) Solanum tuberosum* cv. Desiree induced from stems, *v) Nicotiana rustica* from stems, *vi) Medicago sativa* cv. Sabilt from germinating seed and 2) fungi *Botrytis cinerea* and *Altenaria* sp. All the tissues were kept on solid media in 9 cm diam. Petri dishes.

Immature embryo tissues had been excised 10-14 days after fertilisation and placed scutellum upwards on Murashige and Skoog's (1962) medium, pH 5.8, supplemented with sucrose (3 % w/v) and 2,4-dichlorophenoxy acetic acid (0.1 mg/l) and solidified with agar (0.6 % w/v) (MS).

Immature inflorescences upto 20 mm in length were excised from young shoots, chopped into 1-2 mm pieces and placed on MS. The outer leaves were removed and the inner ones swabbed with ethanol (70 % v/v) before excision of the enclosed inflorescence (Maddock *et al.*, 1983). Mature embryos of rice were obtained from hulled, surface sterilised seed (mercuric chloride 0.1 % w/v for 30 min). Surface sterilised seeds were rinsed in sterile distilled water and placed on water agar (1 % w/v). Embryos were excised after 18-24 h and placed on both

MS and Gamborg's (1968) B5 media, pH 5.8, supplemented with sucrose (2 % w/v); $CaNO_3$, 440 mg/l; NH_4NO_3, 1650 mg/l; glycine 2 mg/l and 2,4-D 10 mg/l, and solidified with agar (1 % w/v) (Plowright, 1988). Callus of *M. sativa* was obtained by placing germinating surface sterilised seed (25 M H_2SO_4 for 30 min) (Krusberg 1961) on Gamborg's B5 medium pH 5.8, supplemented with sucrose (2 % w/v), 2,4-D (2 mg/l) and kinetin (0.5 mg/l), solidified with agar (1 % w/v). Callus from stems swabbed with ethanol (70 % v/v) were maintained on Uchimaya and Murashige's (1974) medium, pH 5.8.

The fungi were grown on potato dextrose agar.

The callus tissues and fungi were maintained at 25 °C in darkness and were sub-cultured every 8-12 weeks. Rice mature embryo callus tissues were sub-cultured once prior to this work though all of the other tissues had undergone several cycles of culture throughout a period of 2-3 years. Tissues for this study were finally prepared by sub-culture 30 days before inoculating *D. angustus*.

Surface sterile inocula recovered from the Petri dish lid of a rice seedling culture were concentrated by cooled (5 °C) centrifugation (7500 *g*, 10 000 rpm) for 10 min. in a sterile Eppendorf microtube (1.5 cm^3). After volume adjustments, five replicates of the seedlings, fungi, and selected tissues (approx. 10 mm diameter) of each callus, were inoculated with 20-30 nematodes in 5 µl of SDW. The inoculum was cooled in ice to prevent aggregation and the homogeneity of the inoculum was checked regularly.

The cultures were examined at weekly intervals and numbers of *D. angustus* (eggs and vermiform stages) were estimated at 60 days after inoculation. Tissues were cut into 2 mm lengths, macerated for 5 s in 100 cm^3 of water, washed through a 313.5 µm mesh sieve and collected on a 5 µm mesh sieve. Lid and surface fractions were rinsed directly.

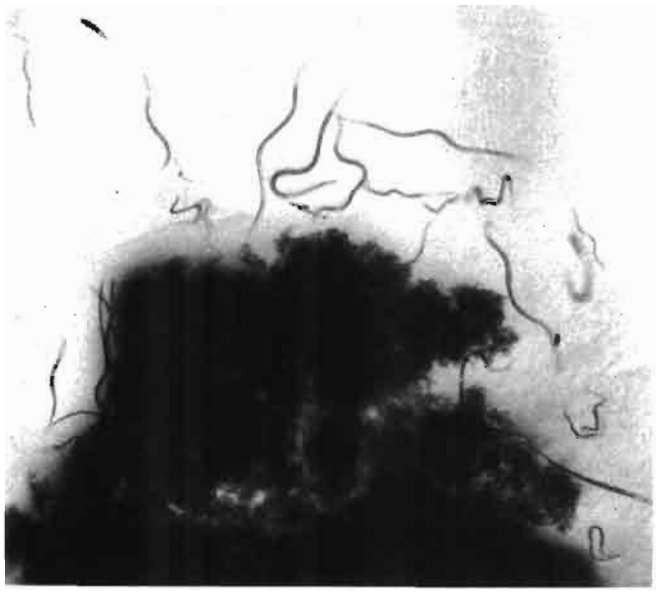

Fig. 3. *Ditylenchus angustus* cultured on callus of wheat cv. Copain.

Results and discussion

Callus tissues varied in their " host " status to *D. angustus*. Callus from *T. aestivum* cv. Copain (Fig. 3) was an extremely good " host " tissue yielding between 13 500 and 128 000 nematodes and eggs/culture. Callus from *O. sativa* cv. IR36 and *M. sativa* cv. Sabilt also supported nematode reproduction but at much lower levels (Table 1). *D. angustus* did not reproduce on callus from *T. monococcum*, *S. tuberosum*, *N. rustica* or *O. sativa* cvs NC492 (a very susceptible variety) and Speaker, neither did it reproduce on fungi.

Table 1. Reproduction of *Ditylenchus angustus* on different tissues.

Species	Basal medium	Number of cultures (pf : pi)		Callus weight (g) (pf : pi)		Change in callus diameter (pf : pi)		Nematodes + eggs (pf : pi)	
		> 1	< 1	> 1	< 1	> 1	< 1	> 1	< 1
Triticum aestivum cv. Copain	MS	3	2	3.15	0.92	+ 20	+ 11	79 996	8
Medicago sativa cv. Sabilt	B5	2	2	0.50	0.11	+ 8	+ 1	1060	3
Oryza sativa cv. IR36	MS	0	5	—	0.36	—	+ 4	—	14
Oryza sativa cv. IR36	B5	1	4	0.93	0.65	+ 9	+ 8	3710	2
Oryza sativa cv. NC492	MS	0	5	—	0.19	—	+ 4	—	2
Oryza sativa cv. NC492	B5	0	5	—	0.66	—	+ 7	—	2
Oryza sativa cv. Speaker	MS	0	5	—	0.59	—	+ 4	—	2
Oryza sativa seedling cv. IR36	B5	5	0	—	—	—	—	21 982	—

Within a " host " callus type, culture success was related in part to the growth of the tissue since only healthy dividing callus of both *T. aestivum* and *M. sativa* supported nematode reproduction. The diameter of successful *T. aestivum* callus doubled during the culture period and did not have the necrotic, watery appearance, displayed by *O. sativa* and *M. sativa* callus, which is normally associated with a heavy nematode infection. Gamborgs B5 supplemented medium supported better growth of rice mature embryo callus than MS media but nematode reproduction occurred in only one of 25 rice callus cultures (Table 1). There was no relationship between callus weight and number of *D. angustus*. Differences in " host " status of tissues may be influenced by differences in explant derivative and the genetic variability of the callus since the stability of tissues is known to vary. Callus is likely to become uniform and predominantly aneuploid after several passages through sub-culture (Pental & Gunckel, 1979) and the variability in *D. angustus* reproduction between *O. sativa* cv. IR36 cultures may reflect the heterogeneity of cell populations in relatively young callus from separate embryos.

O. sativa sterile seedling cultures were more reliable (100 % successful) than callus cultures (20-60 % successful) and yielded more nematodes than either *O. sativa* or *M. sativa* callus. However, mean nematode numbers on *T. aestivum* callus were almost four times those on seedlings and it is likely that careful selection of viable callus before inoculation will improve reliability. Experience with seedling cultures has indicated that live populations can decline to zero by 110 days after inoculation, but there are indications that *T. aestivum* callus cultures have a greater longevity and hence would require less frequent sub-culture.

Clearly both the seedling and *T. aestivum* callus techniques provide excellent means of producing large numbers of nematodes for experiments. The former is a simpler technique and is easier to monitor since inoculated plants exhibit symptoms after 7 days, hence confirming the infection. The techniques facilitate the control of the age of cultures from which nematodes are extracted for use in experiments and hence can reduce variations in infectivity which could result from differences in population viability. They also provide a means for more detailed studies of the host parasite relationship and, for example, the seedling technique has confirmed Ufra symptom development concluding with plant death, in the absence of any other organism. Perhaps the most important aspect of both techniques is the certainty of the eradication of insidious contamination of cultures by other nematodes and in particular seed borne *Aphelenchoides besseyi* which has always threatened conventional cultures.

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