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In Vitro Culture of *Subanguina picridis* in *Acroptilon repens* Callus, Excised Roots, and Shoot Tissues

X. Ou and A. K. Watson[1]

Abstract: The knapweed nematode *Subanguina picridis* is a foliar parasite that is of interest as a biological weed control agent of Russian knapweed. Attempts were made to culture the nematode in callus, excised roots and in shoots derived from roots of Russian knapweed. In callus tissues, the nematode developed from second-stage juvenile to adult but failed to reproduce; it developed only to the fourth stage in excised roots. However, *S. picridis* was successfully cultured in vitro in shoots derived from roots. The nematode induced galls on the leaves, petioles, and shoot apices and developed and reproduced inside the galls. Gibberellic acid increased the development rate of the nematode and promoted the formation of males. This is the first gnotobiotic culture of a nematode used for biological weed control.

Key words: Acroptilon repens, biological weed control, culture, gibberellic acid, knapweed nematode, nematode, Russian knapweed, *Subanguina picridis.*

Russian knapweed, *Acroptilon repens* (L.) DC, is an economically important perennial weed, introduced from Asia Minor (10) into North America in the early 1900s (9). It is now widely distributed in western Canada and in the central regions of the United States, as well as being considered a noxious plant in Australia, New Zealand, and parts of Europe and Asia (15,20). Control is difficult by cultural and chemical methods (20,21).

The nematode *Subanguina picridis* (Kirjanova) Brzeski induces galls on the stems, leaves, and root collars of Russian knapweed and reduces growth and reproduction of this weed (10). The nematode has a restricted host range (22) and was imported from the Soviet Union for the biological control of Russian knapweed in North America (21,23). The current effectiveness of *S. picridis* for biological control of Russian knapweed is limited in North America and other parts of the world because the nematode completes only two generations per year and has limited natural dispersal. Thus a large quantity of nematode inoculum that could be readily disseminated would increase the success of *S. picridis* as a biocontrol (23).

No species of *Subanguina* nor any species of related *Anguina* and *Nothanguina* have been successfully cultured in vitro. The objective of this study was to propagate *S. picridis* in vitro in callus tissues, excised roots, and shoots derived from cultured roots of Russian knapweed.

Materials and Methods

Callus tissue: Callus tissue was cultured on B5KD, B5KDG, or B5KDGL medium; B5KD was Gamborg's B5 medium (6) supplemented with 0.1 mg/L kinetin and 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), while B5KDG and B5KDGL were B5KD supplemented respectively with 1.0 mg/L or 10.0 mg/L gibberellic acid (GA_3).

Petioles from Russian knapweed plants grown in a greenhouse were washed with tap water for 10 minutes, surface disinfested in 1% sodium hypochlorite solution for 15 minutes, rinsed with sterile distilled water three times, and then cut into 5-mm segments. The petiole segments were placed onto B5KD, B5KDG or B5KDGL medium, and callus developed after 7 days.

A dry gall was placed in a petri dish containing sterile distilled water, cut into small pieces and left in the water overnight. The gall debris was removed, and the liberated second-stage juvenile (J2) nematodes in water were concentrated by centrifuging for 5 minutes at 2,720 *g*, and washed in sterile distilled water by centrifuging three times. Nematodes were surface disinfested

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[1] Graduate Research Assistant and Professor, Department of Plant Science, Macdonald Campus of McGill University, 21,111 Lakeshore Rd., Ste.-Anne-de-Bellevue, Quebec H9X 1C0, Canada.

in 100 ppm $HgCl_2$ for 1 minute, followed by three 2-hour surface disinfestations in a solution of 100 mg/L novobiocin + 30 ppm malachite green + 1000 ppm streptomycin sulfate. Surface disinfested nematodes were concentrated by centrifuging and transferred with a pipette to 6% agar medium amended with 300 mg/L novobiocin. Nematodes could be maintained in the agar medium in a refrigerator at 4 C for 3 months.

Fifty disinfected J2 were applied to the callus derived from petioles in B5KD, B5KDG, and B5KDGL media. The cultures were maintained at 22 C day (16 hours)/18 C night (8 hours) for 50 days.

Each week, callus tissue from three cultures of each medium was carefully broken up to release nematodes from the callus, and developmental stages of *S. picridis* were identified using a stereo microscope.

Excised root tissues: Shoot apices 1.5-cm long were excised from Russian knapweed plants grown in a greenhouse, surface disinfested in 1% sodium hypochlorite solution for 15 minutes, and washed three times in sterile distilled water. The shoot tips were excised under a stereomicroscope and were cultured using a filter-paper bridge technique (4) in MSIBG (Murashige and Skoog's MS medium as modified by Linsmaire and Skoog [13] supplemented with 2.0 mg/L indole-3-butyric acid [IBA], 2.0 mg/L 6-benzylaminopurine [BAP], and 1.0 mg/L gibberellic acid [GA_3]). Shoots formed after 1 week and were transferred to the rooting MSIG medium (MS medium as modified by Linsmaire and Skoog [13] + 2.0 mg/L IBA + 1.0 mg/L GA_3). One month after transfer, root tips 2-cm long were excised and transferred to B5G medium (Gamborg's B5 medium [6] + 1.0 mg/L GA_3).

Fifty disinfected J2 were applied onto the medium near the excised root in each of 10 replicated petri dishes. Shoot tip, root, and nematode cultures were maintained at 25 C under a 16-hour photoperiod at 3,000 lux.

The development of nematodes inside and outside roots was observed under a microscope daily for 50 days, and the mean percentage penetration of 10 replicates was calculated.

Shoots derived from excised roots: Twenty excised root segments about 4-cm long were transferred from MSIG to MSIBG medium. Ten segments were placed on the surface of the medium and 10 were inserted below the surface. The cultures were maintained under the same conditions as the root cultures.

To determine the shoot formation rate, 34 additional root segments, also 4 cm long, were inserted into MSIBG and observed daily for shoot formation.

When shoots were 2–3 mm long, 50 surface-disinfected J2 were applied onto B5G or MSIBG medium near roots with new shoots. Treatments were replicated seven times. The cultures were maintained at 20 C, with a 16-hour photoperiod at 3,000–4,000 lux.

Results

Callus tissues: In B5KD and B5KDG media, nematodes developed from J2 to J4 within 3 weeks. In B5KD nematodes did not subsequently develop beyond the fourth stage, and no male J4 were found. Nematodes developed faster in B5KDGL medium than in B5KDG medium (Table 1). Adult females and males developed in B5KDG and B5KDGL. No sperm were found in spermathecae of females; however, sperm were present in male reproductive systems. No eggs were found in the uteri of females. Numbers of nematodes decreased with the culture time, and nematodes did not reproduce in the callus culture.

Excised root tissue: Five days after inoculating cultures, many nematodes (87%) had penetrated root tissue (Table 2), most entering the root tip region. However, the nematodes moved in and out of the root tissue frequently and also moved from one root cell to another. When migrating between cells, the nematode body was squeezed flat, and the intestinal constituents moved forward. Root hairs were de-

TABLE 1. Development of *Subanguina picridis* in callus tissues of Russian knapweed (*Acroptilon repens*) on three culture media.

Days after inoculation	Life stage	Medium† B5KD	B5KDG	B5KDGL
7	J2	+‡		
	J3	+		
14	J2	+	+	
	J3	+	+	
	J4	+	+	
21	J2	+	+	
	J3	+	+	
	J3 molting	+	+	
	J4 (♀)	+	+	
	J4 (♂)	−	+	
28	J2	+	+	+
	J3	−	+	+
	J4 (♀)	+	+	+
	J4 (♂)	−	+	+
	adult (♀)	−	−	+
	adult (♂)	−	−	+
35	J2	+	+	+
	J3	+	+	+
	J4	−	+	+
	adult (♀)	−	−	−
	adult (♂)	−	+	−
42	J2	+	+	+
	J3	+	+	+
	J4	−	+	+
	adult (♀)	−	+	+
	adult (♂)	−	+	−
46	J2	+	+	+
	J3	+	+	−
	J4	−	+	+
	adult (♀)	−	+	−
	adult (♂)	−	+	−
50	J2	+	+	+
	J3	+	+	−
	J4	−	−	+

† B5KD medium was Gamborg B5 medium (5) supplemented with 0.1 mg/L kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D); B5KDG and B5KDGL were B5KD supplemented, respectively, with 1.0 mg/L or 10.0 mg/L gibberellic acid (GA_3).

‡ The first observations of B5KD, B5KDG, and B5KDGL were made 1, 2, and 4 weeks after inoculation. The symbols + and − indicate presence or absence of nematodes.

stroyed by nematode movement and were scattered around the roots.

The nematode developed from J2 to J3 10 days after inoculation and to J4 15 days after inoculation (Table 3). Twenty days after inoculation, the J2, J3, molting J3, and J4 were found inside or outside the root tissue. Further development of the nematodes did not occur beyond 28 days after inoculation. Reproductive systems developed normally during the first 20 days but became shorter and optically unclear after 28 days. At this time, nematode growth subsided and nematode activity also decreased. No nematodes developed beyond J4 during the 50-day period.

TABLE 2. Penetration of *Subanguina picridis* into excised root tissue of Russian knapweed (*Acroptilon repens*).†

Days after inoculation	Penetration‡ (%)
5	87
7	87
12	91
26	93
34	86

† Root tissue cultured on Murashige and Skoog's MS medium as modified by Linsmaire and Skoog (13), supplemented with 2.0 mg/L indole-3-butyric acid + 1.0 mg/L gibberellic acid.

‡ Data presented are means of 10 replicates.

Shoots derived from excised roots: Shoots formed only on roots inserted into the medium, whereas roots placed on the surface of the medium formed callus. Between 4 and 53 days after transfer, shoots formed on 56% of the roots, with most shoots forming during 28–49 days.

Three days after inoculation, nematodes were found inside shoot apices. The first galls were observed 13 days after inoculation on shoots derived from roots on both B5G and MSBIG media. About 50% of the

TABLE 3. *Subanguina picridis* development in excised root tissue of Russian knapweed.†

Days after inoculation	Life stages J2	J3	J4	adult (♀)	adult (♂)
0	+‡	−	−	−	−
10	+	+	−	−	−
15	+	+	+	−	−
20	+	+	+	−	−
25	+	+	+	−	−
35	+	+	+	−	−
50	+	+	+	−	−

† Root tissue cultured on Murashige and Skoog's MS medium as modified by Linsmaire and Skoog (13), supplemented with 2.0 mg/L indole-3-butyric acid + 1.0 mg/L gibberellic acid.

‡ The symbols + and − indicate presence or absence of nematodes.

shoots became galled with from one to four galls per shoot (Fig. 1). Galls formed on the leaves, shoot apices, and the petioles. One month after the first galls were observed, all life stages were found inside galls. Young adult females with one to three eggs inside the uterus and sperm inside the spermatheca were observed. The maximum number of nematodes per gall was 211, a 4.2-fold increase.

Discussion

In this study, we developed a unique method of using the shoots derived from roots to culture *S. picridis*. The development of the nematode was quite different in various types of Russian knapweed tissue. In the callus tissues derived from petioles, males and females developed from J2 but did not reproduce, while in the excised root culture, nematodes did not develop beyond J4. However, in the shoots derived from excised roots, the nematodes induced galls on the leaves, petioles, and shoot apices and developed and reproduced inside the galls. This is the first time *S. picridis* has been propagated in vitro.

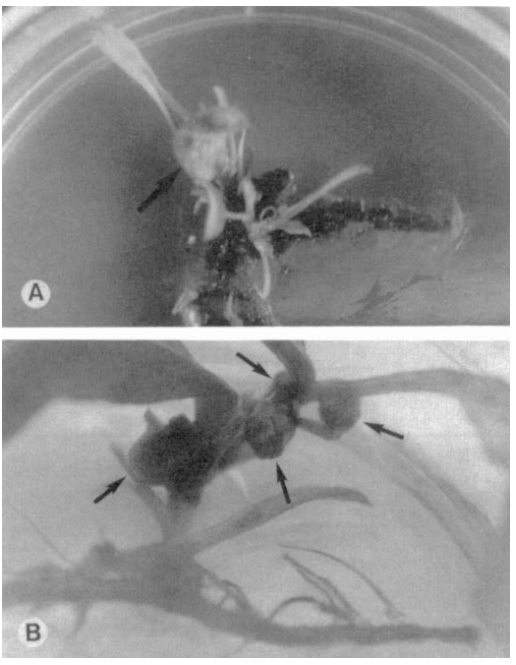

Fig. 1. *Subanguina picridis* gall on Russian knapweed (*Acroptilon repens*) shoot derived from root segment in culture. A) One gall per shoot (arrow). B) Four galls on one shoot (arrows).

Various genera of plant-parasitic nematodes, such as *Meloidogyne, Heterodera, Anguina,* and *Subanguina,* have evolved an intimate relationship with their hosts by the development of specialized feeding sites in plant tissues (5,11). The failure of a plant-parasitic nematode to complete its life cycle may be attributable to: 1) inability of the nematode to enter the host, 2) failure to induce the feeding site, or 3) the lack of elements required for nematode reproduction (12).

The active metabolic status of callus tissue (1) seems to satisfy the developmental requirements of *S. picridis*. Nematodes developed normally in callus from J2 to adults. The hypertrophy, hyperplasia, meristematic cellular morphology, and large increases in protein and nucleic acid content that occur during callus tissue growth (1) are similar to anatomical and physiological changes that generally occur during the development of galls induced by plant-parasitic nematodes (5,11,16–19,21), and various nematodes have been cultured in plant callus tissue. However, *S. picridis* did not reproduce in callus. The absence of sperm in the spermathecae of females and the presence of large numbers of sperm in male reproductive systems indicate that fertilization did not occur in the callus. This failure may result from inactivity of the nematodes in the callus, or a lack of reproductive function of female nematodes in the callus.

In the excised root, *S. picridis* frequently moved in and out of the root tissue, and apparently did not establish a permanent feeding site. The nematode did not develop beyond J4. These results indicate that the root tissue is not suitable for culture of this nematode. In addition, the nutritional or environmental requirements for development from J4 to adult may differ from those required for development from J2 to J4.

The specificity of a plant-parasitic nematode is not only manifested by the selection of its host but is also manifested by the

parasitic position in the plant. Specific parts of the plant and specific growth pattern of a plant tissue are required for nematode development and reproduction (21). Like most gall-forming nematodes, the invasive juvenile of *S. picridis* penetrates into meristematic tissue and induces galls at the penetration site. Though both excised roots and shoots derived from roots of Russian knapweed have meristematic tissues, *S. picridis* can only form galls and reproduce in shoot apex tissues. In monoxenic culture of some cyst and root-knot nematodes (16,17), similar tissue specificity was exhibited; these root-parasitic nematodes were successfully cultured only in the root and induced galls there only.

It is well known that high concentrations of GA_3 (1–8 mg/L) induce the growth of callus in plants (2,3,7,14), but little research has been done on the effects of GA_3 on the culture of plant-parasitic nematodes. Gibberellic acid increased the numbers of the nematode *Aphelenchoides ritzemabosi* in callus tissue culture (24). In our study, for culture of *S. picridis* in callus, the addition of GA_3 to B5KD medium was essential for nematode development beyond the fourth stage. The effect of GA_3 on formation of *S. picridis* males suggests that GA_3 may have male hormonal function. Also, the development of nematodes was more rapid at the higher concentrations of GA_3; adults of both sexes developed in 4 weeks at 10 mg/L, whereas the males were found after 5 weeks and females found after 6 weeks at 1.0 mg/L GA_3. Because GA_3 often produces effects on plants similar to those of auxin (8), the primary effect of GA_3 on nematode development was probably through stimulation of the growth of callus tissue.

Callus and excised root cultures have been used for monoxenic culture of various plant-parasitic nematodes. Establishment of most tissue cultures depends on an exogenous supply of plant growth regulators, but it has been shown that cultured tissue can also synthesize endogenous plant growth regulators (8). Neither auxin nor cytokinin was needed to culture the nematode in shoots derived from the roots in these experiments. It seems that shoots derived from roots contain enough endogenous plant growth regulators for nematode gall induction.

An economical mass rearing system, which can provide a large quantity of nematodes to augment the natural population increase and dispersal, is required to utilize *S. picridis* to control Russian knapweed. Following the success of propagating *S. picridis* in shoots derived from the excised roots, we have established a *S. picridis* mass rearing system that can supply a large number of nematodes for use as a bioherbicide to control Russian knapweed (unpubl.).

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