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Mass Culture of *Subanguina picridis* and Its Bioherbicidal Efficacy on *Acroptilon repens*

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Abstract: A Russian knapweed (*Acroptilon repens*) shoot culture system, initiated from shoot tip culture, was used to generate a source of host plant tissue for the rearing of the nematode *Subanguina picridis*, a biocontrol agent for Russian knapweed. Young shoots growing on solid B5G medium in petri dishes developed galls on leaves, petioles, and shoot tips 7 days after release of 50 nematodes onto the surface of the medium. After 3 months of culturing, each petri dish yielded 7,000–10,000 nematodes. In vitro cultured *Subanguina picridis* were virulent on greenhouse-grown Russian knapweed plants. Galls were first found on seedlings 12 days after infestation; after 2 months, 90% of seedlings were galled on leaves, petioles, and shoot tips, with 1–6 galls per seedling. Three months after shoot emergence, 64% of vegetative shoots originating from root segments were also galled by the cultured nematodes. Similarly, vegetatively regenerated shoots of Russian knapweed were also susceptible to infestation by cultured nematodes.

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

Russian knapweed, *Acroptilon repens* (L.) DC, shoot tissue derived from excised roots was recently demonstrated to be suitable for the propagation of *Subanguina picridis* (Kirjanova) Brzeski (8,9). The entire procedure for root initiation from shoot tip culture and subsequent new shoot initiation from root segments required approximately 3 months. Also, the shoot formation rate was low in that system.

In the field, host penetration by *S. picridis* occurs only in the early spring when young shoots emerge. Nematodes enter the shoot apex and induce galls in the host tissues. This characteristic of *S. picridis* suggested to us that the excised shoot apex from shoot tip culture may be a suitable plant source of tissue for mass culture of this nematode.

The shoot tip culture technique has been widely used to propagate many economically important plants (2). With this technique, a large number of plantlets can be obtained by multiplication of axillary shoots in culture. If *S. picridis* could be cultured in the shoots derived directly from shoot tip culture, the nematode culture time would be shorter, and the cost of the culture process would be greatly reduced. Therefore, experiments were conducted to evaluate the utility of young shoot apices derived from shoot tip culture for the propagation of *S. picridis*.

A potential bioherbicide pathogen should be able to be cultured on artificial medium, should be highly virulent, and should have the capacity to damage its host plant (12). *Subanguina picridis* is the first nematode used as a biological weed control agent to be successfully mass propagated in vitro. Nematodes cultured in the laboratory are grown under artificial but favorable conditions. As a result, the cultured plantlet may be more susceptible to the attack of the nematode. To determine the potential application value of this monoxenic nematode culture system, tests should be conducted to determine the efficacy of the cultured nematodes on whole plants and to determine appropriate application techniques.

Therefore, a subsequent objective of this study was to evaluate the ability of cultured *S. picridis* to penetrate host tissues, to initiate galls, and to suppress the growth of Russian knapweed plants grown in greenhouse conditions.

Materials and Methods

Surface disinfection of shoot apices: Shoot apices 1.5 cm long were excised from

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plants grown in a greenhouse, washed with tap water, immersed in 75% ethanol for 1 minute and 1% sodium hypochlorite solution for 15 minutes, and washed three times in sterile distilled water.

Shoot tip culture: A strip of Whatman No. 1 filter paper (18 × 100 mm) was folded into a letter "M" shape to form a filter-paper bridge. The arms of the paper strip were immersed in MSIBG liquid medium (8) in the bottom of a culture tube (25 × 150 mm) (Fig. 1A).

The surface-disinfected shoot apex was placed on a petri dish. After removal of leaves and leaf primordia with fine needles, the meristematic dome with two to three primordia, approximately 2 mm long, was carefully excised with a scalpel fitted with a pointed tip blade (No. 11) under a stereomicroscope. This shoot tip was then carefully transferred with the blade of the scalpel and positioned upright in the "V" of the filter-paper bridge. This experiment included 10 replicate tubes, each tube containing 1 shoot tip. The culture tubes were capped with plastic covers and maintained at 25 C with a 16-hour photoperiod and a light intensity of 60 $\mu mol \cdot M^{-2} \cdot s^{-1}$. Small shoots developed from the shoot tips 1 week after being placed on the filter-paper bridge (Fig. 1B).

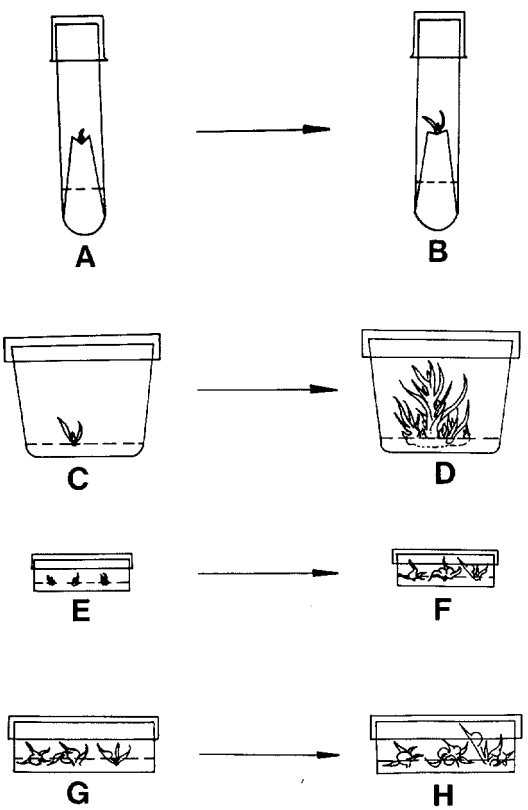

FIG. 1. Flow diagram of the *Subanguina picridis* culture process. A) Russian knapweed (*Acroptilon repens*) shoot tip on the filter-paper bridge. B) Small shoot derived from the shoot tip. C) Small shoot from the shoot tip culture transferred to MSIBG solid medium. D) Cluster of shoots. E) Three young shoots in B5G medium infested with 50 mixed-stage nematodes. F) Galls formed on shoots in culture. G) Galled shoots subcultured on MSIG rooting medium. H) Maintenance of the gall culture. Note that new galls were formed.

Shoot culture and maintenance: Small shoots derived from the shoot tip culture were transferred to MSIBG medium (8,9) in a 600-cm^3 plastic container (Fig. 1C). One month after transfer, a cluster of shoots formed by outgrowth of bud primordia (Fig. 1D). This cluster was divided by a scalpel into smaller clumps (three shoots per clump) in a sterile petri dish. Multiplication of shoots was achieved by transferring these smaller clumps to fresh MSIBG solid medium. Shoot cultures were maintained under the same conditions as the shoot tip cultures.

Plant nematode infestation: Nematodes were obtained from cultured galls (8,9). Galls were opened and the nematodes, in mixed stages of development, were released into sterile water. Nematode suspensions were obtained by removing gall debris with forceps. Fifty mixed-stage nematodes were transferred with a pipette onto B5G medium (8) in a petri dish (60 × 20 mm) with three young shoots (Fig. 1E). Infested shoots were placed in an incubator at 20 C with a 16-hour photoperiod at a light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Gall culture and maintenance: When galls formed in culture (Fig. 1F), the galled shoots were subcultured to MSIG rooting medium (8) in 100 × 25 mm petri dishes (Fig. 1G,H). The gall cultures were maintained at 20 C with a 16-hour photoperiod at a light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and subcultured monthly. Five replicate

galls were dissected at each of several intervals after inoculation, and the numbers of adults, each juvenile stage, and eggs were determined using a stereomicroscope.

Evaluation of virulence of cultured Subanguina picridis: Russian knapweed seeds were germinated on moistened filter paper in petri dishes. Two seedlings, approximately 1.5 cm long, were carefully planted just below the surface of a prepared substrate (Pro mix) in 12-cm-d pots. Approximately 1,000 *S. picridis* of mixed stages of development from a 4-month-old gall produced in culture (8,9) were applied to the substrate surface in each pot. Uninfested seedlings were used as controls. Ten replicates of the two treatments were placed in a completely randomized design on a bench in the greenhouse maintained at 20 ± 3 C day/15 ± 3 C night.

Root segments were excised from Russian knapweed plants grown in the greenhouse. Two 8-cm segments with a 2–5 mm stem bud were transferred to a 12-cm-d pot containing a prepared substrate (Pro mix). The soil in each pot was infested with nematodes, as in the seedling experiment. The infested roots were covered with 1 cm of Pro mix. Uninfested segments were used as controls. Fourteen replicates of the two treatments were placed in a completely randomized design on a greenhouse bench at 20 ± 3 C day/15 ± 3 C night.

The susceptibility of 1-mm, 2-mm, and >5-mm stem buds was also examined. Segments of a young root with buds of these sizes were placed on prepared substrate (Pro mix) in 12-cm-d pots, one root segment per pot, inoculated as previously described. The infested root segments were covered with 1 cm of Pro mix. Controls consisted of root segments not infested with nematodes. Seven replicates of these treatments were placed in a completely randomized design on a greenhouse bench at 20 ± 3 C day/15 ± 3 C night.

To test the ability of the nematodes to move within the soil and locate their host, the surface of a box (50 × 40 × 30 cm) containing greenhouse-grown Russian knapweed plants was divided into two areas. All plants in each box were cut at soil level. Approximately 10,000 nematodes of mixed stages of development from 4-month-old cultured galls were applied to one half of the surface, in each of five boxes (replicates). Gall formation on regrowth was monitored for several months.

Results

Seven days after inoculation, galls were visible on the leaves, petioles, and apices of the shoots, and one or more galls were found on each shoot (Fig. 2A). Thirty-five days after inoculation, the leaves had become yellow, and no nematodes were found in the medium.

Nematodes developed and reproduced inside the galls (Table 1). At the first sign of galling, J2, J3, J4, and molting J4 were found. Five days after galls appeared, both males and females were found. Females had eggs in their uteri and had laid numerous eggs. In addition, newly hatched J2 and eggs in various stages of development were observed. Young adults and eggs in different stages were found in galls

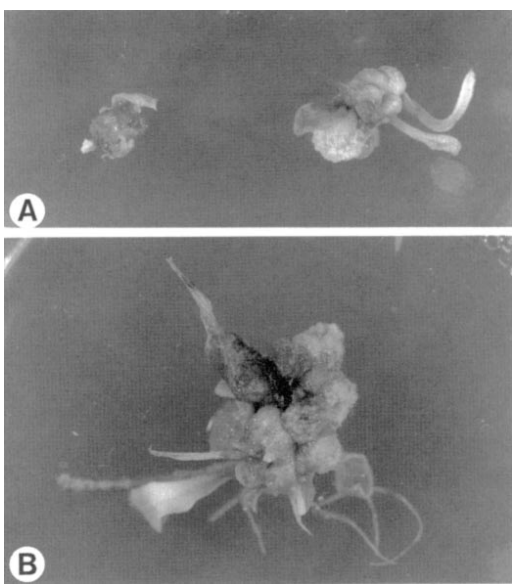

FIG. 2. *Subanguina picridis*-containing galls on Russian knapweed shoots in culture. A) One or more galls per shoot. B) Numerous large galls on a shoot after 5 months in culture.

TABLE 1. Number of *Subanguina picridis* inside cultured *Acroptilon repens* galls.†

Days after gall appearance	Number of *S. picridis*						
	Adults (♂)	Adults (♀)	J4	J3	J2	Eggs	Total
1	0	0	2	2	2	0	6
5	3	2	0	0	3	81	89
10	7	9	5	2	23	288	334
15	3	5	29	54	135	418	644
17	21	23	68	87	192	231	622
30	111	151	105	256	994	3771	5388

† Mean of five replicates.

17 days after the first galls appeared. Nematode numbers increased with culture time. One month after culture, each gall contained an average of 1,078 nematodes. Three months after culture, the nematode number had increased from the initial 50 to 7,000–10,000 per petri dish, a 140–200-fold increase.

New galls were formed after galled shoots were transferred to the rooting MSIG medium (Fig. 2B). Five months after infestation, the gall number reached 18 galls per shoot.

Twelve days after inoculation, two galls were observed on leaves of Russian knapweed seedlings. After 2 months, 90% of the seedlings were galled on the leaves, stems, and the shoot tips at 1–6 galls per seedling (Fig. 3A,B). The growth rate of galled seedlings was reduced visibly in comparison with the controls (Fig. 4A). Eighty days after inoculation, when uninfested control plants were flowering, galled plants were still in the rosette stage (Fig. 4B).

Vegetative shoots originating from root segments were successfully galled by cultured nematodes also (Fig. 3C). Three months after shoot emergence, 64% of the shoots developed galls. These galls were found on the shoot tips, leaves, and stems.

Fifteen days after inoculation, galls were found on 100% of shoots derived from 1-mm-long stem buds, 71% of shoots from 2-mm-long stem buds, and 80% of shoots from >5-mm stem buds.

Similarly, greenhouse-grown Russian knapweed plants that had been cut off at the soil level were susceptible to infection

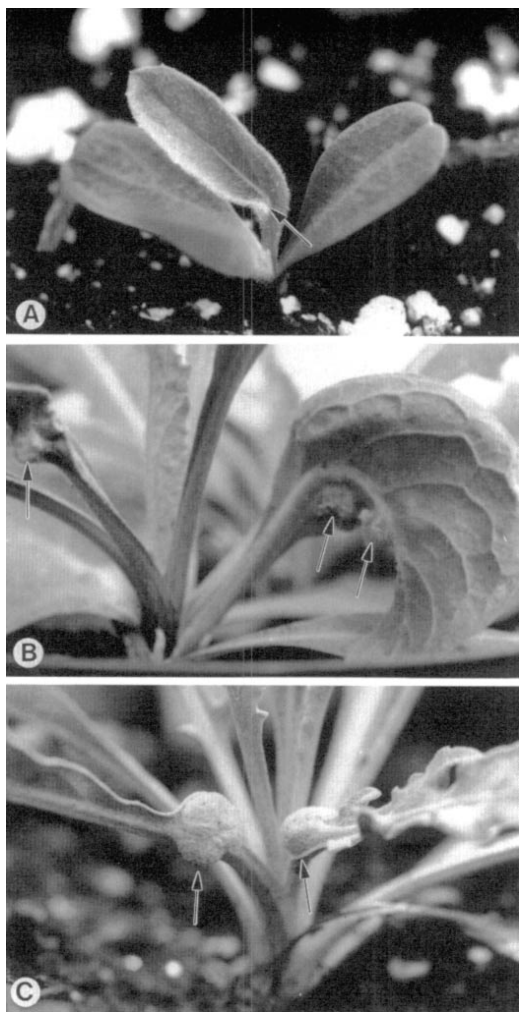

FIG. 3. A) Greenhouse-grown Russian knapweed seedling, 12 days after infestation of substrate with *Subanguina picridis* from shoot culture. Note gall formation on leaf (arrow). B) Numerous galls (arrows) on a Russian knapweed seedling grown in the greenhouse, 40 days after infesting substrate with nematodes from shoot culture. C) Galls (arrows) formed on a vegetative shoot that originated from root segments.

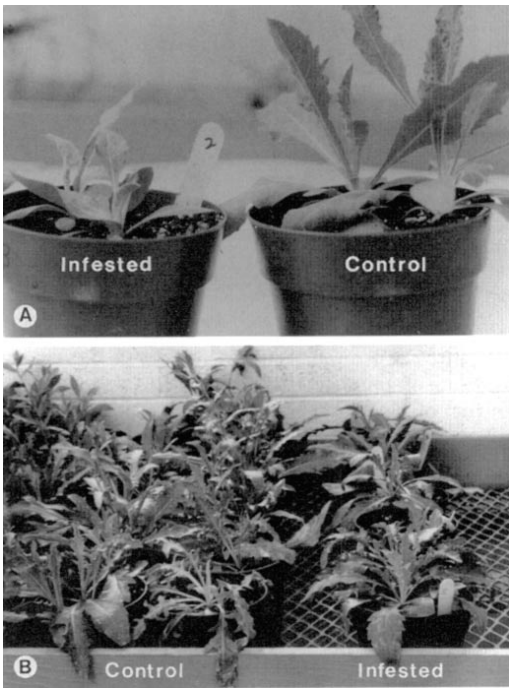

FIG. 4. Growth of uninfected (control) Russian knapweed and *Subanguina picridis*-galled plants. A) 40 days after infesting substrate. B) 80 days after infesting substrate.

by soil-applied cultured nematodes. Galls were first observed on leaves and stems of shoots 19 days after soil inoculation, but only on the plants growing in the inoculated region.

DISCUSSION

A mass rearing system, which can provide large quantities of infective *Subanguina picridis*, is required for the successful biological control of Russian knapweed. This study demonstrates that *S. picridis* reared in vitro are virulent and can induce gall formation on greenhouse-grown Russian knapweed plants.

Our results indicate that the life cycle of *S. picridis* in culture at 20 C is about 12 days. Males and females were found inside the galls 5 days after the galls appeared, and the young adults of the second generation were found after 17 days. Because no nematodes beyond the J4 were found the first day that galls were visible, the J4 may be a critical point in nematode development; gall formation appears necessary for further development and reproduction.

Nematode galls are caused by hypertrophy and hyperplasia of parenchyma tissues. Hypertrophied cells in the nutritive zone, called nutritive cells, contain abundant granular protoplasm and are thought to supply food to nematodes within the gall (1,3,4). These cells act as physiological sinks inducing translocation of assimilates to the gall and gall former (3,6). Shoot galls observed in culture were morphologically similar to those formed in nature (11); they had a central cavity and a well-defined zone of nutritive cells. They also appeared to function as physiological sinks. Once galls were induced, they developed rapidly, and the remaining part of the shoots was stunted. When galls were initiated on the shoot tip, shoot formation was usually stopped, and the shoot produced only gall tissue. Thus, nutrients within the plantlet appear to have been translocated to the gall preferentially, thereby depriving other developing tissues of nutrients required for growth.

The continual formation of new galls on the galled shoots after transfer to fresh rooting medium and failure to observe nematodes in the medium indicated that *S. picridis* migrates inside the cultured tissue. Dispersal through the tissue enabled propagation of the nematode by separating the individual lateral galled shoots and transferring them to fresh medium.

The mass rearing *S. picridis* system we developed provides a favorable artificial environment that overcomes limitations encountered in the field. Host penetration by *S. picridis* normally occurs only during the relatively short period when young shoots of Russian knapweed emerge through the upper soil layer during early spring. The nematode develops and reproduces inside the host during the vegetative growth stage. As the plant matures, however, the nematodes stop developing, and only two generations are completed each year (5). The culture system we developed can continually provide young shoot tissue, enough nutrients, and a con-

stant physical environment for nematode development and reproduction throughout the year. Thus, the system could be modified to produce sufficient numbers of nematodes to augment populations as a bioherbicide.

Successful biocontrol of weeds often depends on comprehensive knowledge of the biology of the target weed and the natural enemy. The endoparasitic nature of *S. picridis* hinders research on its biology. Successful mass rearing of *S. picridis,* however, will facilitate studies needed to identify factors affecting its usefulness as a biocontrol agent. For example, mass rearing could be used to explore the effects of moisture, temperature, tissue nutrition, etc. on infection, to determine factors involved in dormancy induction of J2, and to determine behavioral cues involved in host recognition.

Although Russian knapweed does not propagate extensively from seed in North America (10), seed production plays an important role in propagation in its native range (5). The influence of cultured *S. picridis* on young seedlings suggests that this nematode can be applied when seeds germinate. In addition, the galling of greenhouse-grown perennial Russian knapweed plants demonstrated that control of this weed may be enhanced by removing the above-ground parts of the plant before application of the cultured nematodes.

Soil infestation with nematode–water suspensions has been effectively used by Kovalev et al. (7) in the weed's native range. The nematode–water suspensions are easily prepared and readily applied to weed-infested areas. The nematodes can survive in sterile distilled water for at least 2 months. For long-term storage of cultured *S. picridis,* galled shoots can be maintained in the medium and subcultured monthly.

The successful establishment of the *S. picridis* mass rearing system and the high incidence of infection of Russian knapweed in efficacy tests suggest that the utilization of cultured nematodes as bioherbicides is possible. This may open new prospects for the inundative biological control of weeds with plant-parasitic nematodes.

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