IN VITRO MASS REARING OF THE KNAIPWEED NEMATODE, 

Subanguina

picridis AND ITS USE AS A BIOHERBICIDE

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Research in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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ABSTRACT

A culture system was established for mass rearing of the Russian knapweed nematode, Subanguina picridis (Kirjanova) Brzeski. This system consisted of two parts; a shoot culture system used as the host plant source for the nematode culture, and a monoxenic nematode culture system. The nematode developed and reproduced in this system. Galls were induced on the leaves, stems, and shoot tips of cultured Russian knapweed (Acroptilon repens (L.) DC.) shoots. After 3 months in culture, the nematode number per petri dish increased from the initial 50 inoculated to 7,000-10,000, a 140 to 200 fold increase. This study represents the first time that an above-ground gall forming nematode has been propagated in vitro. It also represents a unique monoxenic nematode culture system to mass rear above-ground endoparasitic plant nematodes.

Various factors, including tissue type, tissue age, medium, and temperature, which affect the formation and development of galls, were examined. The nematode failed to reproduce in callus tissues, and it could not develop beyond the 4th stage in excised root cultures. The optimum incubation conditions determined were: 60-80 μmolm⁻²s⁻¹ light intensity, 20 C temperature, and 4-8 mm for shoot length.

The virulence of cultured nematodes was tested in the greenhouse on Russian knapweed seedlings and vegetative shoots from root segments. The results demonstrated the feasibility and application of this novel mass production system. Nematodes produced in this system were virulent and the growth rate of infested Russian knapweed were reduced.
RÉSUMÉ

Un système de culture a été édifié pour l'élevage en masse du nematode Subanguina picridis (Kirjanova) Brzeski. Ce système se divise en deux parties; un système de culture de pousses utilisé comme matériel-hôte pour l'élevage de nématodes, et un système d'élevage de nématodes. Le nematode s'est développé et reproduit dans ce système. Des galles ont été induites sur les feuilles, les tiges et le bout des pousses de plants de Centauree de Russie (Acroptilon repens (L.) DC) cultivés. Après mois de culture, le nombre de nématodes a augmenté d'un inocula initial de 50 nematodes jusqu'à 7000-10,000 nématodes. Cette étude est la première démontrant qu'un nematode aérien formant des galles peut être propagé in vitro.

Plusieurs facteurs affectant la formation et le développement des galles ont été examinées. Ceux-ci incluent le type et l'âge du tissu, le milieu et la température. Le nématode n'a pas réussi à se reproduire dans le tissu de cal et n'a pas pu se développer au-delà du 4e stade dans la culture de racines excisées. Les conditions optimales telles que déterminées sont: intensité lumineuse de 60-80 μmol m⁻² s⁻¹, température de 20 °C et longueur de pousse de 4-8 mm.

La virulence des nématodes cultivés a été évaluée dans la serre sur des plantules de centauree de Russie et sur des pousses végétatives issues de segments de racines. Les résultats démontrent la faisabilité et la possibilité de mise en application de ce nouveau système de production en masse. Les nématodes produits en ce système sont virulents et le taux d'accroissement et de reproduction des plants de centauree de Russie infestés sont diminués.
Suggested short title:

*In vitro* mass rearing of *Subanguina picridis*
PREFACE

Studies on mass rearing of the knapweed nematode, *Subanguina picridis*, were carried out from May, 1986 to June, 1990. Since *S. picridis* had not been successfully cultured *in vitro*, and no other above-ground gall forming nematodes had been cultured, the experiments in this study included all steps in developing a nematode culture system including the culture of plant tissue, the infestation of established plant tissues with the nematode and the optimization of culture conditions for gall formation and nematode development.

The introduction and the literature review are in chapter 1. The preliminary experiments (chapter 2) included the culture of different types of Russian knapweed plant tissues, the choice of suitable tissue for *S. picridis* culture, and the determination of culture conditions. As a result of the preliminary experiments, *S. picridis* was, for the first time, successfully cultured in shoot tissues from excised roots. The experiments continued to simplify and optimize the culture procedure (chapter 3). In these experiments, young shoots obtained directly from the shoot culture system were used to culture *S. picridis*, and the effects of tissue age, medium, and temperature on *S. picridis* culture were determined. A unique monoxenic nematode culture system which could provide large quantities of *S. picridis* was established. Since the overall goal of this study was to mass rear *S. picridis* for its use as a bioherbicide, the last experiment of this study was the virulence test of cultured nematodes (chapter 4). The data demonstrated the feasibility and application of this novel mass production system. The nematodes produced in this system were virulent and the growth rate of infested Russian knapweed was reduced. The successful culture of *S. picridis*...
picridis in vitro makes the use of this nematode as a bioherbicide possible. Extended application of this culture system to culture other above-ground weed parasitic nematodes may help to open new avenues for the use of cultured nematodes as effective bioherbicides. Discussion of the utilization of cultured nematodes as bioherbicides including a general review about bioherbicides, the advantages of the established S. picridis culture system, and the prospects of this biocontrol program are presented in chapter 5. Two other weed parasitic nematodes have been selected as potential biological weed control agents. In this chapter, the biology and the distribution of these two nematodes, and the current status of their application as bioherbicides are also discussed. The final overall conclusions of this study are provided in chapter 6. References are given at the end of chapter 6.
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I am very grateful to my parents, my husband, my brothers and sisters for their encouragement, support and love.
AUTHOR'S CLAIM OF ORIGINALITY

The following aspects of the research, described in this thesis, are original contributions to scientific knowledge of biological weed control using plant parasitic nematodes, plant nematode culture, and plant nematology:

(1) This is the first time that an above-ground gall forming nematode has been successfully cultured in vitro.

(2) This is the first nematode used as a biological weed control agent to be mass propagated in vitro.

(3) The culture system developed in this study is a unique monoxenic nematode culture system to mass rear above-ground endoparasitic plant nematodes.

(4) For the first time, the effect of various factors including tissue type, tissue age, medium, and temperature on S. picridis culture were examined.

(5) This is the first report that giberellic acid affected the formation of male nematodes.

(6) The hypothesis that the nematode galls function as "physiological sinks" was supported by the evidence from the galls cultured in vitro.
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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Russian knapweed, *Acroptilon repens* (L.) DC is a serious noxious weed which was introduced into Canada in the early 1900's with contaminated alfalfa seed (Groh, 1940) and is now widely distributed in western Canada and the central region of the United States (Reed and Hughes, 1970). It is also a problem in Australia, New Zealand, and parts of Europe and Asia. The plant has an extensive root system, and its strong competitive ability reduces the yield of desired plants and decreases the quality of produce. Control of this persistent perennial weed is difficult by cultural and chemical methods (Watson and Harris, 1984).

The plant parasitic nematode, *Subanguina picridis* (Kirjanova) Brzeski produces galls on the stems, leaves, and the root collars of Russian knapweed, and reduces reproduction and plant growth of this weed. The nematode has a restricted host range (Watson, 1986a) and has been imported from the Soviet Union and released in Canada and the United States for the biological control of Russian knapweed.

The current use of *Subanguina picridis* for the biocontrol of Russian knapweed is limited in North America and other parts of the world because this nematode has only two generations per year and it has limited natural dispersal. Field populations of this nematode require a long time period to build up sufficient numbers to have any effect on the knapweed populations. Thus a large quantity of nematodes which can be readily disseminated are required for the success of this biocontrol program.
The use of plant nematodes as biocontrol agents for the biological control of weeds is new and less proven than insect-nematode biological control strategies. One reason for this is that the insect nematodes can, in several instances, be mass produced on artificial media (Parker, 1986). Attempts to mass produce plant parasitic nematodes have met with very limited success and no above-ground gall forming nematodes have been mass produced in vitro.

Achievement of a successful monoxenic nematode culture depends on three components, the plant tissue, the nematode, and the culture environment. The nematode and the plant tissue are the biological units and the culture environment must be suitable to both plant tissue growth and to the nematode development and reproduction.

Primary factors affecting the culture of plant nematodes in vitro include: biological (plant tissue), chemical (medium), and physical factors of the culture environment. The nature of the plant tissue directly influences the behaviour of the nematodes. Plant tissues which have been used are callus and excised roots in which the callus is used by the nematodes as their food source. Many migratory nematodes have been successfully propagated on callus tissues (Krusberg, 1961; Khera and Zuckerman, 1962; Inserra and O'Bannon, 1975; Corbett, 1970). Excised roots have been used to culture some root-knot and cyst nematodes (Reversat, 1975; Moriarty, 1964; Dropkin and Boone, 1966). For these sedentary parasites, the root tissues are used as their habitat and source of food. The age of plant tissue influences the penetration and the rate of development of the nematodes with tissues becoming resistant to plant nematodes when they are too old.
The chemical contents of the medium indirectly influence the nematode culture throughout the growth and development of the plant tissue in culture. A medium which provides good growth of plant tissue does not necessarily support nematode production. Plant growth regulators play very important roles in plant tissue and plant nematode culture. Different levels and types of plant growth regulators in the medium directly affect the plant tissue culture and nematode penetration.

The main physical factors in plant/nematode culture are temperature, light intensity, and the pH of the medium. Temperature affects both the response of the host to the nematode and the reaction of the parasite to the host.

*S. picridis* is a sedentary plant parasitic nematode. Infective 2nd stage larvae infest young Russian knapweed shoots in the soil, penetrate meristematic tissues, and initiate gall formation. No species of the genus *Subanquina* nor any species of related genera including *Anguina*, *Orrina*, and *Nothanguina* have been successfully cultured *in vitro*.

The objectives of this study were: 1) to establish a plant tissue culture system for the culture of *S. picridis*; 2) to choose the best medium for *S. picridis* culture; 3) to determine the influence of plant growth regulators on cultures; 4) to examine the effects of temperature and plant tissue age on the development of *S. picridis*; and 5) to test the virulence of *S. picridis* from culture. The overall goal was to develop a culture system for mass rearing the nematode, *S. picridis*. 
1.2 Literature review

1.2.1 The biology of Russian knapweed


The native distribution of Russian knapweed includes southern Russia, Mongolia, Western Turkestan, Iran, Turkish Armenia, and Asia Minor (Ivanova, 1966; Moore and Frankton, 1974). Russian knapweed was introduced into Canada in the 1900's with imported Turkestan alfalfa and is now distributed in southwestern Manitoba, Saskatchewan, Alberta, south central British Columbia and southern Ontario (Frankton and Mulligan, 1970; Moore and Frankton, 1974). This weed is also widespread in the western and central regions of the United States (Reed and Hughes, 1970) and has been introduced into Australia (Burbidge, 1963).

Russian knapweed is able to survive in almost any crop in any tillable soil (Rogers, 1928). It is found in both irrigated and arid regions (Selleck, 1964). Russian knapweed forms dense infestations in cultivated fields, grain and alfalfa fields, pastures, and waste places (Watson, 1980). This weed has an extensive root system. It competes primarily for soil moisture and nutrients (Berezovskii and Raskin 1971; Popov et al., 1973). The strong competitive ability of Russian knapweed reduces yields of desired
plants and decreases the quality of the produce. Grain yields of wheat have been reduced by 28-75% with densities of Russian knapweed at 11-64 shoots/m² (Popov et al., 1973). Russian knapweed is more competitive than other weeds and is able to spread steadily and rapidly in good pastures (Rogers, 1928). In addition, Russian knapweed is poisonous to horses, causing a neurological disorder and even death (Young et al., 1970). Russian knapweed is listed as a prohibited noxious weed in the Seeds Act (Agriculture Canada, 1967) and is considered a noxious weed in many provinces of Canada.

The morphology of Russian knapweed has been described by Frankton and Mulligan (1970), Reed and Hughes (1970), Moore and Frankton (1974) and Watson (1980). Russian knapweed is a perennial herb reproducing by seeds and by creeping horizontal roots. In Canada, Russian knapweed does not appear to reproduce extensively from seed (Renney and Hughes, 1969). The root system consists of the original root, one to many permanent lateral roots and their vertical extensions (Frazier, 1944). These roots produce stem buds that develop into leafy shoots.

The life cycle of Russian knapweed is briefly summarized as follows: Shoots emerge early in the spring shortly after soil temperatures remain above freezing. After emergence, the plants form rosettes and bolt in late May to mid-June. Flowering occurs from early July and continues into September (Watson, 1980).
1.2.2 The biological control of Russian knapweed using the plant parasitic nematode, *Subanguina picridis*

1.2.2.1 General review of biological weed control

Biological control of weeds is the deliberate use of natural enemies to suppress the growth or reduce the population of a weed species (Watson, 1989). The use of biotic agents to control weeds is based on the fact that some plant-attacking organisms can reduce their host plant populations to non-noxious levels of abundance. This form of weed control has developed primarily during the last 30 years. Two primary approaches have evolved for biological weed control. These are the classical or inoculative approach and the inundative or bioherbicide approach.

Early studies on the biological control of weeds most often utilized the classical approach, with phytophagous insects being main biotic agents used. The classical approach involves the introduction of a biotic agent from its native range or from elsewhere where it occurs into a region where an exotic weed exists at noxious levels. The biotic agents are simply released after the demonstration that the agents are safe to crop and other economically important plants. There are several steps in developing a classical biological weed control program. They include: (1) determination of suitable target weed species, (2) conduction of overseas surveys for suitable natural enemies in the weed's natural range, (3) selection of the effective natural enemies, (4) determination of host specificity, (5) introduction and the establishment of selected agents, and (6) evaluation of the effect on the weed population (Harris, 1977; Schroeder, 1983). The progress of classical
biological weed control is described in the articles of Wapshere (1982), and Schroeder (1983).

Recently, the inundative approach has become increasing more important. The pioneer work was in Arkansas with the application of a spore suspension of an endemic fungus (*Colletotrichum gloeosporioides* f. sp. *aeschynomene*) to control the serious weed, Northern jointvetch (*Aeschynomene virginica*), in rice. The inundative approach involves mass rearing large numbers of a biological control agent and releasing massive numbers of the agent into the region where the weed is at noxious levels, thereby artificially increasing the abundance and hence effectiveness of the agent (Wapshere, 1982). Both endemic and exotic pathogens can be used in this approach (Hasan, 1988). Generally, fungi are the most common organisms used inundatively, but other pathogens including nematodes, bacteria and viruses are being evaluated. Insects are generally not included in the inundative approach. The procedure for selecting pathogens used inundatively to control weeds was described in detail by Wapshere (1982), Templeton (1982), Hasan (1988), and Watson (1989). Since the pathogen is applied in a manner analogous to that of a chemical herbicide, the inundative approach is also called the bioherbicide approach. The pathogens used in this approach are termed "bioherbicides". More detailed information on the development of bioherbicides will be described in chapter 5.

1.2.2.2 The biological control of Russian knapweed with *S. picridis*

Information on the selection and use of *S. picridis* to control Russian knapweed can be found in the articles written by Ivanova (1966), Kovalev et
al. (1973), Watson and Harris (1975, 1984), Watson (1977, 1986 a,b,c) and Mortensen and Molloy (1989).

Several methods have been tried to control Russian knapweed. Cultural methods for the control of Russian knapweed include crop rotation, clean cultivation, and flooding. Chemical herbicides used to control Russian knapweed are 2,4-D (2,4-dichlorophenoxyacetic acid), picloram (4-amino-3,5,6-trichloropicolinic acid), dicamba (3,6-dichloro-o-anisic acid), and simazine [2-chloro-4,6-bis(ethylamino)-s-triazine]. The combination of cultivation and chemical methods have been employed with varying success (Mordovets et al., 1981; Zharokova and Kidrishev 1984; Popovich et al., 1985). However, the control of Russian knapweed is extremely difficult since its extensive root system is generally not adversely affected by cultivation and the weed is relatively tolerant to commonly used herbicides. Cultural methods are not very effective and existing chemical herbicides are generally not satisfactory for most infestations (Ivanova, 1966; Watson and Harris, 1984).

Since the early 1960's, there has been an intensive search for specific phytophages of weeds in Central Asia (Kovalev, 1974). The natural enemies of Russian knapweed were investigated in the years 1967-1969 by Kovalev (1974). It was observed that insects, mites, nematodes, and fungi (Kovalev, 1968a,b) played large roles in regulating the reproduction of this weed in its primary habitats of Soviet Central Asia (Table 1). The most effective regulator of the reproduction of Russian knapweed is the knapweed nematode, Subanguina picridis. This species spreads from Central Asia to Kazakhstan and the European area of the U.S.S.R. (Kovalev, 1973).
Kirjanova (1944) provided descriptions of the knapweed nematode and of the appearance of the injury to infected knapweed and also pointed out the useful role played by this nematode. Ivanova (1966) reported that in the regions of Tadzhikistan the knapweed was very susceptible to the knapweed nematode, where growth and seed production were considerably depressed and the number of galls on a single plant were high. Field studies were conducted to evaluate the utility of this nematode for the control of Russian knapweed. The galls of *S. picridis* were introduced into the soil when the Russian knapweed was in the development stage and resulted in 100 percent of the knapweed being affected. Of these, 20 percent were destroyed and up to 30 percent were heavily injured (Ivanova, 1966).

Since the method of redistributing or introducing knapweed nematode galls into the soil can be employed only on small plots, Kovalev (1973) tried to use an inundative method to introduce knapweed nematode larvae into the soil to control Russian knapweed. A water suspension of invasive larvae was prepared and applied to the soil by spraying. By this method, 60.8 percent of infested knapweeds were infected and 27 percent destroyed. This procedure of distributing the nematode in a water suspension made it possible to utilize the infective material more economically and to mechanize the nematode infection method (Kovalev, 1973).

In North America, this weed is relatively free of parasites, but it was reported that this weed was attacked by numerous specialized organisms in its native range (Table 1). An overseas survey of the natural enemies of Russian knapweed was conducted and several potential biological control agents were selected to be introduced from its native range for further study under quarantine conditions. *Puccinia acroptili* and *S. picridis* were
imported into Canada to be studied. Some fungi endemic to Canada have also been tested and include: Physarum cinereum, Phomopsis sp., Sclerotinia sclerotiorum, and Alternaria cichorii (Table 1). Since the rust spores of P. acroptili obtained from the U.S.S.R. would not germinate (Watson, 1977), and none of the fungi were promising as bioherbicides (Mortensen and Molloy, 1989), most of the biocontrol program against Russian knapweed has been concerned with the knapweed nematode, S. picridis. The release of S. picridis into Canada was the first attempt to use an exotic plant parasitic nematode in North America for the biological control of a weed.

As with all exotic biological weed control agents, the host specificity and the effectiveness of S. picridis were determined under quarantine laboratory condition prior to its release. The results of the host range examination and effectiveness tests combined with restricted field trial experiments confirmed that S. picridis was a suitable agent for the biological control of Russian knapweed in North America (Watson and Harris, 1984; Watson, 1986b) and was approved for introduction and release in Canada and the United States for the control of Russian knapweed. S. picridis has been released and established in three locations (British Columbia, Saskatchewan, and Manitoba) in Canada.

The current use of S. picridis for the biological control of Russian knapweed is limited in North America and other parts of the world because this nematode has only two generations per year and has limited natural dispersal (Ivanova, 1966; Watson and Harris, 1984). Field populations of this nematode require a long time period to build up sufficient numbers to have an effect on the Russian knapweed populations. The lack of movement of S. picridis in or on the soil suggests that populations of the biocontrol
agent must be augmented in order to achieve control of the target weed. Therefore, a large quantity of nematodes, which can be readily disseminated is required for the success of this biocontrol program. Just as Ivanova (1966) commented on the need for extensive application of the knapweed nematode in the control of Russian knapweed, it is necessary to establish methods for mass rearing the knapweed nematode in artificial conditions.
<table>
<thead>
<tr>
<th>Name</th>
<th>Biological information</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACARID</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aceria acroptiloni</td>
<td>Monophagous</td>
<td>U.S.S.R.</td>
<td>Kovalev (1975)</td>
</tr>
<tr>
<td>V. Shev. &amp; Kov.</td>
<td>seed-head mite</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>INSECT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larinus bardus Gyll</td>
<td>Stenophagous beetle</td>
<td>U.S.S.R.</td>
<td>Ivannikov &amp;</td>
</tr>
<tr>
<td>Larinus jaceae Fabr</td>
<td>Stenophagous beetle</td>
<td>U.S.S.R.</td>
<td>Tyurebaev (1977)</td>
</tr>
<tr>
<td>Rhynchaenus distans Faust</td>
<td>Stenophagous beetle</td>
<td>U.S.S.R.</td>
<td>Ivannikov &amp; Tyurebaev (1977)</td>
</tr>
<tr>
<td>Dasyneura sp.</td>
<td>Stenophagous bud gall former</td>
<td>U.S.S.R.</td>
<td>Tyurebaev (1972)</td>
</tr>
<tr>
<td>Urophora maura (Prfld)</td>
<td>Stenophagous seed-head former</td>
<td>U.S.S.R.</td>
<td>Tyurebaev (1972)</td>
</tr>
<tr>
<td>Aulacida californica (Spey.)</td>
<td>Monophagous</td>
<td>U.S.S.R.</td>
<td>Tyurebaev (1972)</td>
</tr>
<tr>
<td><strong>FUNGI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puccinia acroptili Syd.</td>
<td>Specific, autoecious rust</td>
<td>Canada</td>
<td>Savile (1970)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U.S.A.</td>
<td>Savile (1970)</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum (Lib.) de Bary</td>
<td>Helotiales</td>
<td>Canada</td>
<td>Mortenson &amp; Molloy (1989)</td>
</tr>
<tr>
<td>Alternaria cichorii Nattrass</td>
<td>Specific leaf hyphomycete</td>
<td>Canada</td>
<td>Mortenson &amp; Molloy (1989)</td>
</tr>
<tr>
<td><strong>Nematode</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subanguina picridis (Kirj.) Brzeski</td>
<td>Oligoxenous shoot gall former</td>
<td>U.S.S.R.</td>
<td>Ivanova (1966)</td>
</tr>
</tbody>
</table>

1.2.3 The biology of *Subanguina picridis*

The Russian knapweed nematode, *Subanguina picridis* (Kirjanova, 1944) Brzeski, 1981, Syn: *Anguillulina picridis* Kirjanova, 1944; *Paranguina picridis* (Kirjanova, 1944) Kirjanova & Ivanova, 1968, belongs to the Anguinidae family of Tylenchidae. Based on the biological characteristics, Chizhov and Subbotin (1985) suggested *Subanguina picridis* as *Mesoanguina picridis*. In their reports, the nematodes in the genus *Mesoanguina* have narrow host specificity, and often form hairy galls with a marked internal cavity. However, the genus *Mesoanguina* is also characterized by two morphologically distinct generations inside the gall and invasive 3rd stage larvae rather than the 2nd stage larvae of *S. picridis*. Fortuner and Maggenti (1987) revised the family Anguinidae and retained *Subanguina picridis* in the genus *Subanguina*.

*Subanguina picridis* was first discovered by Kirjanova in the Gissar Valley in 1942. This species is present from Central Asia to Kazakhstan and the European area of the U.S.S.R. (Kovalev, 1974). It is common in cultivated land in Tadjikistan and has been reported from the Shakhristan, Uzbekistan, and Armenian regions of the U.S.S.R. (Ivanova, 1966; Kirjanova and Ivanova, 1969; Pogosyan, 1966).

The life cycle of *S. picridis* has been described by Ivanova (1966) and Watson (1986a). The invasive 2nd stage larvae of *S. picridis* actively penetrate into the meristematic tissues of the apex and leaf axils of the young shoots in the early spring (March–April). The infections take place at the moment when the young growing shoots break through the upper layers of the soil. The larvae concentrate mostly in the region of the shoot apex, but
there are also many of them in the axils of leaves. Three to four days after infestation, a gall appears at the penetration site. The galls are found on the leaves, stems, and root collars of infested plants. The 2nd stage larvae feed, mature, and reproduce inside the galls. According to the observations of Ivanova (1966), *S. picridis* produces two generations during the growing season. The larvae of the first generation hatch in mid-April. Sexually mature individuals of the second generation were noted at the end of May. The adults of the first generation die off as the nematodes of the second generation mature. Toward the end of the vegetative growth of the plant in August, the invasive 2nd stage larvae become dormant and overwinter inside the galls. The 2nd stage larvae in a cryptobiosis state can remain viable for many years inside dry galls. During the winter and early spring, the larvae are revived by moisture and migrate out of the decayed galls into the soil in search of host plants. Correspondingly, the development of *S. picridis* is synchronized with the development of Russian knapweed (Table 2).

As Table 2 demonstrates, the invasive 2nd stage larvae penetrate the emerging young shoots of Russian knapweed in the early spring. The nematodes develop and reproduce inside the galls during the vegetative growth period of Russian knapweed. As the host plants begin to senesce, the larvae stop developing and become dormant. They overwinter in the invasive larval stage and become active again as the young shoots emerge from the soil the following spring.
Table 2. Comparison of the development of *Subanguina picridis* with Russian knapweed.

<table>
<thead>
<tr>
<th>Time</th>
<th>Russian knapweed</th>
<th><em>Subanguina picridis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>March-April</td>
<td>shoots emerge</td>
<td>invasive larvae penetrate the shoots</td>
</tr>
<tr>
<td>mid-April</td>
<td>shoot growth</td>
<td>first generation hatches</td>
</tr>
<tr>
<td>late May</td>
<td>rosette</td>
<td>mature adults of second development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>generation</td>
</tr>
<tr>
<td>June</td>
<td>vegetative growth</td>
<td>eggs and hatching of 2nd generation</td>
</tr>
<tr>
<td></td>
<td>and bolting</td>
<td></td>
</tr>
<tr>
<td>early July</td>
<td>flowering</td>
<td>development of 2nd stage larvae</td>
</tr>
<tr>
<td>Aug.-Sep.</td>
<td>flowering</td>
<td>larvae become dormant in galls</td>
</tr>
<tr>
<td>Oct.-March</td>
<td>above ground senescence</td>
<td>nematodes overwinter inside galls</td>
</tr>
<tr>
<td>March-April</td>
<td>shoot buds on roots</td>
<td>galls decay and larvae migrate out in</td>
</tr>
<tr>
<td></td>
<td>growth in soil</td>
<td>search of new hosts</td>
</tr>
<tr>
<td></td>
<td>and emerge</td>
<td></td>
</tr>
</tbody>
</table>
Watson (1975) pointed out that the establishment of *S. picridis* and subsequent gall formation is probably dependent upon the environmental conditions, the infectivity of the nematodes, and the suitability of the host tissue. A specific physiological status and growth phase of the host plant are required for nematode gall formation. Suitable undifferentiated meristematic tissues must be available for *S. picridis* galls to be induced. He emphasized that host plants which maintain their vegetative stem buds or apical meristems at or just beneath the soil surface and are not under rapid growth and differentiation are required for *S. picridis* to induce galls (Watson, 1986b). The physiological state of nematodes also determines whether or not successful gall formation occurs. The 2nd stage larvae of *S. picridis* were unable to induce galls on their host until a period of time had elapsed after being revived from a dormant state. Gall formation was obtained only when Russian knapweed was grown in a relative cool, moist, infertile environment and after the nematodes had been in a free-living state for at least 1 month (Watson and Harris, 1984; Watson, 1986b).
1.2.4 Host-parasite relationship of *S. picridis*

1.2.4.1 Host specificity of *S. picridis*

It was reported that Russian knapweed was the only host of *S. picridis* (Kirjanova, 1944; Ivanova, 1966; Kirjanova and Ivanova, 1969). Kirjanova (1944) infested several tens of thousands of wheat (*Triticum* spp.) plants with *S. picridis* with no external symptoms of infection observed in the experimental plants. He also reported that the nematode was not found in flax (*Linum usitatissimum* L.) plants. The host range was investigated by Ivanova in 1966. She infested wheat, barley (*Hordeum vulgare* L.), corn (*Zea mays* L.), flax, sunflower (*Helianthus annuus* L.), cotton (*Gossypium* spp. L.) and pea (*Pisum sativum* L.) seedlings with *S. picridis*. The results showed that larvae could penetrate into wheat, barley, corn, cotton and sunflower, during the early development stages of these crops, but the larvae either perished or abandoned these plants since they apparently did not encounter favorable conditions. Test plants were healthy, and the nematodes were not recovered from those plants 15-30 days after infestation. No larvae were found in the seedlings of flax or pea. From these results, she concluded that Russian knapweed was the only host of *S. picridis*.

In the laboratory, the host range of *S. picridis* is restricted to the Centaureinae and Carduinae subtribes of the Cynareae tribe of the Asteraceae family (Watson, 1986a). As with other prospective exotic biological weed control agents, the host specificity of *S. picridis* was determined before it was released into North America. Watson (1986a) examined the host range of
S. picridis. The experiments were carried out in growth chambers. The test plants included Russian knapweed; plants related to Russian knapweed; host plants of nematode species related to S. picridis; and plants which have vegetative reproduction similar to Russian knapweed. From these studies the host range of S. picridis was found to include Russian knapweed and a few closely related species in the Centaureinae and Carduinae subtribes of the Cynareae tribe of the Asteraceae family. In addition to Russian knapweed, galls were formed on Centaurea diffusa Lam., C. maculosa Lam., C. X pratensis Thuill., Carduus nutans L., Cirsium floridanum (Rydb.) Arthur, Cynara scolymus L., and Onopordum acanthium L. Eggs were observed in dissected galls from all the above plants. Extensive galling and severe damage of infected Russian knapweed revealed that Russian knapweed was the only plant highly susceptible to S. picridis. A few infected Centaurea diffusa plants had more than one gall per leaf, indicating that C. diffusa is moderately susceptible to the nematode. Slight damage occurred on the other hosts, indicating tolerance to the parasite. The histological data also supported the conclusion that Russian knapweed was the best host for S. picridis. The nematode induced elaborate galls well supplied with nutritive cells, and nematode reproduction was copious. Nutritive cells were reduced or absent in galls on other plants compared to the Russian knapweed galls. Galled plants other than Russian knapweed supported low or no nematode reproduction.

The host range of S. picridis under field conditions was narrower than in the laboratory (Watson and Harris, 1984). Crop plants which included flax, lettuce (Lactuca sativa L.), sunflower, wheat, safflower (Carthamus tinctorius L.), carrot (Daucus carota L.), globe artichoke (Cynara scolymus...
and Russian knapweed were infested with *S. picridis* in the field. Galls were found on Russian knapweed in the second year after infestation. Galls did not form on any of the other test crop plants and no nematodes were extracted from these plants (Watson and Harris, 1984).

Safflower and globe artichoke are the only two economic crops that are closely related to Russian knapweed. Safflower is in the same subtribe as Russian knapweed and globe artichoke is in the Carduinae subtribe of the Cynareae tribe. The laboratory test results showed that safflower was not a host of *S. picridis*, but galls were found on the leaves of globe artichoke (Watson, 1986a). Globe artichoke is an economic crop primarily grown in California. Therefore the question of whether or not *S. picridis* will become a pest of globe artichoke was of concern and was discussed by Watson and Harris (1984). The low gall formation rate, the fact that the growth of infected plants was not affected, and the decrease in the population of nematodes within globe artichoke tissue suggested that the nematode would have difficulty in maintaining a population on globe artichoke. Meanwhile, the results from the field trials indicated that the host range under field conditions was narrower than in the laboratory as no galls were found on globe artichokes in the field plots (Watson and Harris, 1984). In addition, the cultivation and cropping practices associated with the production of globe artichoke in California do not favour the development of the nematode (Watson, 1977). So, from a safety point of view, the nematode should be no problem to any plant species except Russian knapweed (Watson and Harris, 1984).

*S. picridis* is a oligoxenous parasite; it has a narrow host range and Russian knapweed is the only plant highly susceptible to *S. picridis*. 

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1.2.4.2 The host-response to the infection of S. picridis

1.2.4.2.1 Nematode galls

Nematodes of the genera *Ditylenchus*, *Anguina*, *Subanguina*, and *Orrina* cause galls on various aerial parts of host plants. According to Mani (1964), a gall is a pathologically developed tissue or organ of plants that has arisen mostly by hypertrophy (over growth) and hyperplasia (cell proliferation) under the influence of these parasitic organisms. Nematode galls are developed by hypertrophy and hyperplasia of parenchyma cells (Dropkin, 1969; Viglierchio, 1971). Galls have a well differentiated structure with distinctive cell types. Nematode galls usually have a central cavity containing nematodes (Goodey, 1934, 1935, 1938; Goodey, 1939, 1948; Dropkin, 1969; Viglierchio, 1971) and a nutritive zone that lines the gall cavity.

The hypertrophied cells in the nutritive zone contain abundant granular protoplasm. They are rich in mitochondria and ribosomes and contain high concentrations of lipids, hydrolytic enzymes, and amino acids and are lacking in chlorophyll and vacuoles (Watson, 1979; Skinner et al., 1980). These nutritive cells exhibit nuclear and nucleolar hypertrophy with each cell containing a large round nucleus with two or three nucleoli (Goodey, 1934, 1938; Mani, 1964; Skinner et al., 1980). The walls of these cells are thin (Goodey, 1934). The presence of nutritive cells near the feeding site is a common character of most nematode galls. The structure of the galls is different for each nematode species. Less highly evolved nematode-induced galls may lack definitive tissue and a constant size and shape, but they
still have the layer of nutritive cells near the nematode feeding sites (Watson and Shorthouse, 1979).

The proposed role of the nutritive cells is to supply food to the nematodes within the gall cavity (Goodey, 1934, 1938; Goodey, 1948). It was suggested that these cells are transfer cell-like and function as a powerful physiological sink (Watson and Shorthouse, 1979; Skinner et al., 1980).

1.2.4.2.2 Nematode gall initiation and development

Gall initiation

Galls are initiated only in actively growing undifferentiated tissues (Norton, 1966; Watson and Shorthouse, 1979; Skinner et al., 1980). Most gall formers attack and modify meristematic or actively growing tissues (Watson and Shorthouse, 1979). The agents initiating the gall formation have been proposed as mechanical factors and chemical. Owens and Specht (1964) suggested that hypertrophy of host tissues could be a response to mechanical pressure exerted by an enlarging nematode or to lateral movement of growth substances around the cavity created by the growth of the nematode. However, this mechanical pressure concept was rejected by Schuster and Sullivan (1960) when they found that gall formation could occur without the entry of the root-knot nematode. Most studies have focused on chemical initiating agents. Goodey (1948) was the first to suggest that the formation of galls is the outcome of a chemical stimulus. The roles of auxins, nematode excretory products, nematode enzymes, and amino acids in gall formation were discussed by several investigators (Goodey, 1939; Myers and Krusberg, 1965; Endo and Veech, 1969). This work was reviewed by Krusberg (1963), Webster (1969, 1975), Dropkin (1969, 1972), Viglierchio (1971) and Veech (1981).
The increase or the change in levels of endogenous growth regulators in galled tissues lead to the postulation that growth regulators are involved in gall formation. There have been many attempts to explain nematode gall formation in terms of auxin action. Bird (1962) examined galls induced in tomato roots by *Meloidogyne javanica* (Treub) Chitwood and detected a growth promoting substance (3-indole derivative) that was not present in healthy roots. Balasubramanian and Rangaswami (1962) infested *Abelmoschus esculentus* Moench with the root-knot nematode, *M. javanica*, and found that extracts of galled tissue contained indole compounds. There are other reports of changes in levels of endogenous growth regulators in the presence of nematode infection. Increased levels of growth regulators were found in nematode galls when compared to non-infected host tissue (Balasubramanian and Rangaswami, 1962; Bird, 1962; Viglierchio and Yu, 1968). Based on those studies, Dropkin (1969) suggested that nematodes may introduce or stimulate the formation of certain plant growth regulators in their hosts, which in turn regulate some of the observed changes.

Amino acids were also considered as a means of gall initiation. The amino acid changes in galled tissues are related to the action of auxin. Amino acids can be released by nematodes into their environment (Lee, 1965; Smith and Ellenby, 1967). Viglierchio (1971) suggested that the host response to nematode attack could be mediated by amino acids of parasite origin. Krusberg (1961) found free tryptophane (an IAA precursor) in galls caused by *Ditylenchus dipsaci* (Kuhn) Filipjev on alfalfa, but not in healthy plants. He discussed tryptophane in relation to the mechanism of galling, and suggested that the nematode might secrete indole acetic acid (IAA) or cause the plant to accumulate IAA in the vicinity of the nematode. Amino
acids occur in greater concentrations in nematode-induced gall tissue than in healthy host tissues (Howell and Krusberg, 1966; Owens and Rubinstein, 1966; Owens and Specht, 1966). It was found that proline accumulates in nematode-induced galls in abnormally high concentrations (Owens and Specht, 1966; Mean et al., 1978; Lewis and McClure, 1975). Mean et al. (1978) proposed that the high metabolic activity of root galls exerts a demand on the proline synthesized in the leaves and protein was basipetally translocated to the sites of nematode activity.

There has been considerable speculation on the role of nematode enzymes in gall formation (Veech, 1981). Proteolytic enzyme secretion has been demonstrated in numerous plant parasitic nematodes (Krusberg, 1960; Miller and Jenkins, 1964; Gysel, 1968) and may be involved with gall initiation. Sayre (1958) postulated that plant nematodes secrete a proteolytic enzyme which hydrolyses IAA-protein complexes and releases IAA and tryptophane. The latter reacts with phenolic acids to give auxin. The released IAA would then contribute to the galling of tissues. The meristematic tissue penetrated by the nematode is high in protein (Sunderland et al., 1957) which suggests that proteolytic enzymes might be the key or "trigger" to successful gall formation.

Krusberg (1963) suggested that the secretion, whether it is protease or some other substance, acts as a "trigger" in gall formation. Similarly, Webster (1975) suggested that the substance which causes the specific galling response is probably secreted by the nematode and functions as a "trigger" or an inhibitor of one of the host plant enzyme systems.

It has not been possible to distinguish between the introduction of a growth regulator by the nematode and the indirect changes in levels or
gradients of endogenous growth regulators of the host, or inhibition of enzymes that degrade growth regulators, though many studies have been conducted in searching for the gall initiation agent. There is so far no direct evidence indicating that plant parasitic nematodes alter the host's normal development by the introduction of a growth regulator or by interfering with synthesis or degradation of growth regulators by plant cells (Dropkin, 1969; Viglierchio, 1971). The literature, also, is not clear on precisely how, or indeed even if, growth regulators are involved in the host response to nematode infection. Similarly, the finding of no difference in auxin content between *Meloidogyne* galled and noninfected tomato plants (Setty and Wheeler, 1968) leaves the auxin initiating gall formation theory in question.

Jones (1981) emphasized that introduction of nonspecific protease seems too crude, considering the delicate balance between host and parasite, and therefore more specific enzymes, regulatory proteins could be involved. Therefore it seems reasonable to suggest that the nematodes inject substances possessing regulatory activity, which tip the balance of a few critical pathways. These could result in increased growth regulator production, which might stimulate mitosis or galling.

**Gall development**

The formation of nematode galls is a type of host-nematode adjustment in which the nematode stimulates changes in host cells resulting in alteration of metabolism. The biochemical interaction between the nematode and host plant ensures mutual development resulting in successful parasite development and various degrees of host tolerance. The plant cells attempt
to continue normal biochemical growth processes either by isolating the parasite, or by accommodating nematode demands to obtain adequate nutrients for its development (Dropkin, 1969; Webster, 1975).

In galls induced by Anguina agrostis (Steinbuch) Filipjev on ryegrass (Lolium rigidum Gaudin), gall formation was a process of stimulation of growth and simultaneous local destruction of plant cells (Stynes and Bird, 1982). Invasive nematode larvae fed actively on the primordia and induced rapid cell division and cell enlargement. Once galls were initiated, the cytoplasm of cells became dense and granular and nuclei enlarged. Gradually, the cells emptied and collapsed. A gall comprising a cavity was then formed. The nematodes continued to feed actively and rapidly molted to adults. During this period of rapid nematode growth and development, a corresponding increase in the density of cytoplasm occurred within the cells adjacent to the cavity and, to a diminishing extent, in cells at increasing distances from the cavity. At the same time, the walls of the cells adjacent to the gall cavity became coated with material that stained deeply with toluidine blue. Directional growth of the galls was associated with increasing elongation of the older outermost cells. During this period, the nematode finished laying eggs, and the 2nd stage larvae were beginning to hatch. When the galls reached a maximum size, all the 2nd stage larvae had hatched and completed their development to infective larvae. At this time, the cells adjacent to the gall cavity had almost completely lost their cytoplasmic contents. With the onset of dry summer conditions and senescence of the ryegrass, the galls gradually dried out. As this occurred, the larvae became closely appressed against each other, and the cells of the gall walls became devoid of cytoplasm and collapsed.
According to Griffiths and Robertson (1984), the galls induced by Longidorus elongatus (de Man) Thorne and Swange progressed through five distinct stages. These were initial hypertrophy; hyperplasia; secondary hypertrophy; ingestion of cell contents; and collapse of the gall. In the first stage, hypertrophy occurred and cells developed enlarged nuclei and nucleoli, accompanied by increased cytoplasm and protein. This stage was followed by hyperplasia; cells divided to give two or four daughter cells, accompanied by reduction in volumes of cytoplasm, RNA and protein. The third stage was secondary hypertrophy with enlarged amoeboid nuclei and nucleoli. The concentration of RNA and protein significantly increased in this stage. In the final two stages, as feeding by nematodes progressively removed cell contents, most cells were devoid of inclusions and galls collapsed.

1.2.4.2.3 The gall of the knapweed nematode:

The knapweed nematode, S. picridis, gives rise to galls on the stems, leaves, and root-collar of Russian knapweed. Galls on the stems often visibly distort plant growth. Galls on the leaves are 5-12 mm diam, compared with the average 0.25 thickness of the normal leaf. The galls are whitish in colour (Ivanova, 1966; Watson, 1986b). The galls have a thick solid wall with a cavity inside filled with numerous nematodes. The cavity of the gall is surrounded by a layer of vacuolated cells interspersed with debris from collapsed cells. Outside the inner layer of cells is a densely staining layer of nutritive cells. The cells in the nutritive layer have a large nucleus, often with two or three nucleoli. These cells are not vacuolated and contain cytoplasm. This nutritive layer is surrounded by the vascular bundles which are laterally displaced and circumvent the gall (Watson,
1986b). There is a broad zone of enlarged, tightly packed parenchyma cells between the vascular bundles and the gall epidermis. These cells are vacuolated and not as densely stained as the cells of the nutritive layer and occasionally they contained chloroplasts in the layers just below the epidermis. The cells in the epidermis of the gall are slightly larger, and the cuticle of the gall is thicker than on an ungaled leaf. The epidermal cells are also slightly larger than those of an ungaled leaf.
1.2.5 Monoxenic culture of plant parasitic nematodes

1.2.5.1 Introduction

By the early 1900's, as sterile techniques were established, plant parasitic nematode culture became possible. Plant tissue culture techniques have been used in the culture of plant parasitic nematodes. Byars (1914) first successfully monoxenically cultured a root gall forming nematode, *Heterodera radicicola* (Greeff) Muller on surface sterilized tomato seedlings growing on an agar-mineral salt medium. Tyler (1933) observed the reproduction of *Meloidogyne* sp. using excised tomato roots aseptically growing on nutrient agar medium. Mountain (1954) first established monoxenic cultures of *Pratylenchus minyus* Sher and Allen on excised tobacco (*Nicotiana tabacum* L.) and red clover (*Trifolium pratense* L.) root cultures. Since then, many cyst nematodes and root-knot nematodes have been cultured aseptically on excised roots in culture. The success of propagating the potato rot nematode, *Ditylenchus destructor* Thorne on several plant callus tissues (Darling et al., 1957) initiated the current period in which many species of plant parasitic nematodes have been propagated on a number of different plant callus tissues.

It was recognized that certain kinds of biological information would be difficult or impossible to obtain with plant parasitic nematodes on plants grown in the soil. Similarly, large quantities of a single nematode species required for research of plant nematode physiology and biochemistry are difficult to obtain since most plant parasitic nematodes are generally not easily collected from natural soil and plant sources. Nematode extraction
techniques are laborious and time-consuming, and the nematodes from these sources are not microbiologically sterile (Krustery and Babineau, 1977; Mai and Riedel, 1987). Many studies with plant nematodes have been greatly handicapped by the above problems. Riedel (1983) pointed out that the inability to establish plant parasitic nematodes in pure culture has seriously retarded the study of these organisms as plant pathogens. Two important areas of phytopathological research, establishment of pathogenicity and crop loss assessment, are particularly difficult to pursue when large populations of microbiologically sterile test organisms are not available.

There are many advantages in using monoxenic cultures to conduct research on plant parasitic nematodes. Monoxenic nematode culture systems provide large quantities of sterile, debris-free and single nematode species for study of a plant parasitic nematode, and provide a very convenient experimental environment for the nematologist. When using monoxenic culture systems, many experiments can be conducted under controlled conditions. This system has been used in plant nematode research for many years with the greatest application of monoxenic nematode culture being its propagation of large numbers of nematodes for various purposes, including studies on the biology, physiology, and biochemistry of nematodes.

A culture system provides a large population of a single species, and makes continuous observation possible. Monoxenic nematode culture systems have been used to study nematode life cycles (Thistlethwayte, 1969; Dasgupta et al., 1970; Lauritis et al., 1983); gametogenesis, oogenesis, and embryology as well as the reproduction (Dolliver et al., 1962; Dasgupta et al., 1970; Tamura and Mamiya, 1975; Koening and Schmitt, 1986); inter- and
Intraspecific variation (Roman and Hirschmann, 1969; Tarte and Mai, 1976); and behaviour (Rebois and Huettel, 1986; Guy et al., 1987); Knowledge of their ultrastructure, physiology, and biochemistry is important to understand nematode basic functions and host-parasite interactions. Large numbers of nematodes are required for these studies. Orion et al. (1980) used Meloidogyne incognita (Kofoid and White) Chitwood from excised tomato root cultures for scanning electron microscope work. Riedel and Mai (1971 a,b) used cultured Ditylenchus dipsaci for studies of cell wall degrading enzymes.

The traditional method for evaluating nematode resistance involves direct observation of root galling on young plants grown in heavily infested soil with a susceptible plant being one with well-developed galls, while the resistant plant is free from infestation. Recently, a great deal of effort has been concentrated in attempts to take advantage of the tremendous array of genetic variation found in progenies of tissue-culture-derived plants (Medina-Filho and Tanksley, 1983). In the selection of resistant breeding material, cultured nematodes have been used for forage legumes (Faulkner et al., 1974; Bingefors and Bingefors, 1976; Eriksson, 1980), wheat (Brown, 1974), onions (Bergquist and Riedel, 1972), soybeans (Lauritis et al., 1982a,b; Huettel, 1986) and potatoes (Rivera et al., 1987).

Monoxenically cultured nematodes have also been used in studies to elucidate the mechanisms of action of nematicides (Mai and Thistlethwayte, 1967; Abawi and Mai, 1978). Similarly, mass rearing of nematodes in tissue culture is a technique used extensively by industry for nematicide screening programs (Riedel et al., 1983).
General reviews on the application of plant tissue culture to plant nematology include articles by Zuckerman (1971), Ingram (1973), Krusberg and Babineau (1977), Jones (1980), Riedel et al. (1983), Medina-Filho and Tanksley (1983), and Mai and Riedel (1987).

1.2.5.2 Establishment of monoxenic nematode culture systems

The monoxenic nematode culture system is comprised of plant tissue, the plant nematode, and the culture environment. Achievement of successful nematode culture depends on the adjustments of these three components. The plant nematode and the plant tissue are biological units and the culture environment must be suitable for both organisms. To establish a monoxenic nematode culture system, studies should include a choice of plant tissue, a selection of culture media, and the determination of optimal culture conditions.

1.2.5.2.1 Plant tissue

The plant tissues used in plant nematode culture systems are callus, excised roots, disc cultures, and seedlings. Among these, the most commonly used are callus and excised root cultures.

Callus culture

Callus cultures are cell aggregates which arise from the disorganized growth of plant organs or detached plant tissues, or of previously cultured cells (George et al., 1984). The term callus tissue, as used in reference to
plant nematology, loosely means a friable mass of plant cells arising from excised plant tissues or whole seedlings that have been placed on agar medium containing plant growth regulators, usually 2,4-D (2,4-dichlorophenoxyacetic acid). These tissues contain many differentiated as well as undifferentiated plant cells (Krusberg and Babineau, 1977). The callus tissue is used by the nematodes as their source of food.

Several nematodes in the genera of Aphenlenchus, Aphenlenchoides, Bursaphelenchus, Ditylenchus, Hoplolaimus, Dolichodorus, Paratylenchus, Pratylenchus, Radopholus and Tylenchorhynchus have been propagated on callus tissue. Alfalfa callus seems to be the most suitable substrate for the migratory ecto- and endoparasites culture, and has been widely used in propagating several nematodes. However, some nematodes in the genus Pratylenchus such as P. crenatus Loof, P. convallarriae Cayrol and Ritter have significantly low reproduction rates in alfalfa callus tissue (Riedel et al., 1983). Other callus tissues used for nematode culture include red clover, tobacco, grape, tomato, citrus, periwinkle, carrot, and onion callus (Krusberg and Babineau, 1977; Reise et al., 1987).

Excised root cultures

Interest in propagating nematodes on plant tissues in culture was stimulated by the success of Mountain (1954, 1955) in obtaining reproduction of the lesion nematode, Pratylenchus minyus, on excised root cultures of red clover, corn, and tobacco. Tiner (1960, 1961a) refined techniques for indefinite propagation of the lesion nematode on corn roots in culture using P. penetrans (Cobb) Sher and Allen. He obtained a maximum of 64,000 nematodes per gram of fresh roots from 3 month old cultures. The method of
excised root culture includes excising root tips about 1-3 cm in length from aseptic seedlings and culturing them on a nutrient agar medium.

The relationship of plant tissue and plant nematode parasitic type has been discussed by Jones (1980) and Riedel et al. (1983). Plant parasitic nematodes are divided into two parasitic types: migratory and sedentary. Migratory nematodes such as Trichodorus spp., Tylencehorhynchus spp., Ditylenchus spp., and Pratylenchus spp. usually puncture cells, withdraw their contents and kill the cells. They can feed at the plant surface or within the tissues. These nematodes can be maintained on suitable callus tissues. The sedentary types are endoparasites, and include nematodes in the genera of Meloidogyne, Heterodera, Globodera, Nacobbus, and Rotylenchulus. These nematodes cause host cell modification, and induce cell syncytia or gall formation which are essential for their growth and reproduction. Nematodes of these genera seem to require vascular development in plant tissue in order to complete their life cycle. In contrast to ecto- and endomigratory parasites, sedentary endoparasites such as Heterodera, Meloidogyne and Nacobbus have been cultured almost exclusively on excised roots and seedlings (Krusberg and Babineau, 1977).

Some nematodes such as D. destructor and P. penetrans have been cultured on both callus and excised root cultures. A number of studies have compared the reproductive rates of various nematodes on root cultures with reproduction rates on callus cultures of the same plants. The reproduction rate of Tylencehorhynchus vulgaris on corn callus is two times greater than on excised corn root cultures (Upadhyay and Swarup, 1972). Similarly, P. penetrans reproduced better on callus than on root cultures of eleven different plants (Schroeder and Jenkins, 1963). D. destructor reproduced
well on undifferentiated callus culture of clover, but failed to reproduce
on root cultures of clover or tomato (Faulkner and Darling, 1961). Barker
and Darling (1965) found that the reproduction rates of Aphelenchus avenae
Bastian were much higher on undifferentiated tissues than on semi-
differentiated tissues (callus tissues with growing shoots). Migratory
nematodes grow better on the undifferentiated callus tissues than on
differentiated root tissues (Riedel et al., 1983)

1.2.5.2.2 Culture media

The media which have been commonly used in monoxenic nematode cultures
are White's (White, 1963), Krusberg's (Krusberg, 1961), Riedel's (Riedel et
al., 1972), B5 (Gamborg, 1975) and MS (Murashige and Skoog, 1962).

Medium containing White's macro- and micronutrient solutions has been
widely used to grow excised roots which have been used for the culture of
some plant parasitic nematodes (Mountain, 1955; Upadhyay and Swarup, 1972;
Perry et al., 1980). Faulkner, et al. (1974) used a modified White's medium
with the addition of 2,4-D and NAA (α-naphthalene acetic acid) to culture
alfalfa callus for mass rearing of D. dipsaci.

Krusberg (1961) modified Hilderbrandt's medium (Hilderbrandt, et al.,
1946) for culturing alfalfa callus on which Ditylenchus dipsaci,
Pratylenchus zeae, and P. penetrans were successfully cultured. Reproduction
of Hoplolaimus coronatus Cobb and Tylenchorhynchus capitatus Allen were slow
on alfalfa callus on this medium (Krusberg, 1962). A simpler medium (Riedel
et al., 1972) was developed for the culture of P. penetrans and D. dipsaci
on alfalfa callus. This 4-component medium contained 5 g/L of yeast extract,
2 mg/L of 2,4-D, 10 g/L of sucrose and 10 g/L of agar. The nematodes in the genus of Pratylenchus and D. dipsaci reproduced well on this medium.

Recently, B5 medium and MS medium have been used for the culture of plant parasitic nematodes (Rebois and Huettel, 1986; Brodie and Spivey, 1988; Huettel, 1987; Huettel et al., 1988). The MS medium has been used extensively for callus culture with the significant feature of MS medium being its very high content of nitrate, ammonium, and potassium. The B5 medium has also been used successfully to grow cells of a large variety of plant tissues. The general concentrations of inorganic nutrients are lower than in MS medium. Both MS and B5 media appear to contain the appropriate amounts of inorganic nutrients to satisfy the needs of plant cells from a wide range of species in culture.

1.2.5.2.3 Factors affecting nematode culture

The nature of plant tissue

The nature of plant tissue is very important in nematode propagation. The age of plant tissue cultures when inoculated with nematodes greatly affects the number of nematodes obtained and the susceptibility of plant tissues to the nematode. Aphelenchus avenae reproduced most rapidly on tobacco callus when it was 2 and 4 days old, and the nematode rarely reproduced on callus that was 2 to 3 weeks old when inoculated (Barker and Darling, 1965). Alfalfa tissue cultures were often inoculated with nematodes at 2 weeks old (Krusberg and Blickenstaff, 1964; Riedel and Foster, 1970). The effect of excised root age on nematode penetration was studied by Johnson and Viglierchio (1969), and their results showed that the
penetration of nematodes was significantly reduced when roots had been incubated for 20 days prior to inoculation.

The type of plant tissue used in nematode culture often determines whether or not the nematode culture is a success. Nematodes of the Heteroderidae family seem to require vascular development in plant tissues to complete their life cycle. Sayre (1958) found that *Meloidogyne incognita* would not grow on purely undifferentiated tissues of tomato, but would grow and complete its life cycle in tissues having some degree of vascular development. Tamura and Mamiya (1975) monoxenically cultured *Bursaphelenchus lignicolus* Mamiya & Kiyohara and found that this nematode reproduced large numbers on alfalfa callus but less or not at all on alfalfa seedling root cultures.

Medium composition

Nutrients

In plant tissue culture, the basis of all nutrient media is a mixture of mineral salts combining the essential macro- and micro-elements together with a source of carbon which is usually sucrose. The usual supplements required are vitamins, amino acids, growth regulators, and a chelating agent such as EDTA (ethylene diaminetetraacetic acid) (Yeoman and Macleod, 1977).

The chelating agents have been demonstrated to affect the culture of nematodes. Addition of EDTA caused severe inhibition of reproduction of *Aphelenchoides ritzemabosi* (Schwartz) Steiner (Dolliver et al., 1962). In contrast, McClure and Viglierchio (1966) reported that the development rate of *M. incognita* was decreased by reducing the concentration of iron chelate.
Reproduction of nematodes in culture is also affected by the concentration of macronutrient salts in the medium. Dolliver et al. (1962) found that when the medium was altered to reduce plant tissue growth, the reproduction of *Aphelenchoides ritzemabosi* was limited. Reducing the concentration of calcium ions resulted in decreasing *A. ritzemabosi* reproduction. McClure and Viglierchio (1966) reported that decreasing the concentration of macronutrient salts as a group resulted in increasing the rate of nematode development. Dropkin and Boone (1966) found that high potassium stimulated *Meloidogyne* sp. egg production.

The effect of carbon source on nematode culture has been determined by several workers. McClure and Viglierchio (1966) reported that the development rate of *Meloidogyne incognita* was decreased at reduced concentrations of sucrose. Johnson and Viglierchio (1969a,b) reported that maximum penetration of *Heterodera schachtii* Schmidt in sugar beet root culture occurred when the concentration of sucrose in the medium was 3.0% and when sucrose was omitted from the medium, roots were not penetrated. Other carbon sources also affect nematode culture. Schroeder (1963) found that the amounts of glucose in White's medium influenced *Pratylenchus penetrans* culture on excised corn roots. The greatest increases in numbers of this nematode occurred when the glucose concentration was 20 g/L, and no nematode reproduction took place when the medium contained 80 g/L of glucose or no glucose.

Changes of vitamins in the culture medium also change the development rate of nematodes in culture. McClure and Viglierchio (1966) reported that decreasing the concentration of vitamins increased the rate of nematode development. In contrast, Johnson and Viglierchio (1969a,b) cultured sugar
best nematodes on excised root tissues and found that the penetration rate of nematodes was increased by the increase of vitamins.

The medium components also affect the penetration rate and the sex ratio of nematodes in culture. The evidence on these aspects can be found in the articles of McClure and Viglierchio (1966a,b) and Johnson and Viglierchio (1969a,b).

Plant growth regulators

Plant growth substances play very important roles in nematode culture. The different levels and types of plant growth regulators in media can effect the growth of plant tissue as well as the development of the nematodes. Each nematode species seems to have its own growth regulator requirements.

2,4-D is commonly used to stimulate callus formation of plant tissue and the influence of 2,4-D concentration on the reproduction of various nematodes in callus has been studied. Dolliver et al. (1962) reported that the reproduction rate was reduced when 2,4-D was omitted from Krusberg's medium. Webster and Lowe (1966) found that alfalfa callus grew best with 2,4-D at 0.125 mg/L. Krusberg and Blikkenstaff (1964) tested the influence of plant growth regulators on the reproduction of D. dipsaci, Pratylenchus penetrans, and P. zeae on alfalfa callus and found that the best reproduction in all tests occurred on medium containing 2,4-D.

Few studies have dealt with the effects of other plant growth regulators on nematode culture. Krusberg and Blikkenstaff (1964) reported that the addition of kinetin to medium containing 2,4-D increased the reproduction of D. dipsaci, but decreased the reproduction of P. penetrans and P. zeae on
alfalfa callus tissues. However, kinetin in the medium at the same concentration did not affect the reproduction of *P. vulnus* on alfalfa callus tissue according to Lownsbery et al. (1967). Webster (1967) found that in medium lacking 2,4-D nematode reproduction increased when kinetin was added to the medium.

Webster (1967) reported that *A. ritzemabosi* reproduction increased when GA₃ (gibberellic acid) or IAA (indole-3-acetic acid) was added to the medium without 2,4-D, and the combination of GA₃ and IAA was better than GA₃ alone. Krusberg and Blickenstaff (1964) suggested that NAA could be omitted from the medium with no adverse effect on nematode culture.

**Temperature**

Temperature is a very important aspect of the incubation environment. It influences nematode development, penetration rate, and the sex ratio of nematodes in culture.

Reproduction of *Aphelenchoides ritzemabosi* was inhibited by lowering the temperature (Dolliver et al., 1962). Lownsbery et al. (1967) reported that *Pratylenchus vulnus* cultured on alfalfa callus increased more rapidly at 25 C than at 20 C and did not increase at 30 C or 35 C. Prasad and Webster (1967) reported that the reproduction of *Nacobbus serendipiticus* Franklin cultured in excised tomato roots was affected by culture temperatures, with the life cycle of this nematode completed in 36 days at 25 C, and 43 days at 20 C or 30 C, and no females produced eggs at 15 C. Faulkner et al. (1974) mass cultured *D. dipsaci* on alfalfa tissue and found that this nematode reproduced best in the darkness at 20-25 C. The nematode did not reproduce well at 5 or 10 C and apparently could not reproduce at 30 C. Each nematode
species also seems to have its own optimal temperature range. For *D. dipsaci*, the optimal temperature for development was 20-25°C (Paulkner et al., 1974) and the optimal temperature for development of *Heterodera schachtii* was 25°C (Johnson and Viglierchio, 1969). Olowe and Corbett (1976) compared the effect of temperature (5, 10, 15, 20, 25, 30 and 35°C) on generation time and reproduction rates of *Pratylenchus brachyurus* (Godfrey) Sher and Allen and *P. zeae* on excised maize roots. Generation time differed between the two species, with *P. zeae* developing faster than *P. brachyurus* at all temperatures.

Temperature also affects the penetration rate of nematodes in culture. Johnson and Viglierchio (1969) observed the penetration rate of *H. schachtii* in sugar beet root cultures and reported that maximum penetration occurred at 25°C. Prasad and Webster (1967) found that many larvae of *Nacobbus serendipiticus* penetrated the excised tomato roots at 15, 20 and 25°C, but fewer entered at 30°C.

Plant tissue culture techniques have been used in plant nematology for many years. Up to now, several nematodes in fifteen genera, *Aphelenchoides*, *Aphelenchus*, *Bursaphelenchus*, *Ditylenchus*, *Dolichodorus*, *Heterodera*, *Hoplolaimus*, *Meloidogyne*, *Nacobbus*, *Paratylenchus*, *Pratylenchus*, *Radopholus*, *Telotylenchus*, *Tylenchorynchus*, and *Tylenchus*, have been cultured using these techniques. However, most of these cultured nematodes have wide plant host ranges and it is still very difficult to culture some host specific nematodes. Some nematodes fail to complete their life cycle in plant tissue cultures due to the lack of males (Widdowson et al., 1958; Eriksson, 1980), and some larvae cannot develop into adults when they are in culture. The insufficient knowledge of the relationships between plant tissues and the
nematodes, the requirements of host tissues and the nematodes, and the effects of physical conditions of culture on nematode reproduction limits the culture of many nematode species. In addition, many plant tissue culture techniques such as cell suspension culture and shoot tip culture have not been used in plant nematode culture.

Attempts to mass rear a plant parasitic nematode for weed control are new. Many weed-parasitic nematodes are in the genera of Anguina, Orrina, and Subanguina. These nematodes form galls on the leaf and stem tissues, and many of them have the potential to be effective biocontrol agents of their respective weed hosts. Mass producing plant parasitic nematodes has met with very limited success and neither above-ground gall forming nematodes nor weed parasitic nematodes have been mass produced in vitro.
CHAPTER 2. CULTURE of S. picridis IN DIFFERENT TISSUES

2.1 Introduction

Monoxenic nematode culture involves the nematode, the plant tissue, and the culture environment. The establishment of a functional nematode culture system depends on the perfect balance of these three aspects. As a result, several research steps are required to develop a plant parasitic nematode culture system, including the culture of plant tissue, the aseptic infestation of established plant tissue with the nematode, and the optimization of culture conditions for nematode development and reproduction.

Plant tissue cultures are initiated from small organs or pieces of plant tissue (explants). To successfully culture a plant tissue depends on the explant, the culture medium, and the incubation conditions. Plant material will only grow in vitro when provided with a specialized medium with a correct balance of inorganic, organic, and growth regulator constituents. The physical factors in the culture environment include temperature, light intensity, and pH of the medium. These factors affect plant cell growth and development.

Since S. picridis had not been successfully cultured in vitro, the experiments included all of the above steps. Preliminary experiments included the culture of different types of plant tissue, the choice of suitable plant tissue for nematode culture, and the determination of culture
conditions. All of the preliminary experiments described in this chapter were conducted three times.

The culture of plant tissue included the choice of basal medium, examination of the roles of different plant growth regulators, and the determination of the optimum culture environment. Explants from many plant species form callus and differentiate adventitious organs on MS and B5 media. These two media were chosen for Russian knapweed tissue culture. It is well known that growth and organogenesis in vitro is highly dependent on the plant growth regulators added to the medium (Gamborg, 1975). Growth regulators, particularly auxins and cytokinins, are very important components of culture media. Many aspects of growth, cellular differentiation, and organogenesis in tissue and organ cultures are controlled by interactions between cytokinin and auxin concentrations (George and Sherrington, 1984). Plant growth regulators also play very important roles in plant nematode culture, these may affect the nematodes directly or indirectly through changes in the plant tissue growth status (Krusberg and Babineau, 1977; Webster, 1967). The roles of 2,4-D, kinetin, and GA3 on the different types of Russian knapweed plant tissue cultures and S. picridis cultures were investigated.

Nematodes have been cultured in several types of plant tissues including callus tissue, excised root tissue, and disc cultures (Krusberg and Babineau, 1977). In attempts to obtain a suitable plant tissue type for S. picridis culture, callus culture, excised root culture, and shoot tissue of Russian knapweed were used in S. picridis culture experiments.

The factors in the culture environment not only affect the plant tissue growth, but also affect nematode penetration, development, and reproduction.
In the monoxenic nematode culture system, the culture conditions should be suitable for both plant tissue growth and nematode culture. The final steps in the experiments were to determine the optimum culture conditions.
2.2 Fresh gall culture

2.2.1 Methods

Fresh galls from Russian knapweed plants grown in the greenhouse were cleaned with tap water for 2 hours, surface disinfected in 1% sodium hypochlorite solution for 20 min, and rinsed in sterile distilled water 3 times. Surface disinfected galls were placed in a sterile petri dish, and their epidermis removed under a stereo microscope.

Three treatments with various plant growth regulators in B5 basal medium (Appendix A; Gamborg, 1975) were used in this experiment. The surface disinfected galls without their epidermis were placed aseptically onto the B5, B5G and B5D (Table 3) media in petri dishes (60 mm x 20 mm) with one gall per petri dish. Each treatment was comprised of 3 replicates. The cultures were maintained at 22 C day/18 C night with a 12 hour photoperiod and 80 \(\mu\)molm\(^{-2}\)s\(^{-1}\) light intensity using cool white fluorescent lamps. One month after culture, the growth status of the nematodes in the excised fresh galls cultured on different media were observed after dissection under the microscope. The growth and development status of the nematodes in the excised galls cultured on B5D were observed for a period of 75 days. Three galls were used for each observation with a total of 15 galls for 5 observations.

In a second experiment, each surface disinfected gall was cut into four equal sized pieces which were transferred aseptically to each of four petri dishes containing KL medium [see Appendix B (c)] cultured under different
temperatures, 15°C day/12°C night, 18°C day/12°C night, 22°C day/18°C night, and 24°C/18°C night with 16 hour photoperiod and 80 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) light intensity using cool white fluorescent lamps. Three gall pieces were used for each observation with a total of 15 gall pieces being used for 5 observations during the 60 day experimental period.

Both of the above experiments were repeated 3 times.

Table 3. The media used for fresh gall cultures:

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<thead>
<tr>
<th>medium</th>
<th>plant growth regulator (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BA3</td>
</tr>
<tr>
<td>B5</td>
<td>0.1</td>
</tr>
<tr>
<td>B5BNG1</td>
<td></td>
</tr>
<tr>
<td>B5D2</td>
<td></td>
</tr>
</tbody>
</table>

1. see Appendix B (a).
2. see Appendix B (b).
3. BA = 6-benzyladenine.
4. NAA = \( \alpha \)-naphthalene acetic acid.
5. GA3 = gibberellic acid.
6. 2,4-D = 2,4-dichlorophenoxy acetic acid

2.2.2 Results

Excised fresh nematode galls were maintained only on the B5D medium (Table 4). No callus formed on the galls cultured in the B5 medium. By 1 month after culture, the galls had become very hard and dark and no living nematodes were found inside the galls. B5BNG medium also was not suitable for fresh gall culture as 1 month after being cultured, the galls were hard and green, no callus had formed, and the nematodes inside the galls were
dead. Compared with B5 and B5BNG media, B5D medium was better for fresh gall culture. The nematodes survived and developed inside the galls. Ten days after culture, callus had formed on the surface of cultured galls and nematodes survived inside the galls. Thirteen days after culture, females with eggs inside their uteri, males, different stages of larvae, and eggs were found inside the galls cultured in B5D medium (Table 5). Young females appeared 20 days after culture and the eggs and larvae in different stages were also found, but old females and males had degenerated and were less active at this time. No females with eggs inside the uteri were found. Forty days after culture, old adults, young adults, eggs in the two to three cell stages, some young females with eggs inside their uteri were found, but of the larval stages only 2nd stage larvae were found at this time. Sixty days after culture, old adults were dead, the young females had not developed well and only a few eggs, 2nd stage and 3rd stage larvae were found. The nematode population decreased with time in B5D medium.

The second experiment demonstrated that 22 C day/18 C night was a suitable temperature for S. picridis culture (Table 6). No nematodes were found in the gall pieces cultured at 15 C/12 C after 25 days of culture. No nematodes were found in the gall pieces cultured at 18 C/12 C after 30 days of culture. Nematodes were not found in the gall pieces after 57 days of culture at 24 C/18 C. However, The 2nd stage larvae still could be found in B5D medium under the 22 C/18 C condition after 57 days of culture.
Table 4. The status of galls and nematodes in different media 1 month after culture initiation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Gall status</th>
<th>Nematode status</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5</td>
<td>dark, hard, no callus</td>
<td>none</td>
</tr>
<tr>
<td>B5BNG</td>
<td>green, hard, no callus</td>
<td>none</td>
</tr>
<tr>
<td>B5D</td>
<td>light green, soft, callus</td>
<td>adults, larvae, eggs</td>
</tr>
</tbody>
</table>

Table 5. Nematode status in galls cultured on B5D medium.

<table>
<thead>
<tr>
<th>Duration of culture (days)</th>
<th>Nematode stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>adult (old)</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>75</td>
<td>-</td>
</tr>
</tbody>
</table>

1+ = nematodes in this stage were found.
2- = nematodes in this stage were not found.
Table 6. The effect of temperature (day/night) on the culture of gall pieces.

<table>
<thead>
<tr>
<th>Duration of culture (days)</th>
<th>15°C/12°C</th>
<th>18°C/12°C</th>
<th>22°C/18°C</th>
<th>24°C/18°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>+¹</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>-²</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>39</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>57</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ + = nematodes survived.

² - = no nematodes survived.
2.3 CULTURE of *S. picridis* IN CALLUS TISSUES

2.3.1 Methods

Petioles from Russian knapweed plants grown in the greenhouse were cleaned with tap water for 10 min, surface disinfected in 1% sodium hypochlorite solution for 15 min, rinsed with sterile distilled water 3 times, and then cut into 5 mm length segments. These petiole segments were placed onto KL, KL3, and KL3I media (Table 7). The cultures were maintained at 25 C, 16 hour photoperiod with 60 μmol m⁻² s⁻¹ light intensity using cool white fluorescent lamps. Seven days after culture, callus was derived from the petioles in culture (Fig. 1A).

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 nematodes with water were transferred with a pipette into small tubes and centrifuged for 5 min. at 400 rpm. The top water supernatant was discarded, and the nematodes were washed using sterile distilled water 3 times by the centrifugation method mentioned above. This step was followed by surface disinfection of nematodes with 100 ppm HgCl₂ for 1 min, followed by surface disinfection in a solution of 100,000 ppm novobiocin + 30 ppm malachite green + 1000 ppm streptomycin sulfate. This step was conducted 3 times with each time being at least 2 hours. Surface disinfected nematodes were collected and concentrated by the centrifugation method and transferred with a pipette to 6% agar medium which contained 300 mg/L novobiocin. Nematodes were maintained in the agar medium in the refrigerator at 4 C.
Callus derived from petioles were placed on the KL, KL3, and KL3I media (Table 7) with one callus per plate. Fifty surface disinfected 2nd stage nematodes were then applied to each callus. The infested callus were maintained at 22°C day/18°C night, 16 hour photoperiod with 80 μmol m⁻² s⁻¹ light intensity using cool white fluorescent lamps.

The experimental period was 7 weeks. In this period, three infested callus were used for each observation each week with a total of 21 infested callus being used for 7 observations. This experiment was repeated three times. For observation, callus tissues were carefully broken up to release the nematodes from the callus and the stages of recovered nematodes were identified under a stereo microscope.

Table 7. Media used for the culture of S. picridis in callus:

<table>
<thead>
<tr>
<th>medium</th>
<th>2,4-D</th>
<th>Kinetin</th>
<th>GA₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL₁</td>
<td>1.0</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>KL3₂</td>
<td>1.0</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>KL3I₃</td>
<td>1.0</td>
<td>0.1</td>
<td>10.0</td>
</tr>
</tbody>
</table>

1: see Appendix B (c).
2: see Appendix B (d).
3: see Appendix B (e).
2.3.2 Results

In KL and KL3 media, the nematodes developed from 2nd stage larvae into 4th stage in the first 3 weeks in both media, although female 4th stage larvae were found, no male 4th stage larvae were found and nematodes did not develop beyond the 4th stage in the KL medium. The nematodes developed faster in the KL3I medium than in the KL3 medium (Table 8). Adults developed in the KL3 medium and KL3I medium with females and males being found in both media (Fig. 1B, C), but no sperm were found in the spermatheca of females (Fig. 1D) even though sperm were present in the male reproductive systems. Eggs also were not found in the uteri of females. Nematode numbers decreased with culture time and the nematodes did not reproduce in the callus cultures.
Table 8. The development of Subanguina picridis in callus tissues grown on KL, KL3, and KL3I media.

<table>
<thead>
<tr>
<th>Time</th>
<th>Stage</th>
<th>KL</th>
<th>KL3</th>
<th>KL3I</th>
</tr>
</thead>
<tbody>
<tr>
<td>one week</td>
<td>2nd</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>two weeks</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>three weeks</td>
<td>2nd</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd molting</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th (F)</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4th (M)</td>
<td></td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>four weeks</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th (F)</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4th (M)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (F)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>adult (M)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>five weeks</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (F)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>adult (M)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>six weeks</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (F)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (M)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>46 days</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (F)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adult (M)</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>50 days</td>
<td>2nd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4th</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

1The first observation started two weeks after inoculation for the cultures in the KL3 medium and four weeks after inoculation for the culture in the KL3I medium.
2+ = nematodes in this stage were found.
3- = nematodes in this stage were not found.
Fig. 1. A. Callus initiated from a petiole segment of Russian knapweed. X 5.

B. A _Subanguina picridis_ male from callus culture. X 150.

C. The spermatheca section of a _S. picridis_ female from callus culture. Note no sperm in the spermatheca. X 240.

D. A _S. picridis_ female from callus culture. X 150.

(SP = spermatheca).
2.4 CULTURE OF S. picridis IN EXCISED ROOT TISSUE

2.4.1 Methods

Young shoots from shoot tip culture (see chapter 3) were transferred to the rooting MSIG medium [Appendix D (a)], and maintained in the culture room at 25 C, 16 hour photoperiod with 60-80 μmolm⁻²s⁻¹ light intensity using cool white fluorescent lamps. One month after transfer, root tips 2 cm in length were excised and transferred to MSG medium (Fig. 2A).

Fifty 2nd stage larvae were applied onto the medium near the excised root in the petri dish. The experiment had ten replicates. The nematode cultures were maintained at 20 C, 60-80 μmolm⁻²s⁻¹ light intensity using cool white fluorescent lamps and 16 hour daylength.

The development of the nematodes inside and outside the root was observed under a microscope every day and the percent penetration was determined by subtracting the number of nematodes free in the medium from the total number initially placed in the medium.

2.4.2 Results

Five days after infestation, most of the nematodes had penetrated into the root tissue with most penetrating the root tip region (Fig. 2B, C). However, the nematodes did not remain inside the roots constantly, as it was observed that nematodes moved in and out of the root tissue frequently. They also moved from one root cell to another. When passing through from one cell.
to another cell, the nematode was squeezed, the body was flattened, and the constituents in the intestine moved forward. Root hairs were destroyed by the movement of the nematodes and were spread around the roots. About 87% of the nematodes penetrated into the roots (Table 9).

The nematodes had developed from 2nd stage to 3rd stage larvae by 10 days after infestation, and post-3rd stage larvae were also found (Table 10). Fourth stage nematodes were found 15 days after infestation. Twenty days after infestation, 2nd stage, 3rd stage, post-3rd stage and 4th stage larvae were found inside or out of the root tissue, and molting 3rd stage larvae were also found. Four weeks after infestation, further development of the nematodes did not occur. The reproductive systems developed normally during the first 3 weeks, but they became shorter and obscure 4 weeks after culture. Nematode growth subsided 4 weeks after culture and the activity of the nematodes also decreased over time. No nematodes which had developed beyond the 4th stage were found during the 50 days observation period.
Table 9. Proportion of nematodes that penetrated the roots.

<table>
<thead>
<tr>
<th>Time after infestation (days)</th>
<th>Penetration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>87</td>
</tr>
<tr>
<td>7</td>
<td>87</td>
</tr>
<tr>
<td>12</td>
<td>91</td>
</tr>
<tr>
<td>26</td>
<td>93</td>
</tr>
<tr>
<td>34</td>
<td>86</td>
</tr>
</tbody>
</table>
Table 10. Nematode development in excised root tissue (N=10).

<table>
<thead>
<tr>
<th>Days after infestation</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>adult (F)</th>
<th>adult (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+1</td>
<td>-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1+ = Nematodes in this stage were found.

2- = No nematodes in this stage were found.
Fig. 2. A. Excised root culture of Russian knapweed. X 3.

B. Subanguina picridis aggregated on the excised root tip in culture. X 20.

C. Close-up view of the root section from B (black box section). Note the nematodes have penetrated the root. X 100.

(N = nematodes).
2.5 CULTURE of S. picridis IN SHOOTS DERIVED FROM EXCISED ROOTS

2.5.1 Methods

Excised root segments about 4 cm in length from the rooting medium (MSIG) were transferred to the shooting MSIBG medium [Appendix D (b)]. For the shoot formation experiment, 20 roots 4 cm in length were used. Ten root segments were placed on the surface of the medium, the other 10 roots were inserted into the medium. The cultures were maintained at 20 °C, 16 hour photoperiod with 60-80 μmolm⁻²s⁻¹ light intensity using cool white fluorescent lamps.

To determine the shoot formation rate, 34 root segments 4 cm in length were inserted into shooting medium and observed for the formation of shoots every day.

Fifty surface disinfected 2nd stage larvae were applied onto the B5G medium near the roots with new young shoots when the shoots were about 2-3 mm in length. There were 7 replicates for each treatment. The cultures were maintained at 20 °C, 16 hour photoperiod with 60-80 μmolm⁻²s⁻¹ light intensity using cool white fluorescent lamps.

2.5.2 Results

Only the roots inserted into the medium formed shoots, while the roots placed on the surface of the medium formed callus. Four to 53 days after being transferred, 56% of the roots formed shoots with most of roots forming shoots during 28 to 49 days of the culture period (Table 11).
Three days after inoculation, the nematodes were found inside shoot apices. The first galls were observed on the shoots derived from roots 13 days after infestation. The gall number on each shoot ranged from 1 (Fig. 3A) to 4 (Fig. 3B). About 50% of the shoots were galled. The nematodes formed galls on the leaves, shoot apices, and the petioles in culture.

The nematodes developed inside the galls. One month after the galls appeared, females, males, different stages of larvae, and eggs were all found inside the galls (Fig. 3C, Fig. 4C). Young females with one to three eggs inside the uterus (Fig. 4A) and with sperm inside the spermatheca (Fig. 4B) were observed. The number of nematodes reached 211 per gall, a 4.2 fold increase.
Table 11. Shoot formation from the roots in shoot induction medium (N=34).

<table>
<thead>
<tr>
<th>Time after transfer (days)</th>
<th>Total number of shoots</th>
<th>Shoots/total roots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>28</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>36</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>43</td>
<td>14</td>
<td>41</td>
</tr>
<tr>
<td>49</td>
<td>18</td>
<td>53</td>
</tr>
<tr>
<td>53</td>
<td>19</td>
<td>56</td>
</tr>
</tbody>
</table>
Fig. 3. A. A nematode gall on a Russian knapweed shoot derived from a root segment in culture (one gall per shoot).

B. Nematode galls on a shoot derived from a root segment in culture. Note the presence of four galls on one shoot.

C. Nematodes were released from an excised gall in culture.

(Arrows point to galls, N = nematode).
Fig 4. A. A *Subanguina picridis* female from a nematode gall on a Russian knapweed shoot derived from an excised root. X 130.

B. Close-up view of the spermatheca section of a female. Note the sperm inside the spermatheca. X 270.

C. A *S. picridis* male from a nematode gall on a Russian knapweed shoot derived from the shoot culture. X 130.

(SP = spermatheca, S = sperm).
2.6 Summary and discussion

Excised fresh galls were maintained only on the B5D medium, the nematodes survived and developed inside the excised galls. However, the nematode populations decreased with time in this medium. Seventy-five days after culture, only 2nd stage larvae were found inside the excised galls.

The development of *S. picridis* was quite different in various types of Russian knapweed tissues. The nematode developed from 2nd stage larvae to adults in the callus tissues derived from petioles and both males and females were found. However, although the males developed normally and contained lots of sperm in their reproductive systems, no females with eggs in their uteri, nor with sperm in the spermatheca were found. The nematode did not reproduce in the callus tissues. Nematodes did not constantly remain inside the excised root, as they migrated in and out of the root tissue frequently. The nematode did not develop beyond the 4th stage larvae in the excised root culture. However, *S. picridis* was successfully cultured in the shoots derived from excised roots. The nematode induced galls on the leaves, petioles, and shoot apices and developed and reproduced inside the galls, with eggs and each stage of the nematode found within the galls. Lots of sperm were found in the spermatheca of the females and the females had one to three eggs in their uteri. This is the first time *S. picridis* has been propagated *in vitro*.

A complete life cycle of a plant parasitic nematode includes three phases: penetration, development, and reproduction. The metabolic requirements of a nematode generally vary with its developmental changes, even within one host. A compatible host and plant parasitic nematode interaction allows for considerable development and reproduction of the
nematode. The failure of a plant parasitic nematode to complete its life cycle may be due 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 (Lewis, 1987).

According to Griffiths and Robertson (1984), the gall induced by 
Longidorus elongation (de Man) Thorne and Swanger progressed through five distinct stages including initial hypertrophy, hyperplasia, secondary hypertrophy, ingestion of cell contents, and collapse of the gall. In the first stage, hypertrophy occurred and cells contained enlarge nuclei and nucleoli, which was accompanied with increasing contents of cytoplasm and protein. This stage was followed by hyperplasia, cells divided to give two or four daughter cells, accompanied by reduction in volumes of cytoplasm, RNA, and protein. These changes may be due partly to utilization during cell division and partly to removal during nematode feeding. The third stage was secondary hypertrophy with enlarged amoeboïd nuclei and nucleoli. The concentration of RNA and protein significantly increased in this stage. The most extensive removal of cell contents by the nematode occurred during this stage. These successive changes in the structure and chemical contents of gall cells results in a better food source than non-infested tissues and results in successful nematode development and reproduction.

Similarly, cell structure and metabolic changes occur in the callus development process. According to Aitchison et al. (1977), the callus development process is divided into three stages; induction, division, and differentiation. Chemical constituent and cytological changes occur at each stage. During the induction stage, cell metabolism is activated to prepare for cell division, the RNA and DNA contents are accumulated. In the
division stage, RNA concentration increases, cells divide and are reverted to the meristematic state. The following continual growth and division of the callus culture accompanies massive increases of protein and nucleic-acid content. This active metabolic status of callus culture seems to satisfy the demand of *S. picridis* developmental requirements. The nematode developed normally from 2nd stage larvae to adults. From the morphological aspect, the nematode developed very well in the callus. However, no sperm were found in the spermatheca of females indicating that fertilization did not occur in the callus, even though the males contained large numbers of sperm in their reproductive systems. As a result, *S. picridis* failed to complete its life cycle as the reproduction phase did not occur in callus culture. This failure may due to the inactivity of this nematode in the callus, or the lack of reproductive functioning of female nematodes in the callus. The special gall environment may be essential for this nematode to reproduce.

Various genera of plant parasitic nematode such as *Meloidogyne*, *Heterodera*, *Anguina*, *Lotanguina*, and *Subanguina* have evolved intimate relationships with their hosts by the development of specialized feeding sites in plant tissues (Dropkin, 1969; Jones, 1981). The feeding site is induced after the nematode penetrates inside the tissue. Once the nematode enters the plant tissue, the nematode stimulates changes in cells resulting in alteration of metabolism vital to the nematode's growth and development. In all types of sedentary nematode feeding sites there is an increase in cytoplasmic density, nuclear and nucleolar hypertrophy, cell-wall thickness, an increase in number of organelles, and disappearance of the central vacuole. A failure to induce a feeding site results in the death of the nematode (Lewis, 1987). *S. picridis* did not establish a permanent feeding
site inside the excised root. This was apparent by the frequent movement of the nematodes in and out of the root tissues. The nematode did not develop beyond the 4th stage. These results indicate that the root tissue is not suitable for this nematode culture. In addition, these results also demonstrate that the nutritional or environmental requirements for the development of the 4th stage larvae is different from the 2nd and 3rd stage larvae.

The specificity of a plant parasitic nematode is not only manifested by the selection of its host, but also manifested by the parasitic position in the plant. Completion of the whole life cycle is dependent on the compatibility of the plant and the nematode throughout the life of the nematode. Like most gall forming nematodes, the invasive larvae of S. picridis penetrates into the meristematic tissue of the Russian knapweed shoot apex and induces galls at the penetration site. Though both excised roots and shoots derived from roots have meristematic tissues, S. picridis can only form galls and reproduce in shoot tissue. Similar results were obtained from monoxenic culture of some cyst and root- not nematodes (Sayre, 1958; Riedel et al., 1983). In nature, they penetrate the meristematic tissue of roots and form galls on the roots. The results of monoxenic culture of these nematodes showed that these nematodes were successfully cultured only in the root and induced the galls on only the root. The root has a different anatomy and a different development pattern compared with the shoot. Specific parts of the plant and specific growth pattern of a plant tissue are required for nematode development and reproduction.

Gibberellic acid (GA₃) is a gibberellin commonly used in plant tissue culture experiments. It was found that the addition of GA₃ to the culture
media influences tissue growth (Belkengren and Miller, 1962). High concentrations of GA$_3$ (1-8 mg/L) induced the growth of undifferentiated callus cells (Murashige, 1964; Altman and Goren, 1974; Gautam et al., 1983). Limited research has been done on the effect of GA$_3$ on the culture of plant nematodes. Webster (1966) reported that gibberellic acid increased the numbers of the nematode, *Aphelenchoides ritzemabosi* Schwartz in culture. In this study different results were obtained when using KL, KL3 and KL3I media for culture of *S. picridis* in callus. The only difference in these media was the amount of GA$_3$. The concentrations of GA$_3$ in KL, KL3 and KL3I medium were 0, 1.0 and 10 mg/L respectively. No nematodes beyond the 4th stage were recovered and no males were found in the KL medium. While the nematodes developed from 2nd stage to adults and males were observed in both KL3 and KL3I. These results indicate that GA$_3$ helps the nematode to develop from 4th stage larvae to adults and it promotes the formation of males. The effect of GA$_3$ on formation of *S. picridis* males suggests that GA$_3$ may have a male hormone function. GA$_3$ also increased the development rate of the nematode in this experiment. The development of nematodes was faster in the KL3I medium containing a higher concentration of GA$_3$ with the nematodes developing into adults, both male and female, in 4 weeks, while the males were found after 5 weeks and females were found after 6 weeks in the KL3 medium containing a lower concentration of GA$_3$. When GA$_3$ is added to the culture media, it often produces effects which are a similar in nature to those of auxin (George and Sherrington, 1984). Therefore, GA$_3$ may have stimulated the growth of callus tissue which increased the nematode development rate.

Establishment of most plant tissue cultures is dependent on an exogenous supply of plant growth regulators, but it has been shown that cultured plant...
tissue can also synthesize endogenous plant growth regulators (George and Sherrington, 1984). Neither auxin nor cytokinin was needed to culture the nematode in the shoots derived from the roots in these experiments. It seems that shoots derived from roots contained enough endogenous plant growth regulators for nematode gall induction.

In this study, *S. picridis* has been successfully reared. This is the first time that an above-ground gall forming nematode has been successfully cultured *in vitro*. Callus and excised root cultures have been used for monoxenic culture of some plant parasitic nematodes. This study, however, developed a unique method of using the shoots derived from roots to mass rear an above-ground endoparasitic plant nematode.
CHAPTER 3. ESTABLISHMENT OF THE S. picridis

MASS REARING SYSTEM

3.1 Culture of Subanguina picridis using young shoots from shoot tip culture

3.1.1 Introduction

The success of propagating S. picridis in shoots derived from the excised roots indicates that shoot tissue is a suitable tissue for this nematode culture. However, one major problem in using this method to culture S. picridis is that it takes a long time to obtain shoots. The culture procedure from shoot tip culture to root initiation, and from root segments to the new shoot initiation requires about 3 months. In addition, the shoot formation rate also is not high.

In nature, the penetration of S. picridis into the host plant only occurs in the early spring when the young shoots emerge. Nematodes enter the shoot apex and induce galls in the host tissues. This biological character of S. picridis combined with the experimental results suggest that the excised shoot apex from the shoot tip culture may be used as a plant source for this nematode culture.

The shoot tip culture technique has been widely applied to propagate many economic plants. Using this technique, many plantlets can be obtained by multiplication of shoots from axillary shoots in culture. If S. picridis can be cultured in the shoots directly from shoot tip culture, the nematode culture time will be shorter and the culture cost of the culture process
will be greatly reduced. Therefore, this experiment was conducted to examine
the utility of young shoot apices from shoot tip culture to propagate S.
pticidis.

3.1.2 Methods

Establishment of the plant tissue (shoot) culture system

Surface disinfection of shoot apices

Shoot apices 1.5 cm in length, excised from plants grown in the
greenhouse were washed with tap water and dipped in 75% ethanol for 1 min,
then surface disinfected with 1% sodium hypochlorite solution for 15 min and
washed 3 times in sterile distilled water.

Shoot tip culture

Whatman No.1 filter paper (18 x 110 mm) was folded in the letter "M"
shape to form a filter-paper bridge. The arms of the paper strip were
immersed in the liquid medium in the bottom of a test tube (25 x 150 mm)
(Fig. 5a).

The surface disinfected shoot apex was placed on a petri dish. After
removing leaves and leaf primordia with fine needles, the meristematic dome
with 2 to 3 primordia, approximately 2 mm in length, was carefully excised
using a scalpel fitted with a pointed tip blade (No. 11) under a stereo
microscope (6 x magnification). This meristematic dome with 2 to 3 primordia
(shoot tip) was then carefully transferred with the blade of the scalpel,
and positioned upright in the "V" of the filter-paper bridge. The medium in
the test tube used for shoot tip culture was MSIDG medium [Appendix D (b)].
This experiment was comprised of 10 replicates (10 tubes), and each tube
contained 1 shoot tip. The culture tubes were capped with plastic covers, and maintained under 16 hour photoperiod; 60 \(\mu\)molm\(^{-2}\)s\(^{-1}\) light intensity using cool white fluorescent lamps and 25 C. One week after being placed on the filter-paper bridge, small shoots developed from the shoot tips (Fig. 5b).

**Shoot culture and maintenance**

The shoot culture system was initiated from the shoot tip culture. Small shoots derived from the shoot tip culture on the filter-paper bridge were transferred to solid medium [MSIBG medium with 6% agar (Anachemia)] in a plastic container (600 ml\(^3\)) (Fig. 5c). One month after transfer, a cluster of shoots was formed by outgrowth of bud primordia (Fig. 5d). The cluster of shoots was subdivided into smaller clumps of shoots (3 shoots/clump) in a sterile petri dish by a scalpel fitted with a No. 11 blade. Multiplication of shoots was achieved by transferring these smaller clumps to fresh MSIBG solid medium. This subculture procedure was repeated monthly for maintenance of the shoot cultures. The shoots were cultured in the same conditions as the shoot tip culture.

**Plant nematode infestation**

Nematodes were obtained from cultured galls. Galls were opened and the nematodes in different stages were released into sterile water. The gall debris was removed and nematode suspensions were obtained. Fifty mixed staged nematodes were transferred with a pipette onto the B5G medium [Appendix B (f)] in a petri dish (60 x 20 mm) with 3 young shoots (Fig. 5e). Infested shoots were placed in an incubator at 20 C; 16 hour daylength and 60 \(\mu\)molm\(^{-2}\)s\(^{-1}\) light intensity using cool white fluorescent lamps.

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Fig. 5. Flow diagram of the *Subarquina picridis* culture process.

(a) 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) The cluster of shoots.

(e) Three young shoots in B$_5$G medium infested with 50 mixed stage nematodes.

(f) Galls formed on the shoots in culture.

(g) Galled shoots subcultured on MSIG rooting medium.

(h) Maintenance of the gall culture. Note new galls were formed.
The gall culture and maintenance

When galls formed in culture (Fig. 5f), the galled shoots were subcultured to MSIG rooting medium [Appendix D (a)] in 100 x 25 mm petri dishes (Fig. 5g, h). The gall cultures were maintained at 20°C; 16 hour photoperiod with 60 μmol m⁻² s⁻¹ light intensity using cool white fluorescent lamps, and subcultured monthly.

Galls produced in culture of various ages were dissected with the aid of a stereo microscope and the numbers of adults, larval stages, and eggs were determined. Six observations were conducted during the 37 days experimental period with 5 galls of the same age being used for each observation.

3.1.3 Results

Visible galls were observed on the leaves, stems, and shoot apices of the shoots 7 days after infestation with one or more galls being produced on each shoot (Fig. 6A, B). The leaves of the shoots became yellow, and no nematodes were found in the medium 35 days after culture.

The nematodes developed and reproduced inside the galls (Table 12). On the first day when a gall appeared, 4th stage molting larvae, 4th stage, 3rd stage, and 2nd stage larvae were found in the galls. Five days after galls appeared, males and females with the eggs in their uteri were found. Females had laid numerous eggs, and in addition, newly hatched 2nd stage larvae and eggs in different stages of development were also observed. The young adults and eggs in different stages were found in the galls 17 days after galls formed. Nematode numbers increased with culture time and 1 month after culture, the nematode number reached 1617 nematodes per gall on average.
Three months after culture, the nematode number increased from the initial 50 used to infest the cultures to 7,000-10,000 per petri dish, a 140 to 200 fold increase. New galls were formed after galled shoots were transferred to the rooting MSIG medium (Fig. 6C) with the gall number reaching 18 galls/shoot 5 months after infestation.
Table 12. Population of nematodes inside the cultured galls
(values were a mean of five galls).

<table>
<thead>
<tr>
<th>Time after gall appearance (days)</th>
<th>Number of nematode in each stage</th>
<th>4th</th>
<th>3rd</th>
<th>2nd</th>
<th>egg total</th>
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<td>adult(F)</td>
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<td>2</td>
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</tr>
<tr>
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<td>2</td>
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Fig. 6. A. A nematode gall on a shoot of Russian knapweed from the shoot culture system (one gall per shoot).

B. Nematode galls on a Russian knapweed shoot from the shoot culture system. Note the presence of four galls on one shoot.

C. Numerous galls were formed after the galled shoot was transferred to the rooting MSIG medium.

(Arrows point to galls).
3.2 Determination of the culture environment

3.2.1 Introduction

Factors which affect nematode culture include biological (plant tissue), chemical (medium), and physical factors in the culture system. The biological factors are the type of plant tissue, the age of plant tissue, and the physical status of the inoculum. The chemical factors are the nutrients and the plant growth regulators in the culture medium. The physical factors include temperature, light intensity, and the medium pH.

Temperature is a major physical factor of the culture environment. Like other factors, temperature may influence nematode culture directly or indirectly. For the plant nematode, temperature directly influences the enzyme activity, metabolic rate, and the movement of plant nematodes. It is well known that each species of nematode has its own optimum temperature for development and reproduction. The lower and upper limits of temperature at which the activity, development, and reproduction of the nematode ceases are important values for the development and behaviour of plant nematodes. Temperature exerts a significant effect on the population density, the sex ratio, and the reproductive rate of plant nematodes (Davide and Triantaphyllou, 1967). Temperature has been used to induce nematode population changes (Prasad and Webster, 1967; Heald and Inserra, 1988; Alston and Schmitt, 1988). From the plant tissue aspect, in monoxenic plant nematode culture, temperature indirectly influences the plant nematodes through the growth status of the plant tissue. For the sedentary endoparasite, the plant tissue is used as its food source and habitat.
Temperature directly affects the growth rate, nutrient synthesis, and the hormone balance of plant tissues in culture, and each of these changes in turn affects nematode development. Temperature also affects the response of plants to infection by plant nematodes (Melton et al., 1986). Plant cellular responses, in proper sequence, are necessary for the survival, development, and reproduction of many sedentary endoparasites. Delay or failure of the plant to respond to the infection of the nematode normally results in resistance to the infection. The gall induced by *Subanguina picridis* on Russian knapweed is greatly influenced by climate under field conditions. Therefore, the determination of the optimum temperature for the culture of *S. picridis* is essential for obtaining large quantities of this nematode and this information also can provide data for application of this nematode in the field.

Penetration, development, and the population of the plant nematode are affected by the age of the plant tissue. Young tissue is generally more susceptible to nematode infection than older tissue (Barker and Darling, 1965). The age of plant tissue also affects the sex determination of nematodes (Trudgill, 1967). In the field, *S. picridis* penetrates young shoots emerging in the early spring. As the knapweed matures over the growing season, the nematode feeds, reproduces, and eventually becomes dormant in the invasive stage at the end of the growing season. Determination of the effect of shoot age on gall formation in *vitro* is important for obtaining maximum penetration, gall formation, and reproduction of this nematode. This information will also likely contribute to determine the optimum timing of nematode application in the field.
The success of a plant nematode culture using plant tissue techniques is greatly influenced by the culture medium used. The medium should be suitable to both plant tissue growth and nematode development and reproduction. The MS and B₅ media are being used extensively in plant tissue culture. Both the MS and B₅ media appear to contain the appropriate amounts of inorganic nutrients to satisfy the needs of many plant cells in culture. In order to determine a suitable medium for *S. picridis* propagation, studies were conducted to evaluate the effect of MS and B₅ media on the nematode culture.

The objectives of this portion of the research program were to determine the effects of temperature, shoot age, and medium on the culture of *S. picridis*. 
3.2.2 Effect of Temperature on Gall Formation

3.2.2.1 Methods

Four month old cultured galls were cut open in a sterile petri dish containing sterile double distilled water. Nematodes were released into the water from the opened galls, gall debris was removed with forceps, and nematode suspensions were obtained. Clusters of shoots were removed from the plastic containers and transferred to a sterile petri dish using forceps. Individual shoots were carefully separated. Only small shoots, ranging in size from 0.3-0.5 cm were used. Three shoots were inserted into the B5G medium in a petri dish (20 x 60 mm). Nematode suspensions, each with 50 nematodes of mixed stages were applied to the surface of the medium with a pipette. Cultures were maintained at 10, 15, 20, and 25 C. Each treatment was comprised of 10 replicates (10 petri dishes), and each petri dish contained 3 shoots. The infested shoots were placed in four separate incubators with 16 hours daylength and a light intensity of 60 μmolm$^{-2}$s$^{-1}$ using fluorescent white light. Treatments were observed over a period of 40 days.

The results were analyzed using regression analysis. This experiment was repeated 3 times.

2.2.2 Results

Gall formation response to culture time was different at various temperatures. There was a linear relationship between the number of galls and culture time at 10 C (Fig. 7) with the number of galls increasing steadily over in the culture period of 40 days. The relationship of the
number of galls and culture time was nonlinear at 15, 20, and 25 C with the number of galls leveling off after 20 to 25 days in culture. Gall formation time was delayed as the temperature decreased (Fig. 7). The first galls formed 5 days after infestation at 25 C, 7 days at 20 C, 9 days at 15 C and 11 days at 10 C. Forty days after culture, the total number of galls in each treatment (30 shoots) were 24, 30, 32, and 31 galls for 10, 15, 20, and 25 C, respectively. The overall frequency of galling (galled shoots/total shoots) was 70% for 10 C, 93% for 15 and 20 C, and 73% for 25 C.

Gall formation was nonlinearly related to temperature from 10 to 25 C (Fig. 8). The number of galls per plate were different among these temperature treatments. Nineteen days after culture, the largest number of galls were formed at 20 C and the lowest number of galls were formed at 10 C.

These results demonstrated that temperature affected gall formation time, and gall formation frequency. Compared with the other treatments, 20 C was the most suitable temperature for this nematode culture. Shoot cultures grew poorly and the galls were small at 10 C.
Fig. 7. The effect of temperature on gall formation.

The gall numbers were cumulated from 10 replicates (10 petri dishes) with a total of 30 shoots.
Y = -15.42 + 1.06X  
($R^2 = 0.99, p < 0.001$)

Y = -32.39 + 6.63X - 0.17X^2  
($R^2 = 0.98, p < 0.001$)

Y = 7.15 + 0.54X^2  
($R^2 = 0.98, p < 0.001$)
Fig. 8. The effect of temperature on gall formation.

Data are from 19 days after infestation.
Y = -7.69 + 1.06X - 0.03X^2

(R^2 = 0.48, p < 0.001)
3.2.3 Effect of Shoot Age on Gall Formation

3.2.3.1 Methods

Individual lateral bud primordia, approximately 4.2 mm in length, derived from cultured shoots were transferred to MSIBG medium. These bud primordia were cultured for 0, 5, 10, and 15 days, after which the shoots averaged 4.2, 7.9, 12.6, and 16.8 mm in length (Fig. 9). Four treatments of 0-day (4.2 mm), 5-day (7.9 mm), 10-day (12.6 mm), and 15-day (16.8 mm) old shoots were compared. Each treatment comprised 10 replicates (10 petri dishes), with each petri dish containing 3 shoots to which 50 mixed staged nematodes were applied on the surface of the B5G medium. Infested shoots were placed in a completely randomized design in an incubator at 20 °C with 16 hours daylength at 60 μmolm⁻²s⁻¹ light intensity using fluorescent white light. Treatments were observed over a period of 26 days.

Data were analyzed by regression. This experiment was repeated 3 times.

3.2.3.2 Results

There was a curvilinear relationship between the number of galls and culture time for the 0-day, 5-day, and 10-day old shoots infested with the nematode (Fig. 10). The relationship of the number of galls and culture time was linear for 15-day old shoots infested with the nematode. Gall formation times varied with the different aged shoots. Seven days after culture, galls were found on the 0-day old shoots. Nine days after culture, galls were found on the 5-day, 10-day, and 15-day old shoots. Significantly more galls
were produced in young (0-day) shoots than in older, 10-day and 15-day old shoots. By 26 days after culture, the total number of galls in each treatment (30 shoots) were 38, 29, 25, and 15 for 0-day, 5-day, 10-day, and 15-day old shoots, respectively. The overall frequency of galling (galled shoots/total shoots) was 90% for 0-day old shoots, 83% for 5-day old shoots, 76% for 10-day old shoots, and 33% for 15-day old shoots.

There was a negative linear relationship between the number of galls and shoot age over the range of 0-day to 15-day old shoots (Fig. 11). The number of galls per plate decreased with increasing shoot age.

These results demonstrate that the age of the shoot influences the rate of gall formation and the number of galls formed. Zero and 5-day old shoots with an average length of 4.2 and 7.9 mm are suitable tissues for nematode development and gall formation. Gall formation decreased as shoot age increased. The 15-day old shoots with an average length of 16.8 mm provided poor tissues for gall formation. S. picridis requires young, undifferentiated shoot tissue to initiate gall formation.
Fig. 9. Russian knapweed shoots at different ages in culture. (A) 0-day old shoots. (B) 5-day old shoots. (C) 10-day old shoots. (D) 15-day old shoots.
Fig. 10. The effect of shoot age on gall formation.

The number of galls were cumulated from
10 replicates with a total of 30 shoots.
For 0-day:

\[ Y = -30.71 + 5.48X - 0.11X^2 \]

\[ (R^2 = 0.99, p < 0.001) \]

For 5-day:

\[ Y = -28.99 + 4.28X - 0.08X^2 \]

\[ (R^2 = 0.98, p < 0.001) \]

For 10-day:

\[ Y = -19.87 + 2.77X - 0.04X^2 \]

\[ (R^2 = 0.98, p < 0.001) \]

For 15-day:

\[ Y = -6.04 + 0.81X \]

\[ (R^2 = 0.98, p < 0.001) \]
Fig. 11. The effect of shoot age on gall formation.

Data are

from 26 days after infestation.
\( Y = 3.77 - 0.15X \)
\((R^2 = 0.44, p < 0.001)\)
3.2.4 Effect of Medium on Gall Formation

3.2.4.1 Methods

Fifty mixed staged nematodes from a 4 month old gall were applied on the surface of two different agar media with 3 shoots per petri dish. MSG [Appendix D (c)] and B₅G media [Appendix B (f)] were used. Each treatment was comprised of 10 replicates. The infested shoots were placed in a completely randomized design in the incubator at 20 °C with 16 hours daylength with a light intensity of 60 μmol m⁻² s⁻¹ using fluorescent white light. Treatments were observed over a period of 26 days.

The results were analyzed by regression analysis. This experiment was repeated 3 times.

3.2.4.2 Results

The relationship of the number of galls and culture time was curvilinear for cultures in B₅G and MSG medium (Fig. 12). The time for gall formation was the same for shoots cultured on both the MSG and B₅G media. Seven days after infestation, galls were found on shoots in both media (Fig. 12). However, significantly more galls were produced in the B₅G medium (4 galls per shoot) compared to the MSG medium (2 galls per shoot). Twenty-six days after infestation, a total of 38 galls were found in the 10 petri dishes of the B₅G medium, while only 21 galls were found on the shoots in the MSG medium. The overall gall formation frequency (galled shoots/total shoots) was 90% in B₅G medium and 70% in MSG medium.
These results indicate that B$_2$G medium is the better medium when compared with MSG medium for nematode gall formation. Shoot tissue which is growing rapidly and differentiating is not suitable for S. picridis to induce galls.
Fig. 12. The effect of medium on gall formation.

The number of galls were cumulated from 10 replicates with a total of 30 shoots.
Y = -17.74 + 2.65X - 0.04X^2
(R^2 = 0.97, p < 0.001)

Y = -31.63 + 5.63X - 0.12X^2
(R^2 = 0.99, p < 0.001)
3.3 Summary and discussion

The successful establishment of the host-nematode association is a result of many aspects from both the host and the nematode. The shoot apex is the continuing embryonic region of the plant and is the site of gall formation by the knapweed nematode. Suitable meristematic tissue must be available for the nematode to induce the gall (Watson, 1975). The results from this experiment go a step further to prove that the shoot apex tissue of Russian knapweed is necessary for *S. picridis* to complete its whole life cycle. Similar to what occurs in nature, the nematode modified the penetrated tissue, induced the galls on the leaves, petioles, and the shoot tips of shoots, and developed and reproduced inside the cultured plantlets. Since no nematodes beyond the 4th stage were found on the first day of visible gall appearance, suggests that the 4th stage is a critical point in the nematode's development, the formation of the gall may be necessary for subsequent reproduction.

The duration of the life cycle of this nematode is about 12 days according to the continuous observations in these experiments. Males and females were found inside the galls 5 days after the galls appeared, and the young adults of the second generation were found 17 days after the galls appeared.

It is evident that *S. picridis* is dependent upon the host plant suitability for the establishment of a successful host-parasite relationship. The host plant suitability may be biochemically oriented or may involve plant growth and developmental patterns (Watson; 1975). The results from these experiments demonstrate that certain host growth patterns
are required for this nematode to develop and to reproduce. The host plants are highly susceptible to the nematode attack only in the early growth stages and when the growth rate is relatively slow.

Krusberg (1963) observed that younger or meristematic cells were more susceptible to modification by nematodes than were older cells, and also that physiologically older tissue, such as cortical parenchyma, exhibited only cell separation rather than a galling response. Watson (1975) suggested that rapidly elongating shoots of perennial plant species are probably immune to attack from *S. picridis*. The increased cell division, coupled with an increased rate of differentiation in the elongation of perennial shoots is probably too rapid for the nematode to alter the normal plant development. The effect of shoot age on gall formation indicates that the young shoots are more susceptible to the attack of the nematode, as the shoot continues to grow, the ability of the host to respond to the nematode's gall initiation decreased. As such, the resistance of the host to nematode infection increases as the plant ages.

A plant in a young physiological status and under slow growth is required for *S. picridis*. It was observed that the foliage growth of Russian knapweed receiving the high nitrogen solution was very vigorous, and plants receiving the low nitrogen solution did not exhibit vigorous growth of the primary shoot. Galls formed on the Russian knapweed growing at the low nitrogen level, but did not form on those growing at a high nitrogen level (Watson, 1986a). Using MSG and B5G media to culture *S. picridis*, the results showed that shoots grew faster and stronger in the MSG medium than in B5G medium. Gall formation rate, however, was lower and gall size was smaller in the MSG medium than in the B5G medium. A significant feature of the MSG
medium is its very high content of nitrate, ammonium, and potassium with the general concentration of inorganic nutrients in the BgG medium being lower than in the MSG medium. The study confirmed the earlier work that young shoots under slow growth are necessary for the nematode to induce galls.

The pattern of development during vegetative growth is influenced by various internal and external factors such as genetic and environmental factors. As mentioned before, temperature has two types of effect in the nematode culture system. It directly influences the behaviour and the development of the nematode, and it also affects the plant tissue growth rate which in turn indirectly affects the host's susceptibility to the nematode attack. Shoots grew faster and the galls formed earlier at 25 C in the beginning, but the gall formation rate was decreased with culture time. Gall formation time was two days later at 20 C than at 25 C, while the maximum number of galls were obtained in the shortest time compared with other temperatures. The final gall numbers were almost the same in the cultures under 15, 20, and 25 C, although the gall formation time was delayed under low temperature conditions. A temperature of 10 C provided less galls compared with other temperatures. These results indicated that 20 C is an optimum temperature for this nematode culture. The gall formation rate decreased with the culture time at 25 C which may be due to the faster growth of the shoots. Susceptibility of the shoot tissue to the attack by the nematode decreased as the growth of shoots was inhibited by cool temperatures. The growth of shoots at 10 C were slow and poor, and subsequent gall number was least which may be due to the poor growth of tissues and the inability to provide enough nutrients to satisfy the demands of the nematode.
Nematode galls are developed by hypertrophy and hyperplasia of parenchyma tissues. Hypertrophied cells in the nutritive zone are called nutritive cells. Nutritive cells contain abundant granular protoplasm, and are thought to supply food to the nematode within the gall cavity (Goodey, 1934; Goodey, 1938, Bird and Loveys, 1975). These nutritive cells apparently act as powerful "physiological sinks" attracting assimilates to the gall and gall former (Jankiewicz et al., 1969).

Goodey (1934) presumed that the stimulating substances cause an inflow of plant nutrients of various kinds into the enlarged cells and these are utilized by the parasite living within the gall cavity. The galls induced by S. picridis on Russian knapweed have a well-defined zone of numerous nutritive cells. This nutritive cell zone is more substantial than those reported in other leaf galls induced by nematodes. The large nutritive zone in S. picridis induced galls suggest that this nematode may be very detrimental to its host (Watson, 1986a). The galls formed on the shoots in culture are similar to the galls formed in nature, they have a central cavity and a well-defined zone of nutritive cells. The physiological sink role of the nematode gall was obvious in the cultured galls. Once galls were induced, galls developed rapidly, but the remaining part of the shoots were stunted in growth. When galls were initiated on the shoot tip, usually the shoot formation was stopped and the plantlet only produced gall tissue in culture, indicating that galls absorbed large amounts of nutrients for their development, and thereby hindered shoot growth.

The penetration of this nematode only occurs at the moment when the young shoots of knapweed pass through the upper layer of the soil in the early spring. The nematode develops and reproduces inside the host plant in
the vegetative growth stage during the growing season. As the host ages, the nematodes stop developing and only the 2nd stage dormant larvae survive inside the host to pass through the overwinter stress condition of the cold winter and the absence of food. In nature, only two generations are completed inside the galls per year (Ivanova, 1966). Unlike in nature, the nematode can continually develop and reproduce inside the cultured galls, old nematodes may die, but new generations are continually formed. By the subculturing technique, nematodes can continue to develop and reproduce unimpeded inside the cultured tissues from generation to generation. The dormancy phenomenon may occur in culture when the nutrients in the medium are exhausted, but this can be overcome by subculturing the gall tissue to fresh medium. The established monoxenic nematode culture system provides a suitable artificial environment without the environmental limitations existing in nature. The culture system can continually provide young shoot tissue, enough nutrients, and the constant physical environment for nematode development and reproduction throughout the year. As such this system can provide large numbers of nematodes for use as a bioherbicide. This system provides a decisive advantage in mass rearing of this nematode since large quantities cannot be readily obtained under natural conditions of field plots nor by greenhouse grown Russian knapweed plants, since the formation of galls is greatly affected by the climate and by the growth status of the host plant.

The continual formation of new galls on the galled shoots after transfer to fresh rooting medium, and the observation that no nematodes were found free living in the medium, demonstrate that the nematode can migrate inside the culture tissue. Therefore, this provided an additional way to propagate
the nematode by subdivided the individual lateral shoots with galls and transferring them to fresh medium.

The endoparasitic characters of this nematode limit many detailed research investigations on the biology of this nematode. The successful use of a natural enemy to control a weed is often dependent upon the comprehensive knowledge of the biological characteristics of the natural enemy, its host, and the host-parasite relationship. What are the main factors affecting the nematode's effectiveness, and what are the factors affecting the susceptibility of the host to the nematode attack? These questions are very important to determine the optimum pathogen infestation time and the infestation number. These studies, however, are very difficult to be carried out in the field due to the difficulty of continually observing the development of this nematode in nature. The success of monoxenic culture of this nematode can now make these studies possible. Factors can be tested under controlled conditions using the culture system developed. The results of which could be utilized to achieve a near maximum biotic potential of this agent in the field.
CHAPTER 4. VIRULENCE OF CULTURED NEMATODES

4.1 Introduction

Virulence is the ability of a biocontrol agent to heavily infest an individual weed plant by overcoming its resistance to attack (Wapshere, 1982). A potential bioherbicide pathogen should be able to be cultured on artificial medium, be highly virulent, and have the capacity to damage its host plant (Watson, 1989). *Subanguina picridis* is the first above-ground endoparasitic nematode successfully cultured *in vitro*, which opens a new avenue for using plant parasitic nematodes as bioherbicide to control weeds. After developing the mass rearing technique subsequent questions are: Can the cultured nematodes effectively suppress the growth of the target weed host? If the cultured nematodes are effective, when and how should these mass reared nematodes be applied to field population of Russian knapweed?

It has been reported that some pathogens lose their virulence after being cultured in artificial medium (Zuckerman *et al.*, 1989). In addition, nematodes cultured in the laboratory, are grown under favorable conditions and the cultured plantlet may be more susceptible to the attack of the nematode. Therefore, testing the virulence of the cultured nematodes is required to determine the potential application value of this monoxenent nematode culture system on whole plants.

The objective of this study was to evaluate the ability of cultured nematodes to penetrate, to initiate galls, and to suppress the growth of Russian knapweed plants grown under greenhouse condition.
4.2 Methods

Russian knapweed seeds were germinated on moist filter paper in petri dishes and two seedlings about 1.5 cm in length, were carefully planted in a prepared substrate (Pro mix) in each of 10 12-cm diameter pots. Approximately 1,000 nematodes of mixed stages from a gall produced in culture which had been maintained on the MSIG medium for 4 months, were applied on the substrate surface of each pot. The infested seedlings were lightly covered with Pro mix and maintained in the greenhouse at 20 ± 3 C day/ 15 ± 3 C night. Non-infested seedlings were used as control plants. Ten replicates of the two treatments (10 pots of infested seedlings and 10 pots of non-infested seedlings) were placed in a completely randomized design on the bench in the greenhouse.

Root segments were obtained from Russian knapweed plants grown in the greenhouse. Two segments, 8 cm in length, with a stem bud (2-5 mm in length), were transferred to each of 14 12-cm diameter pots filled with a prepared substrate (Pro mix). About 1,000 mixed staged nematodes from 4-month old cultured galls were applied on the soil surface around the root segments in each pot. The infested roots were lightly covered with 1 cm depth of soil. Fourteen replicates of non-infested segments were used as the control. The pots were placed in a completely randomized design on the bench in the greenhouse at 20 ± 3 C day/ 15 ± 3 C night.

The susceptibility of various sized stem buds, and buds from relatively old and new root systems to the cultured nematode were also examined. The treatments included: buds of 1 mm, 2 mm, and longer than 5 mm on a new root from 50 days old seedlings, new buds of various size on new roots from 50
days old seedlings and old buds on an old root from greenhouse grown perennial knapweed plants. Root segments of the above were placed on prepared soil mix (Pro mix) in 12-cm diameter pots, each pot contained one root segment. Approximately 1,000 mixed staged nematodes were applied on the soil surface. The infested root segments were lightly covered with 1 cm depth of soil. Controls consisted of root segments not infested with nematodes. Seven replicates of these treatments were placed in a completely randomized design on a bench in the greenhouse at \(20 \pm 3\) C day/ \(15 \pm 3\) C night.

To test the ability of the nematodes to move within the soil and locate their host, a box (50 x 40 x 30 cm) with greenhouse grown perennial Russian knapweed plants was divided into two areas, all Russian knapweed plants in this box were cut at soil level and only a half area (one side) was used to infest with cultured nematodes. Approximately 10,000 mixed staged nematodes from 4-month old cultured galls were applied on the soil surface.

4.3 Results

Twelve days after infestation, two galls were observed on leaves of Russian knapweed seedlings (Fig. 13A) and 90% of seedlings were galled after 2 months. Galls formed on the leaves, stems, and the shoot tips with 1 to 6 galls per seedling (Fig. 13B). The growth rate of galled seedlings was reduced compared to the control plants in this experiment (Fig. 14A). Eighty days after infestation, galled shoots were still in the rosette stage but the control plants already were in the flowering stage (Fig. 14B).
Vegetative shoots originating from root segments were also galled with the cultured nematodes (Fig. 13C). Three months after the shoots emerged, 64% of shoots had formed galls. The galls were found on the shoot tips, leaves, and stems.

Fifteen days after infestation, the galls were found on the shoots from 1 mm, 2 mm and > 5 mm stem buds. Gall formation frequency was 100% for the shoots from 1 mm length stem buds, 71% for the shoots from 2 mm length stem buds and 80% for the shoots from > 5 mm stem buds. Two months after soil infestation, 70% shoots initiated from the new buds on the new roots were galled, but no shoots initiated from the old buds on the old root were galled.

Similarly, greenhouse grown perennial Russian knapweed plants which had been cut off at the soil level were susceptible to infestation by soil applied cultured nematodes. Galls were found on leaves and stems of shoots 19 days after soil infestation. But the galls only formed on the plants growing in the infestation region.

These results demonstrate that the cultured nematodes are virulent on whole plants of Russian knapweed. The nematode induced galls on Russian knapweed which resulted in a reduction in plant growth. The nematodes do not move a great distance in the soil as the galls formed only on the shoots in the infestation zone.
Fig 13. A. A greenhouse grown Russian knapweed seedling, 12 days after soil was infested with nematodes produced in the shoot culture system. Note gall formation on leaf.

B. Numerous galls on a Russian knapweed seedling grown in the greenhouse. Forty days after soil was infested with nematodes produced in the shoot culture system.

C. Galls formed on a vegetative shoot which originated from root segments under greenhouse conditions.

(Arrows point to galls).
Fig. 14. A. Comparison between non-infested (control) 
Russian knapweed and galled plants (infested) 
infested with the cultured nematodes 
40 days after infestation.

B. Comparison of the growth of infested Russian 
knapweed plants (infested) with non-infested 
(control) plants 80 days after infestation.
4.4 Discussion

For many plant pathogens, virulence is a function of pathogen nutrition (Stack et al., 1988). The ability of a nematode to induce galls on the host plant is a clear sign of virulence. The nematode invades the host tissues and induces gall formation, and the physiological sink function of gall cells competes for the nutrition with the non-galled portion of the host so that knapweed growth is reduced.

Cultured Subanguina picridis have been shown to be an effective biological control agent of Russian knapweed. In the greenhouse study, 90% of infested seedlings were galled within 2 months after soil infestation with S. picridis from cultured galls. Sixty-four percent of vegetative shoots initiated from stem buds on root segments were galled 3 months after soil infestation of cultured S. picridis. Similarly, greenhouse grown perennial Russian knapweed plants which had been previously cut off at ground level were also susceptible to infestation by soil applied cultured nematodes. Galls were found on the leaves and stems of shoots 19 days after soil infestation. The growth of knapweed was influenced by the soil infestation of the cultured nematodes. The growth rate of galled seedlings was reduced compared to the control non-infested plants in this experiment. The control non-infested plants had developed to the flowering stage, while the galled plants were still in the rosette stage. These data demonstrate the feasibility of and application of this novel mass production system. Nematodes produced in this system are virulent and the growth rate of infested Russian knapweed was reduced.
The ultimate test of virulence of cultured nematodes would be evaluated under field conditions which has been recommended in chapter 6. Limited supplies of naturally produced nematodes and variability within natural populations would limit the opportunity to compare pathogenicity of natural populations and cultured populations of S. picridis. However, the results of this study clearly demonstrate that cultured S. picridis are virulent.

The application of a biological agent for the control of weeds encompasses the preparation and delivery of the agents, methods for the evaluation of the efficacy of the applied agents, as well as consideration of the potential impact the agents may have upon nontarget organisms (Stack et al., 1988). Information on the epidemiology of the disease such as the production and dispersal of the inoculum, and the reproduction and survival of the biological control agent are required. Determination of the environmental factors which influence the biological control agents' performance, and an understanding of the mechanism of control are very important for the successful application of a biological control agent to control its host plant. If we can understand the factors which influence the biological control agent's activity as well as the susceptibility of the host to its parasite, we will be able to utilize the biological agent and obtain maximum performance of that agent. As such, the agent will be able to reveal its true value as a biological agent in the infested area.

The timing of the application of biological control agents should be considered with respect to crop development, the effect of the environment upon the survival, and activity of the agent and target (Stack et al., 1988). The decisions of application time, the application rates, and the application frequencies should be based on the following information: what
developmental stages of the plant are susceptible, and how long the host plant remains susceptible. With this information, appropriate decisions can be made as to when and how to deliver the biological control agent to the environment or plant. This study provides some of the information on the timing of application of this biological control agent. Although Russian knapweed does not propagate extensively from seed in North America, in its native region, seed production plays an important role in its propagation. The influence of cultured *S. picridis* on young seedlings suggests that this nematode can be applied at the time when seeds germinate. The galls did not form on shoots from old stem buds on the old roots which suggests that control effects may need to be focused on the newly formed stem buds. The nematode should be applied during the time when the new stem buds are emerging from the soil. In addition, the formation of galls on the greenhouse grown perennial Russian knapweed plants showed that the control of this weed may be enhanced by removing the above ground parts of this plant first and then applying the cultured nematodes.

Soil infestation of nematode water suspensions provides a convenient method for the application of cultured nematodes. This method may be adapted to the use of common agricultural equipment to spray the cultured nematode on Russian knapweed infested areas. The nematode water suspensions are easy to prepare by releasing the nematode from the opened galls. The nematodes can survive in sterile distilled water for at least 2 months, which provides a short-term storage method for the cultured nematodes. For the long-term storage of cultured *S. picridis*, the galled shoots can be maintained in the medium and subcultured monthly. Alternatively, the cultured galls could be applied intact as a form of granular application.
A mass production system, which can provide large quantities of effective nematodes, is required for the successful biological control of Russian knapweed using the plant parasitic nematode, S. picridis. The establishment of the S. picridis mass rearing system and the virulence test results suggest that the utilization of cultured nematodes as bioherbicides will open new prospects in the inundative biological control of weeds using plant parasitic nematodes. The utilization of cultured nematode as a bioherbicide will be discussed in the next chapter.
CHAPTER 5. THE UTILIZATION OF CULTURED NEMATODES AS BIOHERBICIDES

5.1 Introduction

Prior to this study, the use of \( S. \) picridis to control Russian knapweed was a part of the classical biological approach. The successful mass rearing of \( S. \) picridis opens a new avenue for the use of cultured nematodes as an effective bioherbicide. Therefore, the biological control program of Russian knapweed using \( S. \) picridis can be switched from the classical approach to the bioherbicide approach. This chapter will describe in detail the use of cultured \( S. \) picridis as a bioherbicide. \( S. \) picridis is the first nematode used as a biological weed control agent to be mass propagated in vitro. The culture system established in this study may also be adapted to other weed parasitic nematodes including Orrina phyllobia and Anguina amsinckiae.

5.2 General review of the bioherbicide approach

Biological herbicides are usually developed from indigenous organisms, which are normally restrained in nature, which are cultureable in fermentation tanks, and which can be applied similar to chemicals as inundative inoculum (Templeton, 1982). Watson (1989) mentioned that the term "bioherbicide" is generally restricted to the use of plant pathogens and does not include attempts to augment populations of beneficial insects, nor does it generally include the use of naturally occurring compounds (phytotoxins) produced by microorganisms. As such, a bioherbicide is a preparation of living inoculum of a plant pathogen, formulated, and applied
in a manner analogous to that of a chemical herbicide in an effort to control or suppress the growth of weed species (Watson, 1989).

Daniel et al (1973) listed three general requirements for the selection of a pathogen as a potential bioherbicide; the pathogen must be: 1) able to produce abundant and durable inoculum in artificial culture, 2) genetically stable and specific for the target weed, and 3) able to infect and kill the weed in environments of reasonably wide latitude. According to Watson (1989), the preferred characteristics of a potential bioherbicide pathogen include: 1) growth and reproduction on artificial media, 2) highly virulent, 3) genetic stability, 4) restricted host range, 5) broad tolerance range, 6) prolific propagule production, 7) capacity to damage its host plant, and 8) innocuous in ecological effects.

The development of a bioherbicide involves three major phases: 1) discovery, 2) development, and 3) deployment (Templeton, 1982). In general, and not only for fungal pathogens, the discovery phase involves the collection of diseased plant material, isolation of the causal organism and identification of the pathogen, culture of the pathogen on artificial media, and maintenance of the pathogen cultures in short-term and long-term storage. The development phase involves the determination of optimum conditions for pathogen production and the determination of optimum conditions for infection and disease development, determination of host range and elucidation of mechanism of action of the pathogen. The deployment phase involves close collaboration between non-industrial and industrial sectors through the formulation, scale-up, field evaluation, and marketing stages of the commercialization process of a new bioherbicide product (Watson, 1989).
Until now, only two biological herbicides have been commercially developed in North America. Abbott laboratories marketed the fungus Phytophthora palmivora Bultler (marketed under the trade name DEVINE) in 1981 for the control of strangler vine (Morrenia odorata Lindl.) in Florida citrus groves. The Upjohn company marketed the fungus Colletotrichum gloeosporioides f. sp. aesculinae (c. g. a.) in 1982 for control of northern jointvetch [Aeschynomene virginica (L.) B.S.P.] in Arkansas rice (Oryza sativa L.) and soybean [Glycine max (L.) Merr.] fields (Templeton, 1982). Besides these two fungi, no other fungal pathogens nor other plant pathogens including bacteria, mycoplasmas, viruses or nematodes are as yet registered for use as bioherbicides (Watson, 1989).

Watson (1989) concluded two major biological factors, low virulence of the pathogen and fastidious environmental conditions, and one technological factor, the difficulty to formulate the bioherbicide pathogen are the main factors that restraint the development of bioherbicides. Basic understanding of the disease cycle, the mechanisms of pathogenicity, and the interaction of pathogen and its host are important for the development of bioherbicides.

5.3 The use of the plant parasitic nematode, Subanguina picridis as a bioherbicide

Populations of Russian knapweed in the southwestern regions of USSR are attacked by a leaf and stem gall nematode, Subanguina picridis. The nematode is reported to be damaging to its host in USSR with up to 100% of shoots infected, 20% destroyed, and 30% severely damaged (Ivanova 1966).
picridis has been sprayed in a water suspension in an augmentation biological control programme in the USSR (Kovalev et al., 1973).

In North America, Russian knapweed is relative free of specialized parasites and is not extensively attacked by polyphagous feeders. Since Russian knapweed populations in Canada do not reproduce extensively from seed, potential biocontrol agents that attack the seed head were not considered. However, the leaf and stem gall forming nematode, S. picridis was imported into North America for the control of this weed. Several experiments have been conducted to test the potential of this nematode as a biological control agent in North America including: a) host range testing (Watson, 1986a) b) the persistence of the nematode under field conditions in the absence of Russian knapweed, and c) the effect of S. picridis on Russian knapweed (Watson and Harris, 1984).

The experimental results demonstrated that S. picridis had a restricted host range. Russian knapweed was the only host plant susceptible to S. picridis. The nematode did not persist into the second season under field condition in the absence of knapweed. Although S. picridis had been established in Saskatchewan, Alberta, and Quebec, the populations of this nematode declined five years after infestation. The nematode did not spread rapidly in the infested areas. From these results, it is recognized that S. picridis would have to be spread thoroughly over a knapweed stand in much the same manner as a granular herbicide to achieve rapid and satisfactory control. Since this nematode has only two generations per year, since it does not disperse rapidly, and since it does not persist in the field, its effectiveness as a biological control agent of Russian knapweed will require
a large quantity of nematodes to augment the natural population increase and dispersal.

The potential advantages of the established *S. picridis* culture system described in this work include:

1) A large quantity of virulent nematodes for the control of Russian knapweed can be provided.

2) The nematodes from cultured galls are easily collected and nematode water suspensions are readily made. Therefore, the nematode can be applied as nematode water suspensions and applied with conventional spray equipment just prior to Russian knapweed shoot emergence from the soil. The galls from culture may also be applied as a granular form.

3) For short term storage, the cultured nematodes can be maintained in the sterile water for at least two months. For longer-term storage, the cultured galls can be kept in the fresh medium or the galls may also be stored as a dry gall form for many years.

4) The large scale production of this nematode in the culture room can continue throughout the whole year without climatic limitations. This can not be achieved in the field nor in the greenhouse since those system are limited by climatic factors. In addition, using the culture system to produce the nematodes should provide space and labour savings compared with field or greenhouse production on whole plants.

and 5) The successful establishment of this nematode culture system should make commercialization of this bioherbicide possible.
The process of development of *S. picridis* as a bioherbicide for the control of Russian knapweed is in the commencement of the deployment phase. The application of this system will likely rely on industrial involvement. From the industrial perspective, large market products and short term economic return are of primary interest. To gain the attention of industry, a bioherbicide should adapt to these requirements. Although Russian knapweed is a localized noxious weed in North America, its effect is not as widespread as in its native regions of the U.S.S.R. and Turkey. The application of this system may not only be limited to the North America, but should also have utility in other parts of the world where Russian knapweed has been introduced such as Australia and New Zealand as well as in parts of Europe, the U.S.S.R. and Turkey. Therefore, the potential market is much greater than just North America. Similarly, if other weed parasitic nematodes can be cultured using this process, the possibility of commercialization of this plant parasitic nematode mass rearing process and application of cultured nematodes as effective bioherbicides will be enhanced. Hence, further studies should focus on:

a) optimising this system in terms of cost/unit to maximize production of virulent nematodes while reducing costs of production.

b) conducting field trials for the evaluation of this cultured nematode to control Russian knapweed,

c) conducting detailed research on the biology of this nematode and interaction of this nematode with its host using this culture system, and
d) applying the culture system to other weed parasitic nematodes which are potential weed biocontrol agents.
5.4 Other nematodes as potential biological weed control agents

Two other weed parasitic nematodes, the silverleaf nightshade nematode, *Orrina phyllobia* (Thorne, 1934) Brzeski, 1980 and the coast fiddleneck nematode, *Anguina amsinckiae* (Steiner and Scott, 1934) have been examined as biological weed control agents in the United States.

Silverleaf nightshade, *Solanum elaeagnifolium* is an economically troublesome perennial weed in cotton production in the Southwestern United States. It is distributed throughout the southwest and adjacent Mexico, northeast to Missouri, Indiana, Ohio and Florida. Herbicides for annual weeds are ineffective for this weed. Thus, silverleaf nightshade has spread rapidly and extensively by both seed and perennial root propagation (Orr et al., 1975).

The silverleaf nightshade nematode, *Orrina phyllobia*, was found in Texas. This nematode produces galls on the leaves and stems of silverleaf nightshade and causes stunting and death of heavily infected plants. The reproduction of infected plants is also influenced by the nematode infestation. This nematode is host-specific in addition to silverleaf nightshade, two closely related species *Solanum carolinense* L. and *S. melongena* L. also developed galls (Orr, 1980; Orr et al., 1975).

Infected larvae (4th stage) penetrate apical meristems of actively growing plants (Orr, 1980). The nematode develops and reproduces inside the gall. As the plant and gall tissues senesce, the adults and other stage
larvae die, but the infective stage larvae become dormant and overwinter inside the gall or in the soil. During sustained moist conditions, these larvae exit abscised galls and penetrate actively growing young tissues. Infective larvae of this nematode are highly resistant to environmental stress. The nematode has good tolerance to high temperature and is anhydrobiotic (Sivakumar, 1982).

The nematode has been shown to be an effective biocontrol for silverleaf nightshade. Injury to the weed is through reduced growth and vigour as well as reduced seed production. In addition to being highly host specific for silverleaf nightshade, it is well adapted for survival under extreme environmental adversity. Nematode populations, when introduced into an area, tend to remain as long as the host plant is present, but generally the natural propagation of the nematode must be augmented to achieve satisfactory control of silverleaf nightshade. Studies on the biology of this nematode, the histopathogenesis of the galls (Robinson et al., 1978; Skinner et al., 1980; Bird and Orr, 1982; Robinson et al., 1984), determination of the environmental factors on nematode survival and activity (Robinson et al., 1981; Sivakumar, 1982) have been conducted. The use of different carriers and formulation of the nematode is also being investigated (Parker, 1989). Corn cob grit was spray coated with nematode water suspensions and forced air oven dried at 34 C for 17 hours. Applications of nematode coated corn cob granules on greenhouse grown host plants resulted in gall formation on the infested plants (Parker, 1989). Presently, this nematode is being mass reared in field plots and redistributed to infested fields (Parker, 1991). For this biological control
program, what is lacking is a method to mass-produce the nematode in an economical manner.

Coast fiddleneck, *Amsinckia intermedia* Fisch. and Mey, is an annual weed of the central valley of California. It is capable of inducing pathological disorders in livestock if ingested in sufficient quantities. The coast fiddleneck nematode, *Anguina amsinckiae*, produces galls on the fruit, leaves, and stem apices, severely stunts the weed and greatly reduces seed production. This nematode has been proposed as a biological weed control agent of coast fiddleneck. The information about the biology of this nematode and the use of this nematode as a biological agent to control Coast fiddleneck can be found in the articles of Steiner and Scott (1934), Nagamine and Maggenti (1980), Pantone et al. (1985, 1989), and Pantone and Womersley (1986).

There are other species in the genus *Subanguina* and other species in other genera of *Anguiniidae* including *Anguina*, which are parasitic to undesirable plants (Table 13). This culture system may also be adapted for mass rearing of these nematodes which would facilitate the biological control of these problem weeds.

Under natural conditions, plant parasitic nematodes have shown negative influences on their host plants by competition for nutrients. The development of bioherbicide technology, so far, is restricted to the use of fungal plant pathogens as mycoherbicides. The utilization of nematodes as biological weed control agents has been met with skepticism, the main reason for this is due to the limited success of mass-production of plant parasitic nematodes. The success of mass production of *S. picridis* may be helpful in opening this avenue of using nematodes as bioherbicides. The procedure for
mass production and use as a bioherbicide as described for *S. picridis* are likely similarly applicable to other nematode/weed host systems mentioned above since these nematodes have a similar life pattern to *S. picridis*. They all penetrate young shoots and form galls on the above-ground tissues. An advantage of using gall forming nematodes as potential biological weed control agents is that the nematode galls can easily be applied as a 'granular herbicide', or the nematodes from galls can be easily prepared as nematode water suspensions and sprayed with commonly available farm equipment. Further studies should be done on the application of the *S. picridis* culture system to other nematode/weed systems, and if applicable, the successful mass rearing of these weed parasitic nematode may result in effective and safe biocontrol strategies for some of these troublesome weed species.
Table 13. Nematode species and their respective weed hosts.

<table>
<thead>
<tr>
<th>Nematode</th>
<th>Host plant</th>
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<tbody>
<tr>
<td><em>Agniina agropypri</em></td>
<td><em>Agropyron repens</em></td>
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<td></td>
<td><em>(quackgrass)</em></td>
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<tr>
<td><em>Agniina amsinckia</em></td>
<td><em>Amsinckia intermedia</em></td>
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<tr>
<td></td>
<td><em>(coast fiddleneck)</em></td>
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<tr>
<td><em>Orrina phyllobia</em></td>
<td><em>Solanum elaeagnifolium</em></td>
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<td></td>
<td><em>(silverleaf nightshade)</em></td>
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<tr>
<td><em>Subangina centaureae</em></td>
<td><em>Centaurea squarrosa</em></td>
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<tr>
<td><em>Subangina chartolepidis</em></td>
<td><em>Centaurea biebersteinia</em></td>
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<tr>
<td><em>Subangina cousiniae</em></td>
<td><em>Cousinia spp.</em></td>
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<tr>
<td><em>Subangina millefolia</em></td>
<td><em>Achillea millefolium</em></td>
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<td></td>
<td><em>(yarrow)</em></td>
</tr>
<tr>
<td><em>Subangina mobilis</em></td>
<td><em>Arctotheca calendula</em></td>
</tr>
<tr>
<td></td>
<td><em>(capeweed)</em></td>
</tr>
<tr>
<td><em>Subangina montana</em></td>
<td><em>Cousinia spp.</em></td>
</tr>
<tr>
<td><em>Subangina picridis</em></td>
<td><em>Agroptilon repens</em></td>
</tr>
<tr>
<td></td>
<td><em>(Russian knapweed)</em></td>
</tr>
<tr>
<td><em>Subangina plantaginis</em></td>
<td><em>Plantago spp.</em></td>
</tr>
<tr>
<td></td>
<td><em>(plantain)</em></td>
</tr>
<tr>
<td><em>Subangina varsobica</em></td>
<td><em>Centaurea integrifolia</em></td>
</tr>
</tbody>
</table>
CHAPTER 6. SUMMARY AND CONCLUSIONS

No above-ground plant gall forming nematode has yet been mass produced in vitro. The current use of the knapweed nematode, *Subanguina picridis* (Kirjanova) Brzeski for the biological control of Russian knapweed, *Acroptilon repens* (L.) DC is limited in North America and other parts of the world because of insufficient numbers of this nematode and its limited natural dispersal. It was suggested that this nematode would have to be spread thoroughly over a knapweed stand in such a manner as a herbicide. To use the nematode to control knapweed in this manner, a large amount of inoculum would be required, but this could not be satisfied by collecting naturally formed galls since this nematode has only two generations per year. Therefore, it is necessary to work out a mass production system for *S. picridis*.

The purpose of this study was to establish a mass rearing system of *S. picridis* for its use as a bioherbicide. Several experiments were conducted in this study including: the culture of *S. picridis* in callus, excised roots, shoots initiated from excised roots, and shoots directly from shoot tip culture of Russian knapweed; the determination of the influence of temperature, shoot age, and the media on the *S. picridis* culture; and evaluation of the virulence of cultured nematodes on greenhouse grown plants.

When using callus, excised roots, and shoots to culture *S. picridis*, the development of this nematode was quite different in various types of Russian knapweed tissues. The nematode developed through the larval stages in the
callus tissue, but failed in the reproductive phase, and did not develop beyond the 4th stage in the excised root cultures. The nematode was, for the first time, successfully cultured and propagated in shoot tissues initiated from excised roots with galls forming on the leaves, petioles, and the shoot tips of the cultured shoots. This study represents the first time that an above-ground plant galling nematode was successfully cultured in vitro, and similarly the first nematode being used as a biological weed control agent to be cultured in vitro. The results indicate that the specificity of this nematode is not only in the plant species, but also in the parasitic position of its host plant. Shoot tissue is necessary for this nematode to induce galls and to complete its life cycle.

Factors which influenced the culture of *S. picridis* were determined in this study. Gibberellic acid (GA$_3$) plays a large role in *S. picridis* culture. It helped the nematodes to develop from the 4th stage to adults, increased the nematode development rate, and promoted male formation. The results from this study suggest that GA$_3$ may function as a male hormone for the nematode. Neither auxin nor cytokinin was needed to culture *S. picridis* in shoot tissues, which suggests that cultured shoots contain enough endogenous plant growth regulators for nematode gall induction. Testing the influence of shoot age, media, and temperature on nematode gall formation, indicated that a certain plant growth pattern is required for the nematode to induce galls. In culture, young shoots were more susceptible to the attack of the nematode, and as the shoots aged, the susceptibility of shoot tissue to the nematode infestation was decreased. Compared with B$_5$G medium, MSG medium contains higher concentrations of inorganic nutrients. The shoots grew faster and stronger in the MSG medium, but the galls were smaller and
the gall formation frequency was lower. Similarly, the results from the culture temperature determination experiment showed that the gall formation frequency was decreased when shoot growth was more rapid. Thus, young shoot tissues, under slow growth, are necessary for the success of this nematode culture.

The life cycle of this nematode in shoot cultured galls was determined in this study. The duration of the life cycle of *S. picridis* is 12 days in the cultured gall. New galls formed after the galled shoots were transferred to the rooting medium indicated that this nematode migrates inside the culture tissues.

The "physiological sink" role of the nematode gall was visibly obvious in the cultured galls. After gall induction, gall growth was very rapid, while the other portions of the shoot were stunted in growth. When the gall was induced on the shoot tip, shoot growth was stopped and only gall tissues developed in culture. These results confirm the hypothesis that nematode gall cells act as powerful "physiological sinks".

As a result from this study, a culture system has been established for mass rearing of the Russian knapweed nematode, *Subanguina picridis*. A shoot culture system, initiated from shoot tip culture, was used as the plant tissue culture system for nematode culture. Fifty surface disinfected nematodes were transferred to each of three young shoots growing on the artificial medium in 60 mm diameter petri dishes. Six days after infestation, visible galls were forming on the leaves, shoot tips, and the petioles of the shoots. The nematodes developed and reproduced inside the galls. After being cultured for 3 months, the number of nematodes increased from 50 to 7,000-10,000. Various factors including light intensity,
temperature, shoot size, etc. which affect the formation and development of the gall have been examined. The culture conditions determined in this system so far are: 60-80 μmolm⁻²s⁻¹ for light intensity, 20 °C for temperature, and 4-8 mm for shoot length.

Virulence of the cultured nematodes was tested in the greenhouse. Nematodes were extracted from 4-month old cultured galls and 1,000 nematodes were applied to the soil around seedlings growing in 12 cm diameter pots. Galls were observed on the Russian knapweed seedlings 12 days after being infested with nematodes. Two months after infection 90% of the seedlings formed galls. The galls were also found on the shoots produced from root segments. These results demonstrate the feasibility and application of this novel mass production system. Nematodes produced in this system are virulent and the growth rate of infested Russian knapweed was reduced.

This established S. picridis culture system can provide large quantities of virulent nematode for use as a biological herbicide to control Russian knapweed. Large-scale production of this nematode can be obtained by using the young shoots from the shoot culture system, and inoculating them with nematodes from cultured galls. Short-term storage of the nematodes is attained by maintaining nematodes in sterile water, and for the long-term storage, the galls can be kept on the medium and subcultured monthly. For the infestation of these cultured nematode to Russian knapweed plants, nematodes can be applied by spraying as nematode water suspensions or as a granular application of galls.

This monoxenic nematode culture method may be suitable to culture other above-ground weed-parasitic nematodes since many genera of Anquiniinae such as Anguina, Nothanguina, Orrina, and Subanguina are weed parasitic
nematodes. These nematodes have similar life cycle patterns to that of *S. picridis*, penetrating young shoots and forming galls on leaf or stem tissues. The nematodes in these genera have potential to be effective biological agents of their respective weed hosts. Presently, three nematodes in this family have been selected to be biocontrol agents for weeds, they are *Subanguina picridis*, *Orrina phyllobia*, and *Anguina amsinckiae*. This culture system, developed for *S. picridis* should also be applicable to and used to mass rear two other important weed infesting nematodes: *Orrina phyllobia* on silverleaf nightshade and *Anguina amsinckiae* on coast fiddleneck.

So far, although several types of plant pathogens such as fungi, bacteria, viruses, nematodes have been considered to be used as bioherbicides, only fungi have been successfully used. The use of wood parasitic nematodes as bioherbicides has been limited due to the difficulty of mass-production of plant parasitic nematodes. Attempts to mass produce plant parasitic nematodes has only met with limited success. In addition, the insufficient knowledge on the biology of plant nematodes, the interaction of the host and parasite, and the physiology of plant nematodes also affect the application of nematodes for weed biocontrol.

The contributions of this study are not only restricted to the biological weed control field, but also to the knowledge of plant nematology. The success of the culture of *S. picridis* has overcome the main problems limiting the use of this biological control agent: the insufficient number and lack of natural dispersal mechanisms of this nematode. In addition, in plant nematology, this is the first time that an above-ground gall forming nematode has been cultured in vitro. This culture system can
also provide large quantities of pure, sterile nematodes for plant
nematologists researching host-parasite relationships, physiology, and
biochemistry of above-ground galling nematodes. The success of *S. picridis*
culture is important in opening the avenue of the use of weed nematodes as
effective bioherbicides.

Based on the results of this study, several questions may be raised. Can
the cultured nematodes effectively suppress the growth of Russian knapweed
in the field? If the cultured nematodes are effective, when and how should
these cultured nematodes be applied to field populations of Russian
knapweed? Further studies should be focused on simplification of this
culture system; optimization of the culture conditions; confirmation of
virulence on field populations of Russian knapweed; field trials to
determine optimum timing, rate of application, and other parameters to
maximize the effects of *S. picridis* on Russian knapweed populations. In
addition, the adaption of this system to the silverleaf nightshade and the
coast fiddleneck nematodes would enhance the utility of nematodes as
biocontrol agents of weeds.
REFERENCES CITED


Schroeder, D. 1983. Biological control of weeds. Pages 41-78 in W. W. L. 150
Fletcher (ed.), Recent Advances in Weed Research, Commonwealth Agricultural Bureaux, Slough.


Watson, A. K. 1977. The biological control of Russian knapweed with a


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## APPENDIX A

### B5 BASAL MEDIUM (Gamborg and Wetter, 1975)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock solution</th>
<th>Amount/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>150 mg</td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>2500 mg</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>134 mg</td>
<td></td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>250 mg</td>
<td></td>
</tr>
<tr>
<td>Ferric EDTA</td>
<td>40 mg</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>20 g</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>15 g/100 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

### Micronutrients

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSO₄·H₂O</td>
<td>1000 mg/100 ml</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>300 mg/100 ml</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>200 mg/100 ml</td>
</tr>
<tr>
<td>Na₂MoO₄·2H₂O</td>
<td>25 mg/100 ml</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>2.5 mg/100 ml</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>2.5 mg/100 ml</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>75 mg/100 ml</td>
</tr>
</tbody>
</table>

### Vitamins

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>100 mg/100 ml</td>
</tr>
<tr>
<td>Thiamine.HCl</td>
<td>1000 mg/100 ml</td>
</tr>
<tr>
<td>Pyridoxine.HCl</td>
<td>100 mg/100 ml</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>10,000 mg/100 ml</td>
</tr>
</tbody>
</table>

Final pH adjusted to 5.5 with 2% NaOH or 0.2 N HCl.
APPENDIX B

Modifications to B₅ Medium

a. B₅BNG medium

Add 0.1 mg/L of BA (benzyladenine), 0.01 mg/L of NAA (α-naphthaleneacetic acid) and 1.0 mg/L of GA₃ (gibberellic acid) to B₅ basal medium.

b. B₅D medium

Add 1.0 mg/L of 2,4-D (2,4-dichlorophenoxyacetic acid) to B₅ basal medium.

c. KL medium

Add 1.0 mg/L of 2,4-D and 0.1 mg/L of kinetin to B₅ basal medium.

d. KL3 medium

Add 1.0 mg/L of 2,4-D, 0.1 mg/L of kinetin and 1.0 mg/L of GA₃ to B₅ basal medium.

e. KL3I medium

Add 1.0 mg/L of 2,4-D, 0.1 mg/L of kinetin and 10.0 mg/L of GA₃ to B₅ basal medium.

f. B₅G medium

Add 1.0 mg/L of GA₃ to B₅ basal medium.
## APPENDIX C
### MS BASAL MEDIUM

Composition of Murashige and Skoog's (1962) medium (MS) as modified by Linsmaier and Skoog (1965)

<table>
<thead>
<tr>
<th>Stock soln.</th>
<th>Constituents</th>
<th>Conc. in stock soln (gm/L)</th>
<th>Volume of stock soln. in final medium (ml/L)</th>
<th>Final conc. in medium (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NH₄NO₃</td>
<td>82.5</td>
<td>20</td>
<td>1650.0</td>
</tr>
<tr>
<td>B</td>
<td>KNO₃</td>
<td>95.0</td>
<td>20</td>
<td>1900.0</td>
</tr>
<tr>
<td>C</td>
<td>Micronutrients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H₂BO₃</td>
<td>1.24</td>
<td>5</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄</td>
<td>34.0</td>
<td></td>
<td>170.0</td>
</tr>
<tr>
<td></td>
<td>KI</td>
<td>0.166</td>
<td></td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Na₂MoO₄.2H₂O</td>
<td>0.05</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>CoCl₂.6H₂O</td>
<td>0.005</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td>D</td>
<td>CaCl₂.2H₂O</td>
<td>88.0</td>
<td>5</td>
<td>44.0</td>
</tr>
<tr>
<td>E</td>
<td>Micronutrients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MgSO₄.7H₂O</td>
<td>74.0</td>
<td>5</td>
<td>370.0</td>
</tr>
<tr>
<td></td>
<td>MnSO₄.H₂O</td>
<td>3.45</td>
<td></td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>ZnSO₄.7H₂O</td>
<td>1.72</td>
<td></td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>CuSO₄.5H₂O</td>
<td>0.005</td>
<td></td>
<td>0.025</td>
</tr>
<tr>
<td>F</td>
<td>EDTA-ferric salt</td>
<td>8.6</td>
<td>5</td>
<td>43.0</td>
</tr>
<tr>
<td>G</td>
<td>Vitamins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine.HCl</td>
<td>0.2</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>0.1</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Pyridoxine.HCl</td>
<td>0.1</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>0.4</td>
<td></td>
<td>2.0</td>
</tr>
</tbody>
</table>

**Addendum:** Sucrose 30 gm/L  
Myc-o-inositol 100 mg/L  
Agar 6 gm/L  

Final pH adjusted to 5.5 with 2% NaOH or 0.2 N HCl.
Appendix D

Modification to MS basal medium

a. MSIG medium

Add 2.0 mg/L of IBA (indole-3-butyric acid) and 1.0 mg/L of GA₃ to MS basal medium.

b. MSIBG medium

Add 2.0 mg/L of IBA, 2.0 mg/L of BAP (6-benzylaminopurine) and 1.0 mg/L of GA₃ to MS basal medium.

c. MSG medium

Add 1.0 mg/L of GA₃ to MS basal medium.