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Cnicin concentrations in spotted knapweed (Centaurea maculosa Lam.) and associated soils

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Cnicin Concentrations in Spotted Knapweed (*Centaurea maculosa* Lam.) and Associated Soils

by

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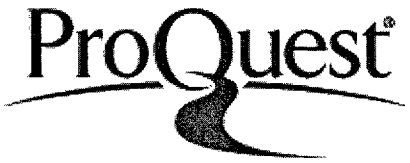

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Cnicin Concentrations in Spotted Knapweed (Centaurea maculosa Lam.) and Assosiated Soils (71 pp.)

Directors: Dr. R.G. Kelsey and Dr. R.J. Fessenden

Spotted knapweed (Centaura maculosa Lam.) was introduction to western Montana in the mid 1920s. It has spread across the state in an epidemic fashion and now infests every county in the state. Many control methods are being tried (biological, chemical, and cultural) with mixed results but more knowledge about the plant is needed to better combat this noxious weed.

Knapweed contains a phytotoxic sesquiterpene lactone, cnicin, in its glandular trichomes. This compound was quantified by high pressure liquid chromatography (HPLC) through plant tissue extracts. Cnicin concentrations for the 1983 and 1984 seasonal samples, dissected plant samples, samples from plants of different ages, and 1984 soil samples were determined. Highest concentrations were found in leaves which correlates with gland density.

In 1983, cnicin concentrations were low in the spring (0.58%) and increased to maximum levels (1.03%) in July. This quantity was maintained in the rosette leaves throughout the rest of the growing season but decreased to 0.48 percent in stems with leaves because of dry matter dilution by the stems. In 1984, rosette leaves maintained a relatively stable cnicin concentration (0.52 to 0.73%) from March to August, peaking in September (1.02%), and decreasing to 0.64 percent in November. Stem and branch leaves contained approximately 2.00 percent cnicin in the summer, increased to 2.76 percent in October, and dropped to 0.96 percent in November. Cnicin content decreased with plant age which correlated with greater quantities of stem tissue in older plants. Soils collected through the 1984 growing season contained no cnicin except for trace amounts during the summer.

Since cnicin remains intact on dead standing tissue, possibly through a combination of its limited water solubility, non volatility, and storage inside a hydrophobic cuticular sac, its allelopathic potential is diminished unless large quantities of spotted knapweed are deposited to the soil through cutting or trampling. This is not to say that knapweed does not contain a compound other than cnicin that is more directly allelopathic. Furthermore, evidence suggests that cnicin may function as a herbivore or disease deterrent. This plus its prolific seed production, competitive ability, and lack of natural enemies all contribute to knapweed's invasion of Montana.

To

Carl, Bonnie

Lisa, Lee, and Lynn

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Chapter One

The Knapweed Problem

"Weed - a plant whose virtues have not yet been discovered"

-Ralph Waldo Emerson-(1803-1882) (Peter 1977)

Spotted knapweed (Centaurea maculosa Lam.) was first collected in North America at Victoria, British Columbia in 1893. It is common thoughout Europe and western Asia and is suspected to have been introduced to the New World as a contaminant of alfalfa seed, either from Asia Minor, or with hybrid alfalfa seed from Germany (Maddox 1982). In Canada spotted knapweed is plentiful in British Columbia, and is common in the eastern provinces of Ontario, Quebec, Nova Scotia, and New Brunswick (Moore 1972, Watson and Renney 1974). It has recently moved into southern Alberta (Ali 1984). There are 1.1 million ha of rangeland in western Canada that have the potential for knapweed infestation with optimum soil and climate conditions (Harris and Cranston 1979). In the United States spotted knapweed can be found coast to coast in the northern states (Reed and Hughes 1970, Moore 1972), but has become a major weed problem in the Pacific Northwest infesting 875,000 ha in Montana, Idaho, and Washington (Maddox 1979). Most of this infestation (810,000 ha) occurs on the rangelands of western Montana (Maddox 1979).

Knapweed was first observed in western Montana in the mid 1920s and at present inhabits every county in the state (French and Lacey 1983, Chicoine 1984). Knapweed is a pioneer species that initially invaded only disturbed sites along roads and railways but then moved into low

value native rangelands. With larger population sizes providing an abundant seed source, knapweed in recent years has moved onto higher value ranges and pastures. It has even penetrated the forests of western Montana. Knapweed can be found at popular recreation sites, along trails, and in areas where forest canopy has been removed by timber harvesting to create an optimum environment for its establishment (Strang et al. 1979, Spoon et al. 1983).

Knapweed is spreading across Montana at a rate of 27.4 percent annually (Lacey 1983). There are several factors contributing to its success. One of the most important is believed to be the lack of natural herbivores and diseases that were left behind in the Old World when the seeds migrated to North America. Environmental conditions in the Pacific Northwest are quite similar to the forest steppe zone in Europe where knapweed is adapted and most aggressive (Harris and Cranston 1979). The plants were well suited for their new habitats and have proven to be strong competitors for nutrients (Belles et al. 1980) and moisture. Knapweed is also known for its prolific seed production (Watson and Renney 1974) and is readily spread by man and his vehicles (Strang et al. 1979). Allelopathy may also contribute to knapweed's ecological success since the leaves are known to contain an inhibitory substance(s) (Fletcher and Renney 1963). This will be discussed in Chapter 3.

As the knapweed density increases, the production of desirable forage decreases. Grass productivity can decline 40 to 80 percent or greater (Watson and Renney 1974, Harris and Cranston 1979, Maddox 1979). This is not to say that animals do not utilize knapweed. Sheep are

known to select knapweed over other desirable forages (Cox 1983), and goats will graze plants right along with other species. Cattle utilize actively growing plants in the spring, but their use seems to be variable and dependent on the availability of other forage and the stocking rates, among other things. Horses generally avoid knapweed (Spoon et al. 1983). Wildlife utilization is probably minimal, although little information is available.

Even though there is some livestock use of knapweed, it is not sufficient to compensate for the losses in grass. Consequently, the carrying capacities of infested sites decrease. Costs of the current infestion in Montana are estimated at $4.5 million annually (French and Lacey 1983). Montana has 13.7 million ha of grazable woodlands and rangeland vulnerable to knapweed invasion, and if completely occupied, could result in $155.7 million in lost livestock forage each year (Bucher 1984). It is also predicted that wildlife populations would be affected, decreasing the elk herd by 220 head annually by 1998 (Spoon et al. 1983), and ultimately affecting hunter success and outfitter incomes.

Property values decline when infested with knapweed. In Oregon rangeland valued at $99 to $148 per ha decreased to $10 to $15 per ha after knapweed invasion (Maddox 1979). Not only do market values decrease but knapweed depletes aesthetic quality and recreational appeal. In the forests, it is beginning to compete with shrubs and young trees for nutrients and moisture which affects seedling growth and survival to the point of slowing timber production (Spoon et al. 1983). Not only are farmers and ranchers experiencing economic losses, but the

state as a whole is also losing.

Many control methods and combinations of control methods are being tried and experimented with to help combat the spread of this noxious weed. Chemical control with Tordon 22K (picloram) has been most widely used with best results at 282 to 561 g ai/ha (Renney and Hughes 1969, Belles et al. 1980, Chicoine 1984). It has the advantages of having a flexible application time, it lasts for more than a year (with a residual control of at least two years, and usually longer), and does not affect other grasses. It also has a relatively low toxicity and is rapidly excreted from animals (Renney and Hughes 1969). Tordon does have the disadvantages of being very costly, $37/ha ($15/acre) and periodic retreatment would be necessary. Since it is extremely stable, it has the potential of being dangerous to cultivated crops and could not be used around waterways (Maddox 1979). Other herbicides such as 2,4-D, asulam, bentazon, buthidazole, dicamba, and glyphosate have been tried with varied results, but reapplication would be necessary because of their short residual activity (Renney and Hughes 1969, Belles et al. 1980, Chicoine 1984). Even though these other chemicals have a lower initial cost they are not cost efficient because of their need for frequent reapplication.

Biological control is the introduction of natural herbivores (usually insects) or diseases, to a weed in order to decrease plant density. In Montana biological control of knapweed was started by introducing the seed head fly, Urophora affinis in 1973 (Story and Anderson 1978). Urophora quadrifasciata is a close relative that was released in Canada in 1970 (Harris 1980). It has migrated to western

Montana and is now more widely distributed and better established than U. affinis (Story and Nowierski 1984). Both species lay eggs in the knapweed flower bud and the larvae feed on the head while the plant works to form a gall around the larva instead of producing seed (Story and Anderson 1978). The procedure to find the proper agent(s) for biological control is a very slow and gradual process, but after this initial cost it is very inexpensive to maintain. Two root mining moths (Agapeta zoegana and Pelochrista medullana) were released in western Montana in 1984 and their overwintering success will be studied in 1985. The larvae of these moths attack and damage the roots of the small knapweed rosettes. A three year screening project was started in 1984 on two or four more of these natural enemies (Knapweed update 1984). Before the plant is reduced below its economic threshold at least four biological agents will have to be established and this means a cost of $1.8 million and 28 scientist years. Before biological control becomes fully effective 10-20 years will pass. During this time other control methods need to be used and experimented with, along with good pasture management, since biological control may not be uniformily successful at all sites or in all years (Harris and Cranston 1979).

Grazing by sheep is a hybrid form of cultural/biological control. Dr. Cox of the University of Montana has seen effective control of knapweed by sheep on his 80 acres in the Clark Fork River bottom west of Missoula and hopes, one day, to rid his entire place of knapweed with sheep alone (Cox 1983). Heavy grazing in the spring keeps the knapweed in a state of perpetual regrowth reducing flower formation and seed production to near zero. Sheep could be useful for control on sites

where the animals can be adequately managed. Further research is needed on the open range to determine stocking levels necessary to impact the knapweed, and to quantitatively measure the responses of native vegetation.

Burning is a management tool with limited application for eliminating knapweed. In order to kill roots and residual seeds in the soil, surface temperatures would probably have to exceed 260 to 316°C. With herbaceous, weedy fuels, like knapweed, surface temperatures would only reach 204°C and only for a brief period (Spoon et al. 1983). Other disadvantages of burning include uncontrollable fire and patchiness. Unburned areas provide seed for rapid reinfestation. Reseeding after the burn would improve the results, but also add to the costs (Renney and Hughes 1969).

Mowing decreases the number of plants that produce seed and it also decreases seed viability (Watson and Renney 1974). The knapweed cuttings might also be used as livestock feed. Spotted knapweed has a protein content of 9-18 percent in the spring which decreases by about 50 percent after flowering (Kelsey, personal communication). This compares favorably to sun cured alfalfa that has 18 percent protein (Ensminger and Olentine 1978). In preliminary feeding trials, sheep, goats, cattle, and horses ate knapweed hay. Silage was prepared by sealing chopped tissue in plastic bags. After a three day introductory period cattle acquired a taste for the silage and began eating it freely on the fourth day. Sheep ate silage immediately the first day it was offered (Kelsey, personal communication). Further research to determine the effects of knapweed hay and silage on livestock health and

reproduction should be conducted before it is recommmended as a reliable winter feed.

Other cultural methods that can help to slow knapweed's invasion include good range management practices, seeding disturbed areas with quick establishing and competitive plants, limiting the transport of weed infested hay, and keeping vehicles free of weedy parts, particularily after seeds have formed. Strong public awareness and extension programs to educate the general public and keep them informed about the problem will also aid in the control of this noxious plant (French 1984, Lacey and Fay 1984).

Chapter Two

Knapweed Biology

Spotted knapweed does well on soils with a wide range of chemical and physical properties but does best on disturbed sites. It flourishes in areas where the soil pH(H_2O) is from 6.4 to 7.4 and the sulfur, carbon, organic matter, and nitrogen percentages range from 0.014-0.047, 1.34-3.64, 2.27-6.19, and 0.102-0.329, respectively. It is associated with soils having a C/N ratio of 11.5 to 16.5, a phosphorus content from 2.20 to 3.03 ppm, and a total exchange capacity (meq/100 g) from 15.34 to 28.32 (Watson and Renney 1974). Douglas fir, ponderosa pine, and the foothills prairie habitats are most susceptible to knapweed invasion (Chicoine 1984) at elevations of 30 to 1200 meters. Knapweed is not common on cultivated land, or irrigated pastures, and it prefers open habitats (Watson and Renney 1974), although it is successful at invading small canopy openings in the forest.

Spotted knapweed produces anywhere from 436 seeds per plant on natural rangelands to 25,263 seeds per plant grown on irrigated plots (Watson and Renney 1974). In northern Idaho it averaged 22,000 seeds/m^2 from 1973 to 1976 (Schirman 1981). If seed production were cut to below 0.1 percent and plant density declined, spotted knapweed would still spread to adjoining land (Roze et al. 1984). Seeds are dispersed by a flicking action of the head which can project the seeds up to a meter from the parental plant. This dispersal usually occurs 2-3 weeks after maturity of the flower (Watson and Renney 1974).

Eighty percent or more of the seeds are viable and will remain viable underground for 12.5 months (Chicoine 1984). It has been

estimated that after completely removing knapweed from an infested site, it would take 60 to 75 months, without any further seed production, before the natural seed reserves in the soils were exhausted (Chicoine 1984). Germination will occur over a temperature range of 7-34°C and soil moisture should be from 55 to 70 percent for optimum results (Watson and Renney 1974, Spears et al. 1980). Seeds can germinated in fall or spring depending on environmental conditions and seedling mortality rate is only 12 percent (Watson and Renney 1974). Canopy cover has no effect on germination. Since knapweed is uncommon in shaded areas, other factors besides low-light intensity must be affecting germination and emergence, or seedling survival after emergence (Spears et al. 1980).

In the first year, plants normally produce only basal rosette leaves and no stems. Growth and development of the root system is probably significant during this first growing season. The second year, in late May or early June, plants bolt usually producing only one stem. Rosette leaves are transformed into stem leaves as elongation takes place, and no rosette leaves remain when stem growth is completed.

Stems are erect or ascending, branched, and can reach a height of approximately 120 cm. Each branch is terminated by a single head that develops into a purple flower. Heads start to appear in June and flowering begins in mid-July, continuing into August and September. Individual flowers bloom for 2 to 6 days (Chicoine 1984) and then reclose while the seeds mature. Two or three weeks later the heads dehydrate and begin to open (Watson and Renney 1974) for seed dispersal. As the heads dry they turn a yellow color. Gradually the other aerial

tissues dehydrate and become yellow, dying back to ground level.

In the fall, as the stems dehydrate, vegetative reproduction occurs with the formation of new rosettes at the base of the stems. These remain attached to the parental root stock (Watson and Renney 1974). The following spring (May/June), some, or all of the vegetative rosettes can bolt, forming multiple stems. In the older plants not all rosettes will bolt, maintaining separate clusters of rosette leaves throughout the summer months. Addition of new vegetative rosettes at the end of each growing season results in a continually expanding root crown just below the soil surface, allowing older plant to produce over 15 stems (Watson and Renney 1974). Thus, spotted knapweed is most often a short lived perennial rather than a true biennial.

Chapter Three

Knapweed Allelopathy and Chemistry

Allelopathy is "any direct or indirect harmful effect by one plant on another through the production of chemical compounds that escape into the environment" (Rice 1974). Some compounds are autotoxic effecting both the producer of the chemical and the species in the surrounding vegetation, whereas other toxins only inhibit the growth of the surrounding plants. Fletcher and Renney (1963) found that soils naturally infested with Russian knapweed (C. repens Lam.), or artificially infested with powdered Russian knapweed tissue inhibited tomato and barley growth. Extracts from spotted knapweed, diffuse knapweed (C. diffusa Lam.), and Russian knapweed all retarded the germination and growth of lettuce and barley. Roots were most severely inhibited. Russian knapweed contained the strongest inhibitor and spotted knapweed the weakest. Of the various plant parts tested, leaves contained the greatest quantities of inhibitor. The active compounds were isolated by paper chromatography but never indentified (Fletcher and Renney 1963).

Duplicating some of these early experiments, Kelsey and Locken (submitted for publication) have isolated and identified a phytotoxin in the aerial tissues of spotted knapweed. A ten percent water extract from fresh rosette leaf tissue showed no effect on lettuce germination, but decreased root growth to 68 percent of control. Water, ether, and chloroform extracts were prepared from five grams of air-dried rosette leaves and bioassayed with lettuce. Germination was only slightly affected and seedling growth, especially in the roots, was severely

retarded by all three extracts. Levels of inhibition were similar to those reported by Fletcher and Renney (1963). An active chloroform extract was concentrated by removing hexane soluble cuticular waxes and then fractionated by column chromatography. Nearly all fractions inhibited lettuce growth, but only a few affected germination. A compound in the most inhibitory column fraction was isolated and identified as cnicin, a sesquiterpene lactone, by TLC, NMR, and direct IR comparison with an authentic sample.

Bioassays of 0.0, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg cnicin per 5 mL water were first tested on lettuce. Germination was not affected at 1.0 mg cnicin, but it gradually decreased as the cnicin concentration increased, and was only 20 to 30 percent of the control at 10.0 mg. Seedling growth was affected more severely, reaching less than 20 percent of the control at only 1.0 mg. Three grass species, rough fescue (Festuca scabrella Torr.), crested wheatgrass (Agropyron cristatum (L.) Gaertn.), and bluebunch wheatgrass (Agropyron spicatum (Pursh) Scribn. & Smith) were bioassayed with the same concentrations of cnicin. Germination was reduced to varying degrees at 4.0 and 6.0 mg. Their root growth was sensitive with significant inhibition beginning at the 4.0 mg level. Two tree species, western larch (Larix occidentalis Nutt.) and lodgepole pine (Pinus contorta Dougl. ex Loud.) were tested. Germination of the latter was not retarded. Growth of both species was inhibited at all concentrations, with significant differences from controls at 4.0 to 6.0 mg of cnicin.

Spotted knapweed was also bioassayed with no effect on germination, but significant retardation of seedling growth at all concentrations.

This indicates that unless knapweed has some mechanism for eliminating, avoiding, or minimizing cnicin's inhibitory effects under natural conditions it would provide the plants with a minimal competitive advantage over the associated vegetation. Autotoxicity could have some value as a mechanism for self regulating population density. It was concluded that cnicin could be allelopathic under the appropriate environmental and biotic conditions, possibly adding to the ecological success of spotted knapweed (Kelsey and Locken, submitted for publication).

Muir and Majak (1983) recently completed a similar study with diffuse knapweed. Aerial tissues contained both a polar and nonpolar inhibitor and root extracts were inhibitory. Ryegrass (Lolium multiflorum L.) bioassays, and column chromatography of a solvent extract from combined leaves and stems led to the isolation of cnicin. A column fraction, and impure cnicin isolated from it, both inhibited ryegrass seedling growth at 0.4 mg/mL, but purified cnicin, at the same concentration, did not affect germination or growth, and was rejected as a major inhibitor by itself. The polar inhibitor was not identified.

Politis (1946 a,b) reported the isolation of bitter tasting cnicin from the glandular trichomes of holy thistle (Cnicus benedictus L.). It was again isolated from this taxa over a decade later (Korte and Bechmann 1958). Two alternative structures were assigned the following year (Suchy et al. 1959). This was reduced to a single probable structure in 1962 (Suchy and Herout 1962, Suchy et al. 1962), that was not correct, and required revision three years later (Suchy et al. 1965). The final, currently accepted structure was published in 1969

(Figure 1) (Samek et al. 1969).

Cavallito and Bailey (1949) in the United States were probably the first to isolate crystalline cnicin from Centaurea maculosa, but did not provide a structure. They did conduct extensive chemical analysis and the data are sufficient to confirm that it was cnicin. Suchy and Herout (1962) isolated it from European C. stoebe (L.) Sch. et Thell, a comprehensive species that included C. maculosa. It has been reported in numerous other species in this genus, C. aspera L., C. bruguerianа DC., C. calcitrapa L., C. eriophora L., C. iberica Trev., C. micranthus I.F. Gmel., C. ovina Pal., C. rocheliana (Heuffel) Dostal, C. sphaerocephala L., and C. sulphurea Willd. (Gonzalez et al. 1978, Rustaiyan et al. 1982, Seaman 1982, Geppert et al. 1983).

In addition to having phytotoxic properties cnicin also disrupts metabolic activities in other biological systems. Cavallito and Bailey (1949) tested it with numerous bacteria and found it to be bacteriostatic toward both gram positive and gram negative organisms. Bactericidal activity was observed at higher concentrations. Similar experiments were repeated recently with comparable results (Vanhaelen-Fastre 1972, Vanhaelen-Fastre and Vanhaelen 1976). Cnicin is cytotoxic toward human carcinoma cells in vitro with an ED_{50} of 3.4 µg/mL for KB cells (Vanhalen-Fastre and Vanhaelen 1976) and an ID_{50} of 0.1 µg/mL for HeLa cells (Gonzalez et al. 1978). In vivo it is active against L-1210 leukemia (Vanhaelen-Fastre and Vanhaelen 1976). These types of activity are not unusual for sesquiterpene lactones (Rodriguez et al. 1976).

Sesquiterpene lactones can cause various responses from plant

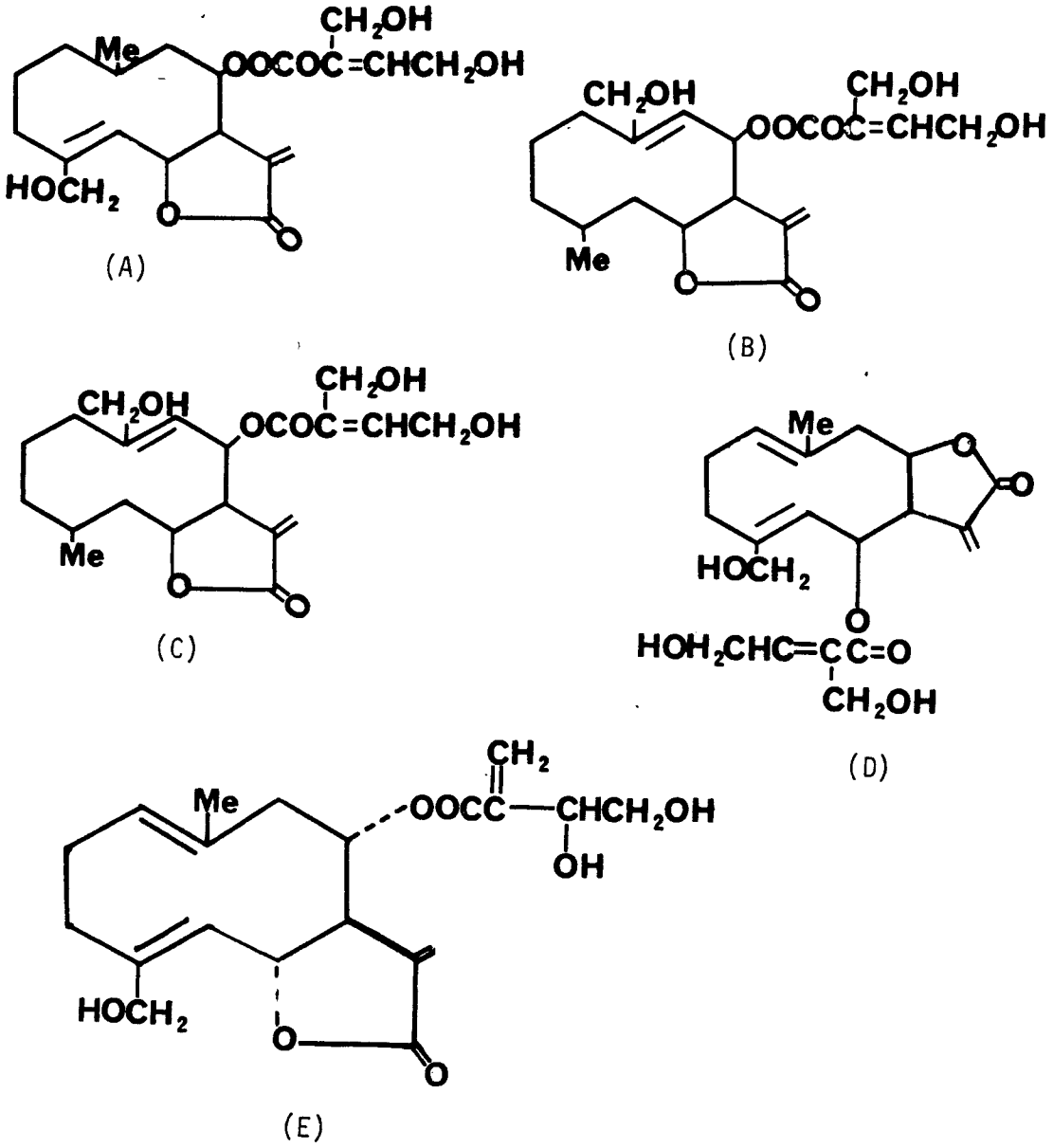


FIGURE 1. Proposed cnicin structures. (A)&(B) Two alternative structures proposed by Suchy et al. in 1959. (C) Single probable structure of cnicin (Suchy and Herout 1962, Suchy et al. 1962). (D) Revised structure as proposed by Suchy et al. (1965). (E) Currently accepted structure of cnicin (Samek et al. 1969).

systems. Root initiation from the hypocotyls of mungbean (Phaseolus aureus Roxb.) is stimulated by various compounds with an α,β-unsaturated lactone (Kalsi et al. 1977, Kalsi 1979). The isomeric form of the exomethylene double bond had a strong influence on the root growth (Kalsi et al. 1981). Amo and Anaya (1978) bioassayed seven sesquiterpene lactones with five native plants species from the ruderal vegetation of a tropical zone in Mexico. Germination and growth was stimulated, or inhibited depending on the lactone, its concentration, and the plant species being tested. Several other studies have reported sesquiterpene lactone inhibition of germination and/or growth of the bioassay species (Dalvi et al. 1971, McCahon et al. 1973, Asakawa and Takemoto 1979, Spencer et al. 1984).

Parthenin is a phytotoxic sesquiterpene lactone that contributes to the allelopathic and autotoxic effects of Parthenium hysterophorus, an aggressive tropical weed. This plant has spread to all parts of India over a period of 20 years, infesting five million hectares. Pure stands are often several hectares in size and nearly devoid of any other vegetation. Phytotoxins are released to the soil through leaching, decomposition of plant tissue, and root exudation (Kanchan and Jayachandra 1979 a,b). The inhibitors remained active in the soil for about 30 days. Dried leaves mixed into the soil inhibited growth and yield of beans (Phaseolus vulgaris L.), cowpea (Vigna sinensis L.), tomatoes (Lycopersicum esculentum L.), and ragi (Eleusine coracana Gaertn.). Surprisingly, leaf mixed soil stimulated growth and productivity of bajra (Pennisetum typhoideum Rich).

Chemical analysis has revealed high concentrations of parthenin in

the leaves (0.30% dry weight), inflorescence (0.30%), and cypsela (0.15%) with lower concentrations in the stems (0.02%) and roots (0.01%) (Kanchan 1975, Kanchan and Jayachandra 1980). Phenolic acids were also abundant in these tissues. Trichomes from the epidermal surface contained parthenin and vanillic acid. Parthenin, caffeic acid, ferulic acid, and vanillic acid were present in leaf washings. Root exudates and rhizosphere soils contained parthenin, anisic acid, vanillic acid, ferulic acid and fumaric acid. All of these compounds are phytotoxic except fumaric acid and possibly anisic acid (Kanchan 1975, Kanchan and Jayachandra 1980). Parthenin at 50 ppm on filter paper inhibited radicle and hypocotyl growth of beans (Garciduenas et al. 1972). In Hoagland's solution with 100 or 200 ppm parthenin, young bean plants grew very little compared to the control. When 0.1 ml of 100 or 200 ppm solutions of this compound was applied to bean cotyledon leaves, trifoliate leaf growth was arrested. Parthenin and coronopilin, also a sesqiterpene lactone in P. hysterophorus, in solution were toxic to P. hysterophorus seedlings and plants. These sesquiterpene lactones in combination with the phenolic acids inhibit the germination of P. hysterophorus achenes. From this evidence Picman and Picman (1984a) concluded that these compounds are not only allelopathic, but also autotoxic, and may help regulate the timing of germination and population density.

Fuerst and Putnam (1983) recently proposed a set of criteria that should be address in order to prove competitive or allelopathic interference between plants. For allelopathy these criteria were (1) identification of the symptoms of interference; (2) isolation, assay,

characterization, and synthesis of the toxin; (3) simulation of the interference by supplying the toxin as it was supplied in nature; and (4) quantification of the release, movement, and uptake of the toxin. It is desired but not essential "to show that the selectivity of the toxin to various species corresponds to the range of species affected by the allelopathic agent".

Because of knapweeds ecological characteristics suggesting that allelopathy may be functioning, and cnicin's phytotoxic properties toward native grasses and trees (Kelsey and Locken, submitted for publication), this study was initiated to determine the concentration of cnicin in knapweed tissues and surrounding soils. This information could then be used to evaluate the role that cnicin plays in knapweed's ecology and successful invasion of western Montana rangelands. It might also find use for improving, or developing new methods of control for these unwanted plants.

Chapter Four

Materials and Methods

A. Study Site

A study site was selected in the Rattlesnake Creek drainage (T13N, R19W, sec 11) about 3 miles north of the University of Montana campus. The area is a large field (roughly 325 by 650 m) with nearly no slope, at an elevation of 1067 m. Soil is a Typic Haploboroll formed on an alluvial terrace and climatic conditions are about the same as the Missoula valley with mean annual temperature of 6.2°C and 32.6 cm precipitation (Cordell 1971). Spotted knapweed was the dominant plant species intermixed with four grasses, cheatgrass (Bromus tectorum L.), Japanese brome (B. japonicus Thumb.), Canadian bluegrass (Poa compressa L.), and Poa bulbosa L. There were a few scattered herbs; one of the most obvious in late June being cinquefoil (Potentilla glandulosa Lindl.).

A local rancher who leases the property from the Montana Power Company thought the field had been seeded to grain in the 50s or early 60s. His personal knowledge dated back to 1964 and knapweed was already quite dense. Residual grasses were heavily grazed by horses for three years, from 1969 through 1971. In 1978 the field was plowed for reseeding, but never seeded. There were no further disturbances between 1978 and 1984.

B. 1983 Seasonal (Monthly) Samples

In 1983 monthly composite plant samples (from several randomly

selected plants) were collected from April to October. Only plants with a previous year's stem attached were sampled in an attempt to minimize any age variation between plants bolting for the first time, and the older ones that had bolted before. Plants were removed from the soil with approximately 15 cm of root attached. Dead stems and leaves were discarded and the roots were separated from the aerial tissue in April, May, and June. Starting in July three tissue types were collected; stems with leaves attached, rosette leaves, and roots. Plants had begun to bolt at the May sampling date. In September, the heads, portions of the branches, and the larger leaves at the base of the stem were yellow and dehydrated. Older rosette leaves were also dead. By October stems were in the final stages of drying and there was new rosette leaf growth among the dead leaves attached to the root crown. Dead leaf samples were collected in these last two months. After air drying, the plant tissues were ground with a Wiley mill to pass a 20 mesh screen, sealed in double plastic bags, and stored in the dark.

C. Dissected Plant Samples

On August 16, 1983 two complete plants were collected and dissected into the following eleven distinct tissue types: live stems, live leaves on stems, dead leaves on live stems (leaves on the lower portion of the stem), flower heads, live rosette leaves, dead rosette leaves, branches, branch leaves, dead stems (one year old from previous growing season), dead leaves (one year old) on dead stems, and roots. These tissues were further treated as above.

D. Samples From Plants of Different Ages

On August 16, 1983 individual plants from three different age groups (less than one year old, two years old, and older than two years) where clipped. These plants were sampled at a study site (T13N, R19W, sec 31) near Fort Missoula where the relative ages of the plants were known. In October of 1981, a small area had been scraped free of vegetation and then rototilled. On this treated area in 1983, most plants with rosette leaves only, should have been less than one year old. Those that produced a single stem were in their second growing season. Adjacent to the treated area, plants older than two years were collected. They were identified by having old dead stems from the previous year, plus multiple stems from the present year. These were air-dried, sealed in plastic bags, and stored in the dark. The entire aerial portion of each plant was ground, excluding the dead stems from the ones older than two years.

E. 1984 Seasonal (Monthly) Samples

In 1984 three individual plants with a previous years stem attached were collected each month from March 1984 until March 1985. These were treated as in 1983, but only leave tissue (stem leaves including those from the branches, rosette leaves, and dead rosette leaves) was analyzed. Because of limited tissue on individual plants composite samples (from several plants) were gathered in March 1984, and January 1985, with no collections in December 1984 or February 1985. Plants were beginning to bolt on the May sampling date, but most stems were still small and considered rosette leaf tissue. In July rosette leaves

were combined with stem leaves from the same plant. During July and August there was little precipitation, and one of the plants sampled in August had a yellow dehydrated stem. New rosette growth was beginning to appear at the base of the dehydrated stems in September.

F. 1984 Soil Samples

Soil samples where also collected monthly simultaneously with the knapweed tissue from March 1984 to March 1985. On each collection day five random soil samples were collected at depths of 0-2 cm, 2-6 cm, and 6-15 cm. These were sealed in plastic containers, placed on ice, and returned to the lab were they were sifted fresh through a number 10 (2 mm) and a number 20 (20 mesh) sieve to remove plant tissues that might contain cnicin. Sieving also made the soil uniform and easy to work with. They were air-dried, placed in plastic bags, and stored in the dark.

G. HPLC Analysis

Prior to analysis the tissue was oven dried at 40°C for 24 hours and a one gram sample of each was extracted for 30 minutes with methanol (15 mL) and constant stirring. This lower oven temperature was used to decrease the possibility of cnicin structural rearrangements. Two milliliters of the extract was filtered through a millipore filter (0.5 µ) before injection (2 µL) into the Perkin Elmer Series 3 high pressure liquid chromatograph (HPLC) equipped with a reverse phase 12.5 cm Alltech Lichrosorb column (5µ particle size). A solvent gradient of 10%-25% acetonitrile in water the first 20 minutes, 25% the next three

minutes, 25%-40% for three more minutes, and 40% for the last three minutes was used with a flow of one milliliter/minute (Marchand et al. 1983). Cnicin was quantified with an ultraviolet detector set at 215 nm and recorded on a Hewlett Packard 3380A integrator/recorder. A standard cnicin curve (Figure 2 was prepared at the start of the analysis and a reference standard was run daily before the extracts. Each plant sample was analyzed in duplicate and if these were not within three percent of one another a third one was run.

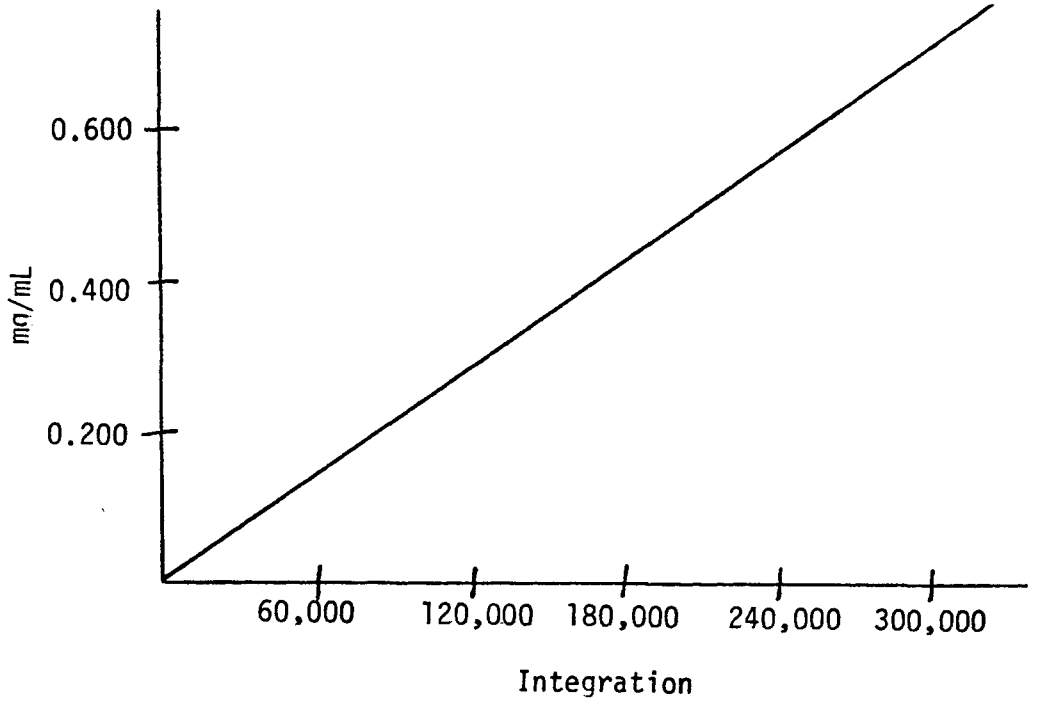


FIGURE 2. Standard cnicin curve

H. Quantitative Thin-Layer Chromatography

Quantitative thin-layer chromatography (TLC) was used to examine

the chemical components in each 1983 plant sample. One gram of tissue was extracted for thirty minutes with constant stirring in fifteen milliliters of methanol. When tissue was limited, smaller quantities were extracted with the same ratio of solvent. After setting aside for a few minutes to allow the particles to settle the solvent was decanted into a vial and sealed. These extracts were spotted quantitatively (50 µL) on a silica gel G plate with a 0.5 mg/mL cnicin standard (50 µL) and developed in a 5:4:1 (chloroform:petroleum ether:ethanol) solvent system. Soils collected in 1984 were also analyzed by quantitative TLC. Twenty grams of soil was extracted with methanol, filtered, and roto-evaporated to dryness at room temperature. This was resuspended in methanol (0.5 mL), quantitatively (50 µL) spotted on two silica gel G TLC plates. One was developed in a 5:4:1 (chloroform:petroleum ether:ethanol) and the other in a 2:2:1 (chloroform:petroleum ether:ethyl acetate) solvent system. Each plate was photographed under ultraviolet light to visualize fluorescent compounds. It was then sprayed with concentrated H_2SO_4, charred at 100°C overnight, and rephotographed.

I. Observations of Glandular Trichomes

Glandular trichomes were examined on the epidermal surface of plant tissues collected in July 1985 using a 45X dissecting microscope.

J. Qualitative TLC of Glandular Trichomes and Gland Bearing Tissues

Glandular trichomes were collected from epidermal stem tissue that had been oven dried overnight at 65°C. Stem glands were more accessible

than the leaf glands in pits. They were sucked into a capillary tube with vacuum supplied from an aspirator. Glands were trapped on a cotton plug in a larger piece of glass tubing (2-4 cm section of Pasteur pipet) attached on the end of the capillary with a small rubber stopper. The larger pipet was connected to the aspirator with rubber tubing. Capillary and pipet trap with glands were extracted in approximately 10 mL chloroform for five minutes, then removed from the solvent and rinsed with 3 mL of methanol. The combined solvents were evaporated under vacuum at room temperature. The entire extract was applied to a TLC plate with a few drops of chloroform. Fresh samples of leaves, stems, branches, phyllaries, and flowers were prepared in triplicate. Replicates of each tissue was taken from separate plants. These were extracted with 20 mL of chloroform for five minutes (except the leaves which were extracted only one minute), filtered through paper, and the solvent removed under vacuum at room temperature. The entire extract (except for leaves that required only a portion of the extract) was applied to a TLC plate, along with a cnicin reference. Plates were developed in the 5:4:1 solvent system and the cnicin visualized by acid spray and charring as described previously.

K. Statistical Analysis

Analysis of variance was used to test for differences in means at the 0.05 level of probability. If the analysis of variance tests showed a significant effect, means were separated using Duncan's new multiple range test (Steel and Torrie 1960). All statistical analyses were performed using SPSSX (SPSSX 1983). Monthly comparisons of the

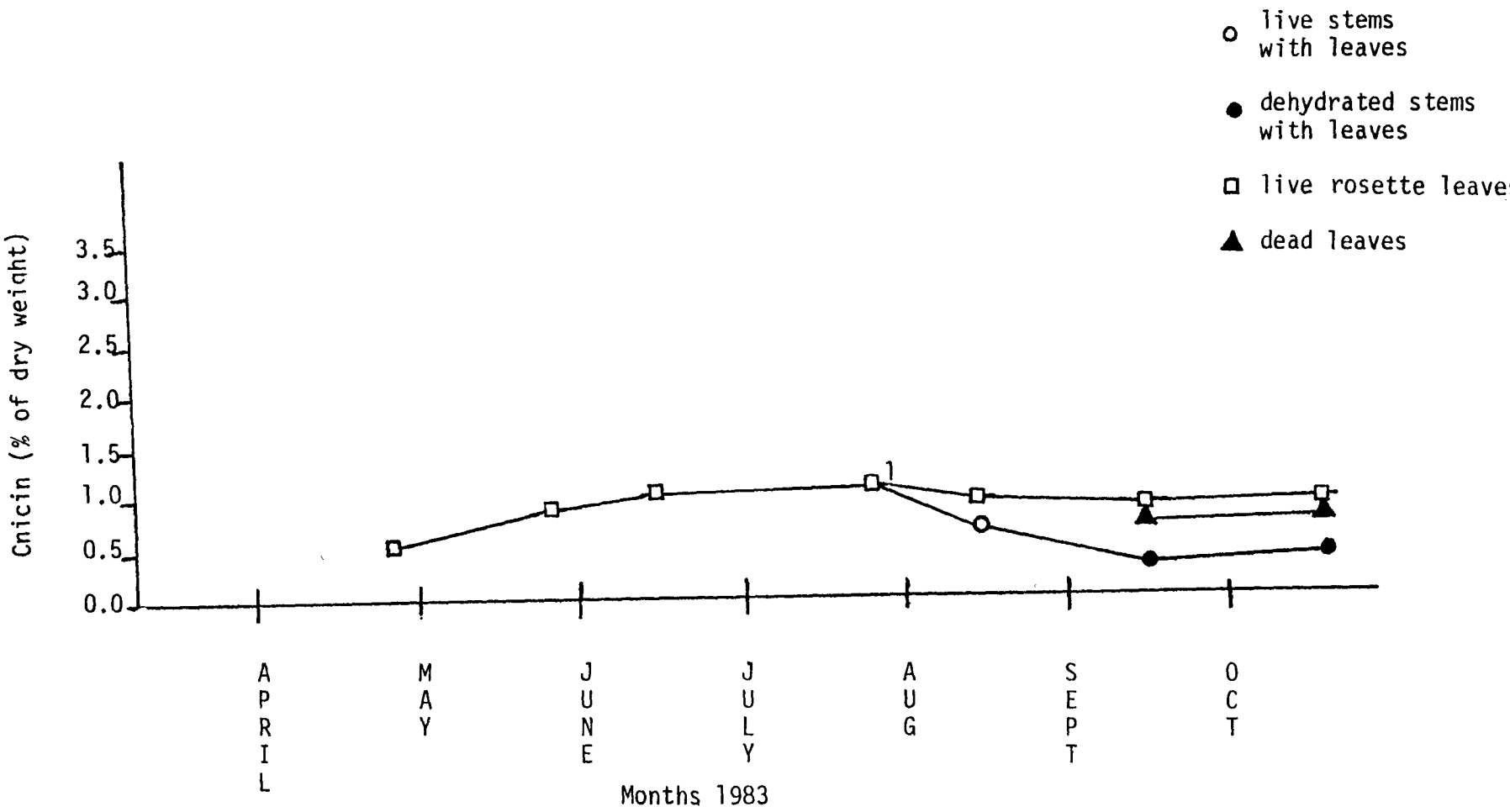
1984-1985 data were made for each individual tissue type over a season and the three tissue types were compared for specific months. The results are summarized in the Appendix.

Chapter Five

Results

A. Cnicin Concentrations on a Seasonal Basis in 1983

Composite plant samples were collected monthly from April to October 1983 and cnicin concentrations were analyzed by HPLC. The tissue was separated into distinct parts (stems with leaves attached, rosette leaves, dead rosette leaves, and roots) when possible. Figure 3 shows the percent cnicin through the growing season by plant parts. The cnicin concentration in rosette leaves increased from 0.58 percent in April to 1.01 percent in June and then stayed fairly constant until October. In stems with leaves, cnicin decreased from a high of 1.05 percent in July to a low of 0.38 percent in September with a slight increase in October. Dead leaves contained 0.80 percent cnicin in September and October; 0.1 to 0.2 percent lower than in the live rosette leaves from the same date. Roots contained no detectable cnicin by HPLC. The detection limit for the HPLC was calculated as 0.85 ppm for a 2 uL injection. Quantitative thin layer chromatography of extracts from these tissues confirmed the HPLC results. Size and intensity of the cnicin spot correlated closely with the measured concentration. When cnicin was undetectable in a sample by HPLC, either no spot, or a faint spot appeared on the TLC plate at the appropriate R_f position for cnicin.

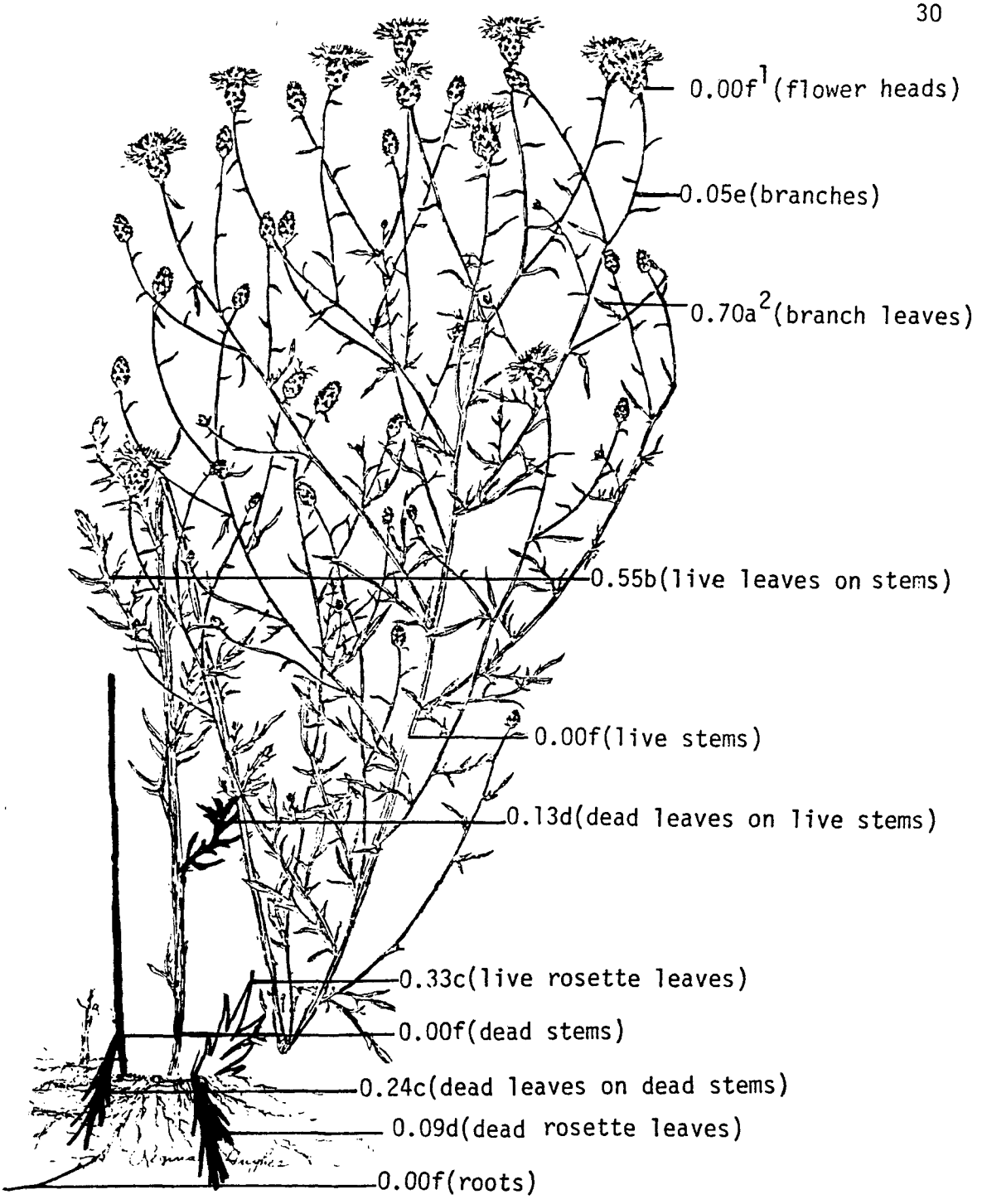


[1]Data point for both stems with leaves and rosette leaves.

FIGURE 3. Cnicin concentration of composite knapweed samples on a seasonal basis for 1983.

B. Cnicin Concentrations in Different Plant Tissues

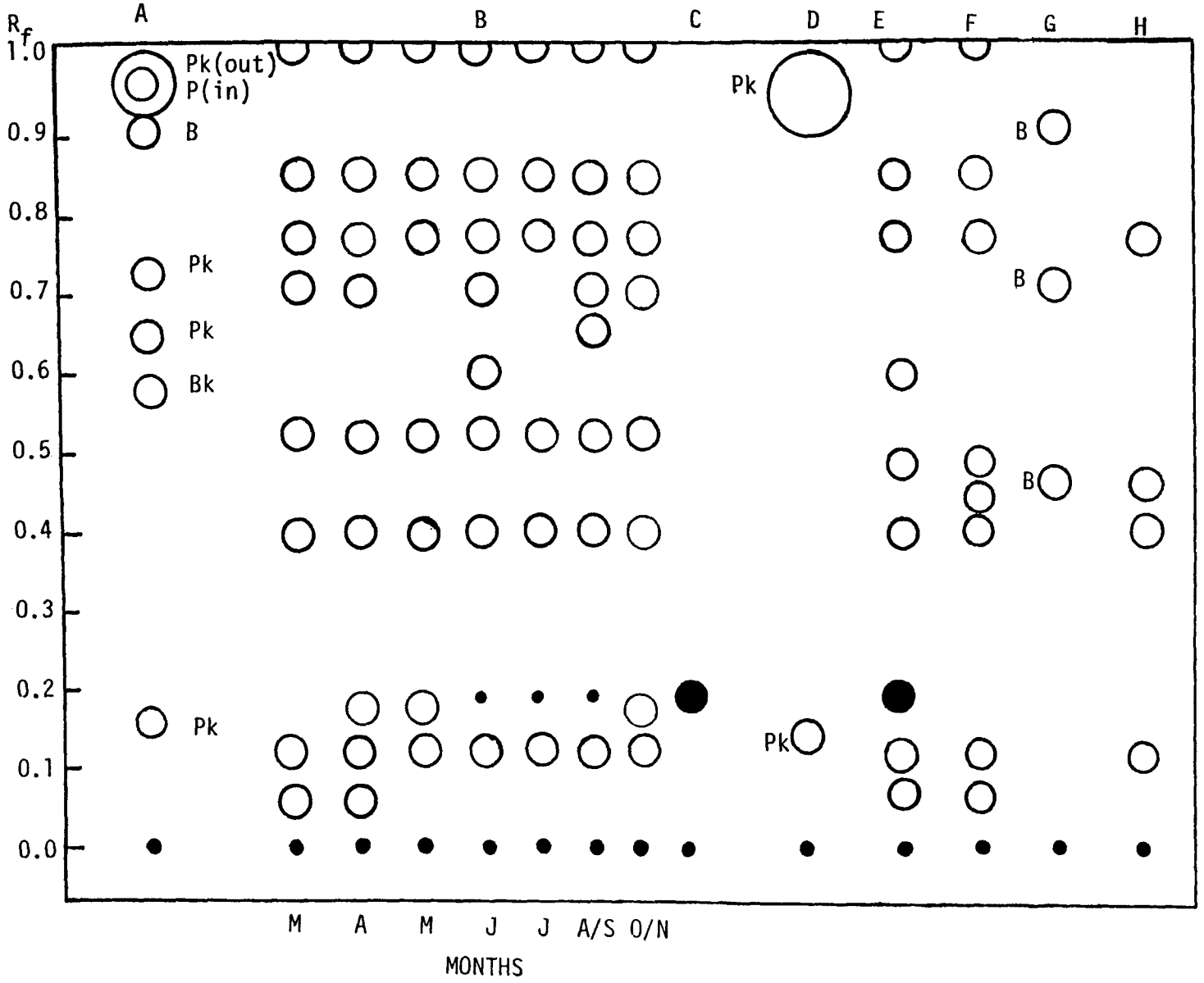
Two plants collected in August 1983 were dissected into eleven distinct tissues and each analyzed for their cnicin concentrations (Figure 4). Branch leaves contained the highest quantities (0.70%) whereas live stems, dead stems (from the previous year), flower heads, and roots had none (see Section G of Results). In live leaf tissue, the cnicin content increased moving up the plant; rosette leaves at ground level contained (0.33%), stem leaves (0.55%), and the branch leaves (0.70%). All dead leaves, including those in rosettes, those on live stems and even the ones on stems from the previous growing season contained detectable quantities of cnicin. Again, the TLC analysis confirmed the HPLC data. There was no cnicin spot from the flower heads or roots. There was a trace amount of cnicin detected in live stems, but apparently it was lower than the detection limit for the sensitivity setting used on the HPLC. Compounds other than cnicin were detected by TLC. In plant tissues containing cnicin (branch leaves, live leaves on live stems, rosette leaves, dead rosette leaves, dead leaves on live stems, dead leaves on dead stems, and branches) there were nine distinct spots (Figure 5). Dead and live stem tissues had the same spots as the tissues above, but less concentrated; the cnicin (R_f 0.20) was just barely visible in the latter. In the roots, six spots were missing (R_f 0.06, 0.20, 0.50, 0.60, 0.85, 1.00) and one new spot (R_f 0.45) appeared. Heads, had no cnicin and one additional large spot at R_f 0.43.



[1]0.00 is equivalent to undetectable by the HPLC

[2]Means followed by different letters are significantly different at a 0.05 level of probability.

FIGURE 4. Cnicin concentration of different plant tissues. Each value is an average from two plants. (Diagram reproduced from Selected Weeds of the United States 1970).


R_f
A
B
C
D
E
F
G
H
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Pk(out)
P(in)
B
Pk
Pk
Bk
Pk
Pk
B
B
B
M
A
M
J
J
A/S
O/N
MONTHS

C. Cnicin Concentrations in Plants of Different Ages

Knapweed plants from different age groups were collected in August of 1983 and their cnicin content determined by HPLC. The highest percentage of cnicin (0.40%) was found in the plants less than one year old, or those that had only rosette leaves and no stems (Table 1). Two year old plants, with a single stem, had the next highest quantity (0.12%) and the lowest concentrations (0.07%) occurred in plants over two years old with multiple live stems. Whole plants, including leaves, stems, and heads, were ground and extracted. Since stem tissue contained no detectable cnicin, increasing the proportion of stem in the sample would dilute the cnicin in the leaves. Consequently, the older plants having more stem tissue, would have a lower cnicin concentration.

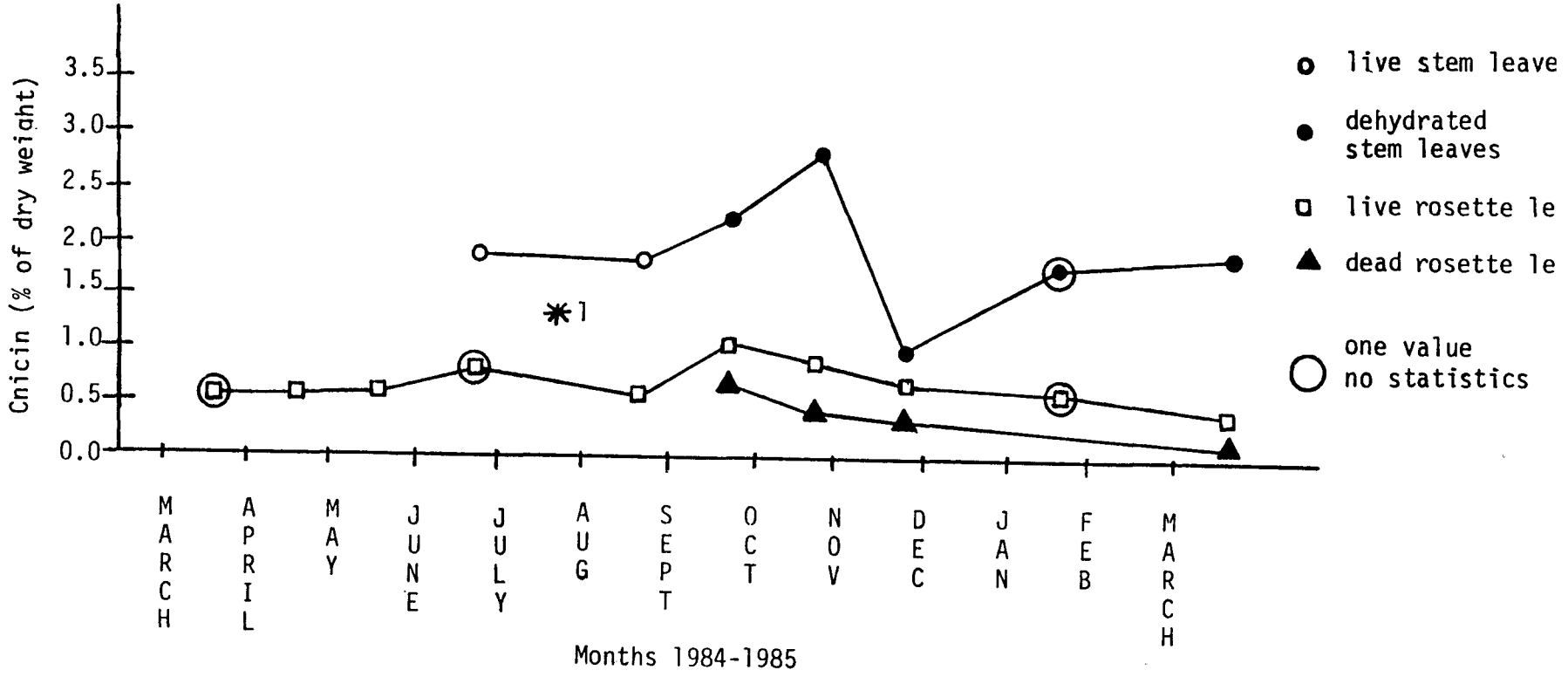
TABLE 1. Cnicin concentrations in plants of different ages.

Age of Plant	Plant no.	% Cnicin on a Dry Weight Basis
Less Than One Year Old	1	0.36
	2	0.43
	3	0.41
	$\bar{x}$	0.40a[1]
Two Years Old	1	0.20
	2	0.06
	3	0.11
	$\bar{x}$	0.12b
Older Than Two Years	1	0.11
	2	0.07
	3	0.03
	$\bar{x}$	0.07c

[1]Means followed by different letters are significantly different at the 0.05 level of probability.

D. Cnicin Concentrations on a Seasonal Basis in 1984

Three individual plants were collected each month from March 1984 to March 1985 with no samples gathered in December or February. Based on the results from 1983, only leaf tissues (stem leaves including those from the branches, rosette leaves, and dead rosette leaves) were analyzed by HPLC. Figure 6 shows the mean concentration of cnicin on a dry weight basis over the season. Stem leaves had the highest cnicin content ranging form 0.96 percent to 2.76 percent. In the spring, prior to bolting, some of these leaves were part of a basal rosette with a cnicin concentration around 0.5 percent. In May-June when the stems elongated they were transformed into stem leaves widely spaced along the lower stem. New stem and branch leaves were formed as the stems grew. In June the cnicin concentration in stem leaves (1.85%) was more than double the quantity in rosette leaves (0.73%). Whether or not there was an actual increase in the leaves that had been part of the spring rosette, was not determined. Cnicin levels remained stable in stem leaves through the summer but then increased as the tissue began to dehydrate and die. The concentration reached a peak in October at 2.76 percent. This was followed by a large unexplained decrease in November to a low of 0.96 percent. By winter the concentration had risen again to the level of the previous summer (1.70%), and it was still near this level in late March 1985. There were only two months with significantly different cnicin concentrations, November (0.96%) and October (2.76%). The greatest interplant variation was observed in these leaves (Table 2), with a two percent difference between the maximum (3.92%) and minimum (1.83%) concentrations in October.



[1]The July sample (marked by an asterisk) was a combination of the stem and rosette leaves.

FIGURE 6. Mean cnicin concentration in the leaf tissue of spotted knapweed on a seasonal basis for 1984-1985 (each date is an average from three plants except those that are circled).

Live rosette leaves had a lower, but more stable, cnicin content (varying from 0.51 to 1.02 percent) throughout the 1984 growing season (Figure 6), and there was also less interplant variation (Table 2). The high occurred in September about the time that new rosette leaves began to appear at the base of the dehydrated stems. This then decreased to near summer levels as winter approached. September's 1.02 percent was significantly greater than the spring and summer levels (see Appendix). Dead rosette leaves also contained cnicin, but at much lower concentrations than in the live rosette leaves sampled on the same day. In September there was 0.60 percent in the dead leaves compared to 1.02 percent in live leaves. By October dead leaves contained only 0.34 percent cnicin and it was nearly gone from this tissue by the following March (0.05%).

When comparing the 1983 and 1984-1985 seasonal data no direct correlation can be made between the stem leave tissues because the 1983 samples contained stem tissue and the 1984-1985 samples did not. Generally, the rosette leaves, which can be compared, had a lower cnicin concentration in 1984 than in 1983. This could possibly be explanined by the difference in the amount of rainfall for the two years as shown in Figure 7 with 1984 being drier than 1983 from June through September. When soil moisture is limited, plant growth is usually inhibited before photosynthesis and this can result in a build up of carbohydrates in the tissue (Trlica 1977, Trlica and Singh 1979). Since cnicin concentrations were calculated on a dry weight basis, a higher carbohydrate level in the 1984 plants would increase the dry matter content causing a decrease in the percentage of cnicin.

Table 2. Interplant variation in leaf cnicin concentrations during 1984.

Leaf type	Plant no.	1984 Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	1985 Jan.	Mar.
Stem	1	--	--	--	1.62	1.29[1]	2.59	2.47	3.92	0.88	1.70	1.53
	2	--	--	--	--	1.27	1.24	2.43	1.83	1.26	--	1.61
	3	--	--	--	2.07	1.40	1.45	1.57	2.54	0.75	--	2.34
	$\bar{x}$	--	--	--	1.85	1.32	1.76	2.16	2.76	0.96	1.70	1.83
Live Rosette	1	0.52	0.49	0.56	--[2]	--	0.35	1.19	0.93	0.88	0.53	0.56
	2	--	0.36	0.47	0.73	--	0.69	1.06	0.91	0.46	--	0.20
	3	--	0.69	0.64	--	--	--	0.82	0.68	0.57	--	0.30
	$\bar{x}$	0.52	0.51	0.56	0.73	--	0.52	1.02	0.84	0.64	0.53	0.35
Dead Rosette	1	--	--	--	--	--	--	1.21	0.47	0.25	--	0.15
	2	--	--	--	--	--	--	0.43	0.27	0.26	--	0.00[3]
	3	--	--	--	--	--	--	0.16	0.29	0.29	--	0.00
	$\bar{x}$	--	--	--	--	--	--	0.60	0.34	0.27	--	0.05

[1] Stem and live rosette leaf tissue was not differentiated here.

[2] This plant did not bolt.

[3] 0.00 is equivalent to undetectable by HPLC.

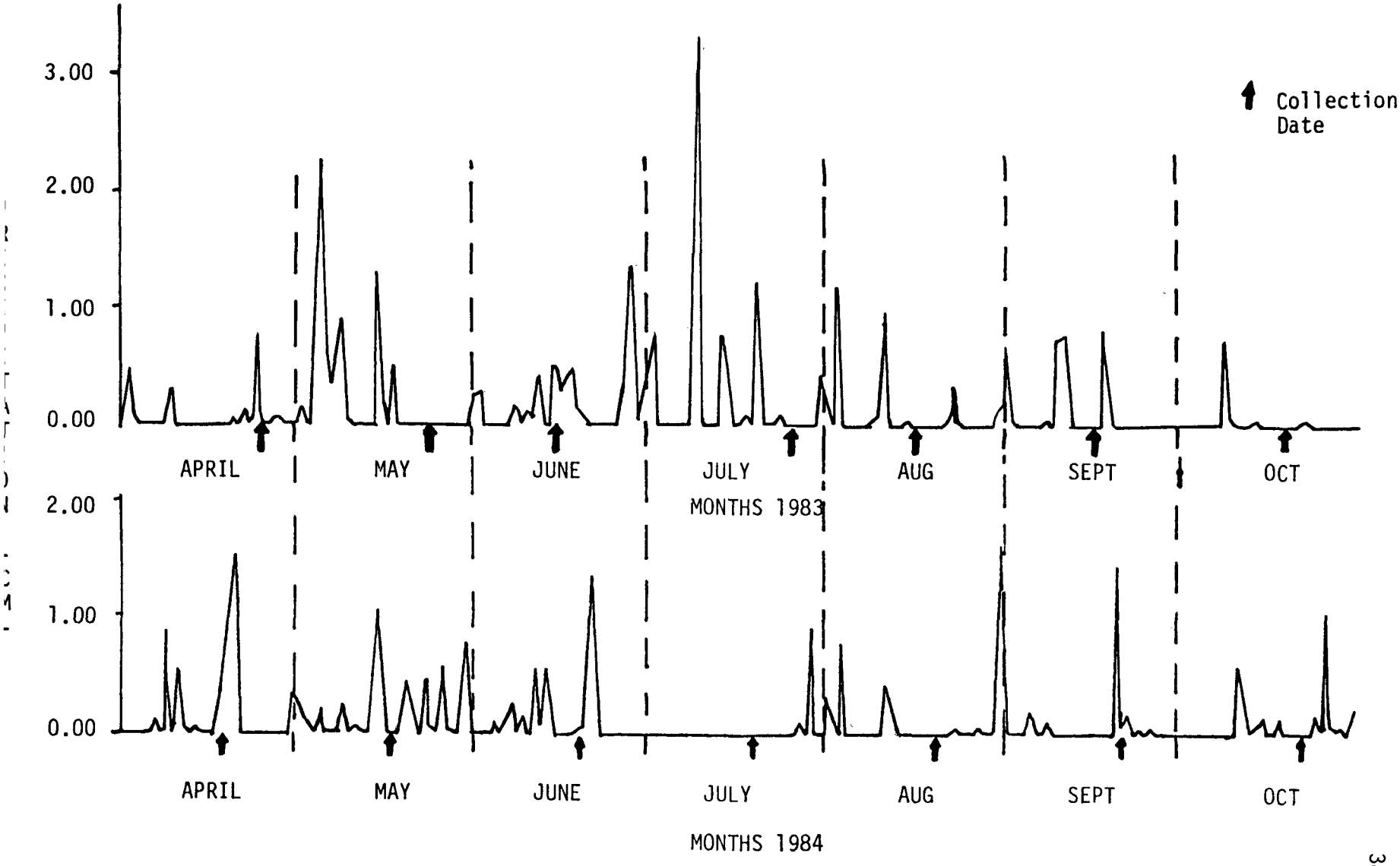


FIGURE 7 Daily precipitation at the Missoula county airport from April 1 to

E. Cnicin Concentrations in the Soils

Since there was a good correlation between HPLC and TLC data for the 1983 seasonal tissue samples, the soils collected monthly in 1984-85 were examined by TLC prior to HPLC to see if there was any cnicin present. Figures 8 and 5 summarize the soil's TLC analysis in the 2:2:1 solvent system and the 5:4:1 solvent system, respectively. Samples collected from June to September (0-2 cm depth) were the only soils showing even a trace of cnicin by TLC, with June and August having what appeared to be the greatest quantities. Subsequently, the 0-2 cm samples for June and August were analyzed by HPLC. The August soil sample had the highest concentration of cnicin at 0.7 ppm. Since this was so low, and all the other soils appeared to have an equal or lower concentration by TLC, no further HPLC was considered necessary. Cnicin was not present in significant quantities, at any time of the year, at this study site.

Soil TLC plates developed in the 2:2:1 solvent system and viewed under UV light were the same throughout the entire season with the exception of one spot at R_f 0.34 that appeared in July (Figure 8). The lower spots between R_f 0.00 and 0.30 were hard to distinguish from one another because they were so close together. There was a fluorescent blue spot under ultraviolet light (R_f 0.64) that charred with acid and heating. This charred spot was present through May but then disappeared. There were not many changes through the season in the compounds visualized by charring. The top spot at R_f 0.93 was not present from March to May, but appeared in June and remained the rest of the year. Another new spot appeared in June at R_f 0.53, this spot's

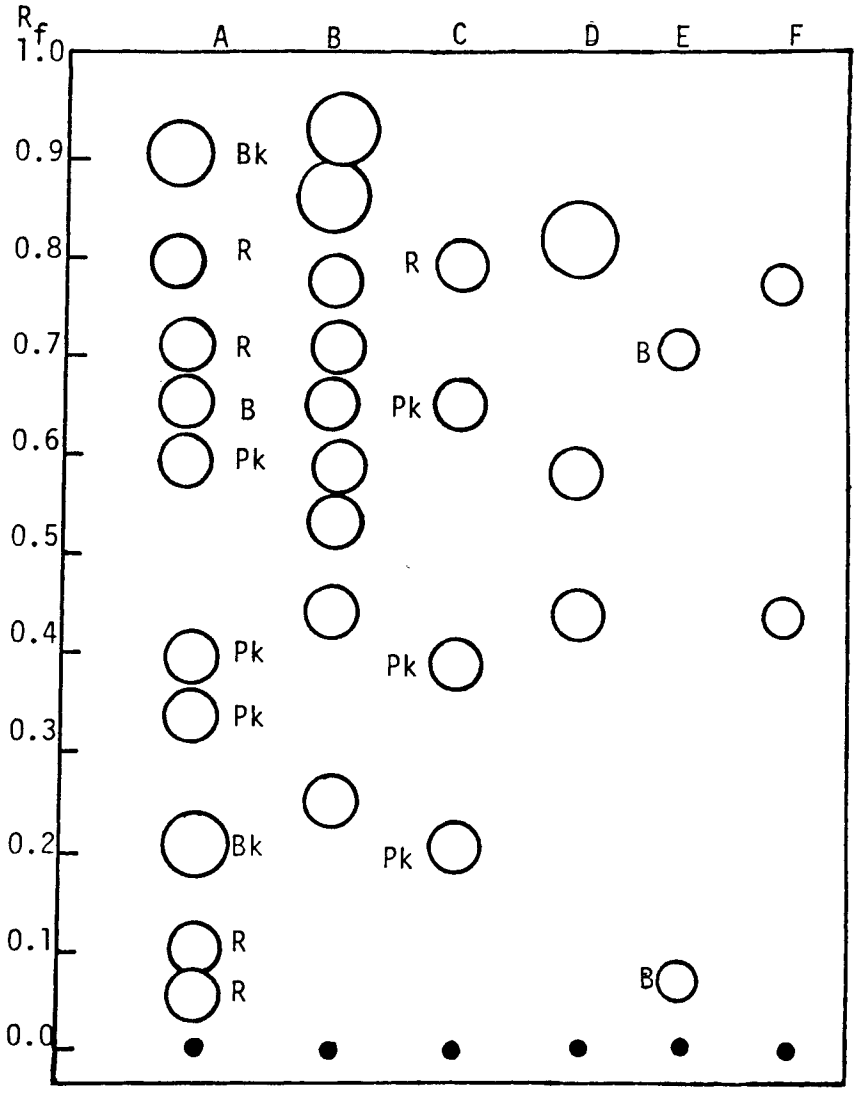


FIGURE 8. TLC patterns of the 1984 soil samples from March to November developed in a 2:2:1 (chloroform:petroleum ether:ethyl acetate) solvent system. (A) Under ultraviolet light. (B) acid sprayed and charred. (C) Rosette leave tissue standard under ultraviolet light. (D) Rosette leave tissue standard acid sprayed and charred. (E) Root standard under ultraviolet light. (F) Root standard acid sprayed and charred. See Figure 5 for UV spot color code.

intensity increased as the depth of the soil increased, whereas the intensities of all other spots either stayed the same or decreased as soil depth increased. Cnicin does not move from the application spot in the 2:2:1 solvent system.

In the 5:4:1 solvent system the UV visible spots were the same for all months with the lowest spot at R_f 0.15 being present only through May. Changes were observed each month when the plates were charred except between August and September and between October and November (Figure 5). Spots at R_f 1.00, 0.85, 0.78, 0.53, 0.40, and 0.13, were present in all months with a trace of cnicin showing up only from June to September (R_f 0.20). The intensity (relative concentration) of all spots either stayed the same or decreased as soil depth increased.

Standard rosette leave and root extracts were run by TLC along with the soils to see if any compounds were present in both plant tissue and soils. In the 2:2:1 solvent system (Figure 8) there were four UV visible spots at R_f 0.80, 0.64, 0.40, and 0.20 for the rosette leave extract. The top spot was distinct whereas the other three were less defined. Compounds at 0.80 and 0.40 appeared to be the same as in the soil. Spots visualized with acid and charring were present in the plant tissue and the soils (R_f 0.44 and 0.59) with the tissue spot at R_f 0.83 overlapping with a couple of spots in the soils. One UV visible spot from roots at R_f 0.07 in the 2:2:1 solvent system was among the hard to distinguish UV soil spots. The blue fluorescent spot in roots R_f 0.70 was not in the soil, there was a red spot instead. Charred spots at R_f 0.43 and 0.80 were present in both roots and soils.

In the 5:4:1 solvent system there were three UV spots in the TLC

patterns of plant tissue that appeared to be present in the soils. One from roots at R_f 0.90 and two from the aerial tissues, R_f 0.95 and 0.15, but the latter was only present in the soil through May. All compounds in plant tissues that charred on plates developed in the 5:4:1 solvent system were present in all soils, except for four. Those absent from the soil were the root compound at R_f 0.43 and the aerial tissue spot at R_f 0.48. There were two spots in aerial tissues that appeared in some soils, the one at R_f 0.06 occurred only in March and April and the one at R_f 0.60 showed up in June.

F. Glandular Trichomes

Observations of knapweed tissues with a 45X dissecting microscope revealed the presence of conical shaped (bristle like) nonglandular trichomes (Ormrod and Renney 1968) and glandular trichomes. Glandular hairs were located in depressions, or pits, on both the adaxial and abaxial leaf surfaces. Their external appearance was similar to glands reported in other genera of the Compositae family (Rodriquez et al. 1976, Blakeman and Atkinson 1979, Vermeer and Peterson 1979 (a&b), Kelsey and Shafizadeh 1980). They have a bulbous head, containing a clear liquid that looked like a water droplet in the pit.

New leaves developing from the center of rosette clusters were covered with a wooly tomentum making it difficult to view the glands. Glands were present in high density on these young leaves indicating that they were formed early in the leafs development. Gland density seemed to decrease with leaf age and size. Blakeman and Atkinson (1979) found the young leaves of Chrysanthemum parthenium Bernh. to have a higher gland density than the mature leaves. They suggested that glands developed early on the young leaves and then spread apart as the leaves expanded without any new glands forming. This may also occur in knapweed. Summer rosette leaves have a long fiberous rachis. Gland density on this portion of the leaf was lower than on the leaflets.

There were glands along the length of the main stem, but the density decreased significantly going from the tip to the base. Gland numbers at the stem base were very low compared to the leaves. Branches were covered with glands at a density equal to or greater than on the upper portion of the main stem. Glands were also present on the

phyllaries and the corollas. Deep pits like those associated with leaf glands were not common on any of the other tissues. Seeds collected at the study site in September of 1983 had a smooth seed coat free of glandular trichomes.

Standing dead stems from the previous growing season still had intact glands attached on all tissues (main stems, branches, leaves, phyllaries) just as they were when alive. In some glands the contents were no longer clear, having turned opaque or white. Recently dead rosette leaves, from the 1985 growing season, also retained their glands with contents intact. Most of the tomentum was gone from the dead tissue making the glands easier to see than on the live material.

G. Cnicin in Glandular Trichomes and Gland Bearing Tissues

Cnicin was detected by qualitative TLC in the extracts from glands removed from the stems and all the extracts from gland bearing tissues; leaves, branches, main stem, phyllaries, and flowers. This differs from the quantitative TLC and HPLC results that found only trace quantities in the stem and none in the heads (phyllaries and flowers combined). Apparently cnicin is not very concentrated on stems or heads and was diluted below detectable levels in the extraction procedure used for quantitative TLC and HPLC. In the qualitative TLC procedure the entire extracts were applied to the plates allowing the detection of less concentrated compounds.

Chapter Six

Discussion

Cnicin was present in glandular trichomes collected from knapweed stems and it was detected by TLC in all tissues bearing glands on their epidermis. There is little doubt that cnicin is a glandular component. Most terpenoids, particularly the monoterpenes and sesquiterpenes, are associated with specialized anantomical structures in plants: secretory cells, cavities, ducts, or trichomes (Loomis and Croteau 1973, Schnepf 1974, Fahn 1979). Various sesquiterpene lactones have been located within glands (Rodriguez et al. 1976, Blakeman and Atkinson 1979, Kelsey and Shafizadeh 1980, Kelsey et al. 1984) and cnicin has been reported in glandular trichomes on the epidermis of Cnicus benedictus (Politis 1946 a,b). Many of these glandular products are biologically active and may provide the plant with an epidermal chemical defense against herbivores and disease (Levin 1973, Kelsey et al. 1984)

In spotted knapweed, gland density and cnicin concentrations were both highest in the leaves. Leaves attached to the branches contained the most cnicin (0.70%) followed by leaves on the main stem (0.55%) and then rosette leaves (0.33%). These differences can probably be explained by leaf structure which differ somewhat between the three types. Leaves are pinnatifid. The largest ones occur in the rosette and at the stem base, decreasing in size as you progress toward the stem and branch tips. In summer rosette leaves, the leaflets are attached at the end of a long and fiberous rachis, which have a much lower gland density than the leaflets. Leaves on the stem have a shorter, wider rachis that is more leaflet like and less fiberous. Leaflets may be

attached along its length. On branch leaves the rachis is quite reduced and is not present when the leaf is entire. Rachis dry matter would dilute the cnicin in the leaflets of the larger leaves from the rosette and main stem. Also, branch leaves, and leaflets were smaller and seemed to have a greater gland density. These two factors contributed to the lower cnicin levels in rosette leaves compared to the branch leaves.

The main stems had glands present along their length with a marked difference in density between the tip and the base. There were very few glands at the stem base, and they were widely spaced. Greatest numbers were observed near the tip. Trace levels of cnicin observed by quantitative TLC, and undetected by HPLC, were caused by dilution from the large quantity of internal biomass per unit area of epidermal surface. Plants used for the dissection analysis were collected in mid-August when the stems had become tough and fiberous. Whole plants (green aerial tissue) are approximately 25 percent neutral detergent fiber (cell wall) in mid-June, increasing to near 50 percent by the first week in August (Mihalovich and Kelsey, unpublished data). Most of this change was probably due to cell wall thickening in the main stem and branches. Stem dry matter diluted the cnicin concentrations in the aerial tissues of plants two years and older (Table 1). It was also responsible for the decreasing cnicin concentrations in the samples of stems with leaves analyzed in 1983 (Figure 3).

Branches had glands at a density that was equal to, or greater than that on the stem tip. Branch size or internal biomass will influence the concentration of cnicin measured. The 0.05 percent in Figure 4 was

an average of 0.00 percent in one plant and 0.10 percent in the other. So cnicin concentrations expressed as percent of dry weight will probably vary considerabley from one branch to another.

Glandular trichomes were observed on the phyllaries between vascular strands, but there were no deep pits like on the leaves. Glands were also present on the corollas. Their cnicin concentrations were considerably lower than in the leaves. Roots normally produce no external glands, so the absence of cnicin in this tissue was not unusual.

When the tissues of knapweed dehydrate and turn yellow in late summer, glandular trichomes persist on the dried epidermis. Dead stems which remain standing with their leaves attached still had intact glands nearly one year after they had died. In 1984, the cnicin concentration of the stem leaves increased as they died and dehydrated going from 1.76 percent in August to 2.76 percent in October. This was most likely caused by losses in leaf dry matter relative to the cnicin. November leaves had a much lower quanitiy of cnicin (0.96%) compared to October and this large decrease in a one month period is difficult to explain. Variation between the three November plants was not excessive and they were analyzed on different days with the HPLC. Dry matter could not have increased to dilute cnicin because the leaves were dead. By late January the concentration was back up to 1.70 percent with a slight increase to 1.83 percent in March, 1985. Dead leaves on standing dead stems, collected in August 1983, had been dead for nearly a year but still contained 0.24 percent cnicin.

The persistence of glandular trichomes and the gradual loss of

cnicin from dead standing knapweed is strong evidence that cnicin is not readily leached from the tissue. In crystalline form cnicin has limited water solubility (Cavillito and Baily 1949). Within the glands, cnicin is very likely mixed with other organic compounds in a liquid, surrounded by a cuticular sac (Potitis 1946 (a,b), Fahn 1979, Slone and Kelsey 1983). Cuticle is hydrophobic and protects the gland contents from water extraction. When air-dried, leaves and stems (collected in mid-June) were soaked overnight in water and then redried, the cnicin concentration in the extracted residue was greater than in the unextracted tissue (Mihalovich and Kelsey, unpublished data). Apparently, water removed the soluble carbohydrates and other cellular components more readily than cnicin. Compounds present in the glands of Chrysanthemum parthenium, including the sesquiterpene lactone parthenolide, could not be extracted by washing mature leaves in water (Blakeman and Atkinson 1979). If cnicin is not readily leached by rain from the dead standing tissue it is very unlikely that it is leached from the epidermis of live tissue during the spring and summer.

Rosette leaves die and dehydrate during the summer, and the glands remain intact on their epidermis. Unlike the stem and branch leaves, their cnicin concentration is lower in the dead tissue than in the live. When rosette leaves dehydrate the leaflets shrivel, becoming brittle and easily broken and dislodged from the rachis. The fiberous rachis, however, is very sturdy and persistent, but this part of the leaf has fewer glands and less cnicin. When leaflets are lost, the remaining dead material will not contain as much cnicin. This may be part of the reason for decreasing concentrations in dead rosette leaves from

September 1984 through March 1985. Also, during the winter, rosette leaves are covered with snow flattening them into a loosely packed litter layer around the plant, with some of the lower tissues in direct contact with the soil. As the snow melts in the spring this litter is constantly saturated with water. Rain will resoak it after the snow is gone. Both of these provide the opportunity for slow leaching to take place. This could be more effective at removing cnicin than the occassional washings that standing dead stem leaves receive from rain and snow. Wet tissues could be subjected to more rapid decay from bacteria and fungi, especially the litter in direct contact with the soil. Loss of high cnicin containing leaflets, and the possibility of extended leaching, reduce the cnicin concentration in the overwintered remnants of rosette leaves to near zero (Figure 6).

Under laboratory conditions cnicin was phytotoxic to all plant species bioassayed including lettuce, crested wheatgrass, rough fescue, bluebunch wheatgrass, western larch, lodgepole pine and spotted knapweed. Although it did inhibit the germination of all species except lodgepole pine and spotted knapweed it was not a strong germination inhibitor. It was most effective at retarding growth particularly in the roots. The growth of lettuce, bluebunch wheatgrass and spotted knapweed was inhibited significantly at all concentrations of cnicin tested (Kelsey and Locken, submitted for publication). In order for cnicin to function as an allelopathic compound it must be able to enter the environment in sufficient concentrations to be toxic. Cnicin is a crystalline solid and can not escape from the tissue by volatilization. As indicated in the above discussion, storage of cnicin within glandular

trichomes on the epidermis may actually protect and prevent leaching from rain. Very little cnicin is probably lost from live green tissue by leaching. Extended soaking of dead rosette leaves and litter by snow melt and rain could remove cnicin from these tissues in the spring. Dead stem leaves might also lose some of their cnicin very gradually by leaching.

Tissue breakdown and decomposition is another mechanism for releasing chemicals from plants (Rice 1974). At the end of the summer when tissues die and dehydrate most cnicin appears to remain in glandular trichomes on the dead plant material. Therefore, when this tissue decomposes the cnicin might be released. Dry matter productivity of spotted knapweed is variable, depending on numerous factors, but quantities of 400 g/m^2, or less, are probably common (Belles et al. 1980, Chicoine 1984, Kelsey in press). A population that produced 100 to 400 g/m^2 dry matter in July, would contain one to four grams of cnicin per m^2, if the concentration were one percent as in 1983 (Figure 3). This quantity added to the soil each year could build up significant concentrations. However, TLC analysis of the knapweed infested soils did not show high concentrations of cnicin at any particular time of year. Highest quantities appeared at the 0-2 cm soil depth during the summer months, but at less than 1.0 ppm. This would be too low to inhibit germination, or root growth, as observed in the laboratory (Kelsey and Locken submitted for publication).

The absence of cnicin in the soil may be the result of several factors. Dead tissues containing cnicin do not drop to the litter or soil surface all at once. Instead there is a gradual deposition over a

one year period, or longer. Dead stems may still be standing upright with some leaves attached, a year after they die. Dead rosette leaves and new litter will be covered with snow during the winter. This can compress the tissue into a loosely packed layer, especially around the plant crown. Patches of litter free soil may occur between plants. Snow melt and spring rains soak the litter and may gradually leach some of the cnicin to the soil. Wetting and drying between spring rains causes the litter tissues to expand and shrivel. Small pieces may break off and drop to the soil surface. During the spring wet period bacteria and fungi may decompose some of the litter. Cnicin is antibacterial (Cavallito and Bailey 1949, Vanhaelen-Fastre 1972, Vanhaelen-Fastre and Vanhaelen 1976) but its activity varies depending on the species of microorganism. Some decomposers might be able to use it as an energy source, or possibly cause it to breakdown to other products. Breakdown or rearrangement in the litter, or soil, is very probable because cnicin belongs to the germacranolide structural class of sesquiterpene lactones with a cyclodecadiene ring structure, which tends to be reactive, unstable, and easily rearranged (Fischer et al. 1979). Cnicin has an ester side chain that can be readily hydrolyzed under mild conditions (Fischer et al. 1979) to give salonitenolide, a derivative that has less bacteriostatic activity than cnicin (Vanhaelen-Fastre and Vanhaelen 1976). In the lab, cnicin was observed to be unstable in ethanol or other weak acid solutions, particularly if heated. The antimicrobial activity has been associated with the presence of an α-methylene-γ-lactone that can be deactivated by a Michael-type addition with the thiol group in cysteine (Vanhaelen-Fastre 1972). Although this

functionality is not always necessary for biological activity (Lee et al. 1977, Picman and Towers 1983, Harmatha and Nawrot 1984), it is an active site in many instances (Szabuniewicz et al. 1974, Calzada et al. 1980, Picman et al. 1981, Spring et al. 1982, Elissalde et al. 1983) and can be deactivated by reactions with certain amino acids and proteins. If Michael-type addition reactions can occur in the litter, or soil, cnicin's phytotoxicity would probably be eliminated. In the litter and soil, exposure to high temperature, UV light, H^+, OH^-, and oxygen could cause structural changes to take place. Any intact cnicin that does become incorporated into the soil could be diluted to concentrations below the level of toxicity. Some combination of these factors were probably responsible for the absence of cnicin in the soil.

These data strongly suggest, that within the environmental and biological parameters of this study site, cnicin was probably not functioning as an allelopathic compound. This, however, does not eliminate the possiblility that other compounds in the plant are allelopathic. TLC analysis of the soils revealed the presence of several knapweed compounds at much higher concentrations than cnicin was ever observed. Toxicity of these substances should be tested. The most efficient method would be to bioassay the soil for toxicity first and then try to isolate the active compounds if the results are positive.

Also, the study site was fenced with minimal disturbance from man or animals. Retention of cnicin in the trichomes on dead standing tissue is probably significant in preventing high concentrations in the soil. Under other circumstances where the plants are subjected to disturances that accelerate the physical breakdown and incorporation of

dead tissue into the litter and soil, toxic concentratons of cnicin, at least at the soil surface, might result. Dead stem leaves, collected from upright stems in the spring, inhibited the germination and growth of lettuce seedlings (Kelsey unpublished results). If all the standing dead tissue, or even the live tissue, in a population was knocked down at once and compacted on the soil surface, or mixed into the upper layer of soil, cnicin could possibly reach sufficient concentrations to be toxic. Consequently, if knapweed is mowed to reduce seed production and plant vigor, then it would be best to remove the cuttings from the site to avoid the addition of any cnicin and other possible phytotoxins to the soil. If this were repeated yearly it could drastically curtail any allelopathic effects from the aerial tissues.

This raises an interesting question. If cnicin and other chemicals in knapweed leaves and stems had evolved through natural selection for their allelopathic activity why isn't there a more rapid or simple method for release into the soil? Cnicin occurs in rather high concentrations on the leaves, it is not volatile or very water soluble. Furthermore, it is sequestered within glandular trichomes beneath a cuticular layer that protects it from leaching. Leaves and stems do not die and immediately drop to the ground so they can release their chemical contents. Stems remain standing and the leaves gradually fall off over an extended period. These are all characters that would have evolved to retain high cnicin concentrations in the tissues, not eleminate them. Maintaining significant chemical components on the epidermal surface would be desirable if they provided protection against diseases or herbivores (Levin 1973, Kelsey et al. 1984). Cnicin is

antimicrobial and could defend against bacterial attack, but this application is limited with the cnicin located in the glands. Epidermal surfaces between glands would not be protected unless the cnicin could be released to spread over the surface. In Chrysanthemum morifolium Ramat gland contents are viscous on mature leaves and not readily released to the epidermal surface (Blakeman and Atkinson 1979). In young tissue the oils are more fluid and may provide antimicrobial protection for the early stage of growth and development.

The failure of North American herbivores, both insects and ungulates, to consume spotted knapweed, has been considered one of the major factors contributing to its success in Canada and the United States. Not having coevolved with knapweed, the North American herbivores might not recognize it as an acceptable food source and/or the glandular chemicals could be feeding deterrents that decrease plant palatability. Like many sesquiterpene lactones, cnicin has a very bitter taste (Politis 1946 a,b, Wagner 1977). In the genus Vernonia, most species contain the bitter tasting sesquiterpene lactone, glaucolide-A. The exception is V. flaccidifolia Small that produces no sesquiterpene lactones. Wild rabbits and whitetail deer avoid eating Vernonia plants that naturally synthesize, or have been artifically coated with glaucolide-A (Burnett et al. 1977, Mabry and Gill 1979). Cows avoid ingestion of all Vernonia species regardless of their sesquiterpene lactone contents, possibly learned by trial and error since most of the plants are bitter (Mabry et al. 1977). Cnicin could provide similar protection for knapweed. If it has evolved for this purpose, then the maintenance of high concentrations on the epidermal

surface until after seed dispersal would be advantageous. Since seed dispersal continues after the stems dehydrate, leaf retention with its bitter components could provide some protection through this period of the four basic taste sensations, sweet, salty, sour, and bitter, bitter was most effective at stimulating responses in domestic ruminants. Cattle responded to lower concentrations of bitter substances than did goats or sheep (Goatcher and Church 1970).

Glaucolide-A has also been extensively tested with insect herbivores. It deters the feeding of various lepidopterous larvae, particularly the southern armyworm, Spodoptera eridania and the fall armyworm, S. frugiperda (Burnett et al. 1974). Their growth and survival is reduced significantly when reared on diets containing this lactone (Jones et al. 1979). Adult fall armyworms avoid ovipositing eggs on any plants containing natural or artifically applied glaucolide-A (Burnett et al. 1978a). Laboratory feeding tests suggested that V. gigantea and V. glauca were protected from insect herbivory by glaucolide-A relative to V. flaccidifolia which synthesizes no lactones. However, field insect feeding trials indicated just the opposite. Vernonia flaccidifolia was fed upon significantly less than the lactone synthesizing species (Burnett et al. 1977). It was concluded that glaucolide-A was most effective as a mammalian feeding deterrent (Burnett et al. 1978b), than an insect deterrent, or V. flaccidifolia has evolved some other defense mechanism against insects (Burnett et al. 1977).

Many other sesquiterpene lactones have been tested with various insects and most of them cause some degree of feeding deterrence (Picman

et al. 1978, Nawrot et al. 1983, Smith et al. 1983, Streibl et al. 1983, Harmatha and Nawrot 1984), growth inhibition (Picman et al. 1978, Nakajima and Kawazu 1980, Smith et al. 1983) and/or reduced survival in the laboratory (Picman et al. 1978, Nakajima and Kawazu 1980, Smith et al. 1983, Picman and Picman 1984b). In contrast, Wisdom et al. (1983) reported almost no effect from five sesquiterpene lactones on the feeding or growth of corn earworms. Further research, particularly in the field, must be conducted before the insect repellent and toxic properties of sesquiterpene lactones can be fully evaluated.

It is interesting to note that the insects that have been introduced to North America from Eurasia for biocontrol of spotted knapweed include no leaf defoliators. They all attack plant tissues with low cnicin concentrations. Urophora affinis and U. quadrifasciata form galls in the heads, Metzneria paucipunctella feed on florettes and seed, and Agapeta zoegana and Pelochrista medullana are root miners of small rosettes (Story and Nowierski 1984).

Localization of cnicin on the epidermal surface has important implication for the plants. Assuming that most terpenoid biosynthesis takes place within the glands, a reasonable assumption (Fahn 1979, Croteau 1981), plants could synthesize and maintain high concentrations of toxic compounds without interferring significantly with normal growth. Only the specialized gland cells would be required to synthesize toxins and this is problably more energy efficient than having all cells produce the compounds. Also it allows the build up of effective concentrations at a site (the epidermis) that assures immediate and maximum contact with the sensory receptors of herbivors,

and possibly disease organisms. A one percent concentration of cnicin on the surface of knapweed tissue might be perceived quite differently than one percent distributed evenly throughout the tissue. Biological and chemical data more strongly support cnicin's evolutionary development as a protectant against herbivores and disease, rather than a phytotoxin. Allelopathic properties may be a secondary benefit derived as a consequence from the synthesis and accumulation of antimicrobial and/or antiherbivory compounds.

Chapter Seven

Summary and Conclusions

Cnicin is a phytotoxic sesquiterpene lactone present in epidermal glandular trichomes on the aerial tissues of spotted knapweed. Highest quantities were measured in the leaves where glands were most dense. Stem tissue did have glandular trichomes but the cnicin concentrations were diluted to low levels by the fiberous dry matter. In the 1983 seasonal sampling of combined aerial tissue, cnicin concentrations were low in the spring (0.58%), increased with the development of stems, and were at maximum levels (1.03%) through flowering in July. This quantity was maintained the rest of the growing season in the rosette leaves, but decreased in the stems with leaves to 0.48 percent. The latter change was caused by dry matter dilution from the stems. Cnicin concentrations in aerial tissues decreased with plant age, which also correlated with the amount of stem tissue. In 1984, rosette leaves maintained a relatively stable quanitity of cnicin (between 0.52 and 0.73%) from March through August, it peaked in September (1.02%) decreasing to near summer levels by November (0.64%). In combined stem and branch leaves cnicin levels were close to 2.00 percent during the summer, then increased as the leaves dehydrated reaching a maximum of 2.76 percent in October. This was followed by a large unexplained drop in November (0.96%). By January and March of 1985 the concentrations had risen back to the previous summer levels. The combined stem and branch leaves exhibited the most interplant variation.

Glandular trichomes and cnicin remained on the dead tissues.

Cnicin was probably not very leachable since relatively high concentrations were still present in the stem leaves six months after they had died. During this same six month period cnicin levels decreased in the dead rosette leaves because the leaflets with higher quantities of cnicin were dislodged from the fiberous and more persistent rachis. Leaching from rain and snow may have also eliminated some of the compound.

There appears to be no ready mechanism for releasing this sesquiterpene lactone from the plant to the soil. It is not volatile, and has limited water solubility. Furthermore, it is stored inside a hydrophobic cuticular sac that restricts its leachability. When the tissues die they dehydrate and remain standing in place. Rosette leaflets shrivel and may gradually drop to the soil, snow helps to press the dead rosettes and other dead tissue into a loosely stacked litter layer around each plant. This gradually breaks up and decomposes. Stems can remain standing for up to a year, or longer, after they die. Leaves gradually drop to the litter and soil during this time. Cnicin has a reactive and unstable structure that is susceptible to rearrangements and decomposition. As a consequence, at the study site, cnicin was detected in only trace quantities in the soil. Under other conditions, such as mowing or trampling, large quantities of live or dead knapweed tissues could be rapidly deposited to the soil surface, or into the soil. In this situation cnicin concentrations might reach toxic levels. Available evidence suggests that cnicin may function primarily as a chemical defense against herbivors and disease rather than an allelopathic agent.

In conclusion, the high cnicin concentrations in the leaves of spotted knapweed make an important contribution to the ecology of these plants. It is very likely part of a chemical defense against disease and herbivory. Its function as an allelopathic compound is probably variable depending on the biotic and abiotic conditions at a given sites. Under the proper set of conditions it could reach toxic levels in the soil. Compared to other knapweed characters that have contributed to its success in western Montana, cnicin's allelopathy should not be considered any more important than any of the others. In fact it may be less so. This does not diminish cnicin's possible role as an herbivore deterrent and the potential contribution it has made to knapweed's competitive ability. Since cnicin is restricted to the aerial tissues, its allelopathic effects could by avoided by removing the tissue every year. This could be achieved by burning, sheep grazing, or mowing. If conducted properly the latter two would provide additional benefits such as reduced seed production, reduced seed viability, and smaller less competitve knapweed plants.

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Appendix

A. Statistical Summary for the 1984-1985 Seasonal Data (Dates not underscored with the same line are significantly different at a $p<0.05$. Rosette has been abbreviated to rose.)

(1) Spring rosette and stem leaf tissue

Oct 1984 stem (2.76%)	Sept 1984 stem (2.16%)	Mar 1985 stem (1.83%)	Aug 1984 stem (1.76%)	June 1984 rose &stem (1.47%)	July 1984 rose &stem (1.32%)	Nov 1984 stem (0.96%)	May 1984 rose (0.56%)	April 1984 rose (0.51%)

(2) Rosette leaf tissue including June and July

June 1984 rose &stem (1.47%)	July 1984 rose &stem (1.32%)	Sept 1984 rose (1.02%)	Oct 1984 rose (0.84%)	Nov 1984 rose (0.64%)	May 1984 rose (0.56%)	Aug 1984 rose (0.52%)	April 1984 rose (0.51%)	March 1985 rose (0.35%)

(3) Rosette leaf tissue excluding June and July

Sept 1984 rose (1.02%)	Oct 1984 rose (0.84%)	Nov 1984 rose (0.64%)	May 1984 rose (0.56%)	Aug 1984 rose (0.52%)	April 1984 rose (0.51%)	March 1985 rose (0.35%)

(4) Stem leaf tissue

Oct 1984 stem (2.76%)	Sept 1984 stem (2.16%)	June 1984 stem (1.85%)	March 1985 stem (1.83%)	Aug 1984 stem (1.76%)	Nov 1984 stem (0.96%)

(5) Dead rosette leaf tissue

Sept 1984 dead rose (0.60%)	Oct 1984 dead rose (0.34%)	Nov 1984 dead rose (0.27%)	March 1985 dead rose (0.05%)

(6) September 1984

stem (2.16%)	rose (1.02%)	dead rose (0.60%)

(7) October 1984

stem (2.76%)	rose (0.84%)	dead rose (0.34%)

(8) November 1984

stem (0.96%)	rose (0.64%)	dead rose (0.27%)

(9) March 1985

stem (1.83%)	rose (0.35%)	dead rose (0.05%)