A GROWTH INHIBITOR FOUND
IN CENTAUREA SPP.

by

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ABSTRACT

The knapweeds were introduced into North America in the nineteenth century. They have now established themselves and are spreading rapidly. In British Columbia's dry interior they grow in dense infestations, tending to exclude as well as out-compete other plants. Because of this characteristic they now present a serious problem. It has been suspected that toxic substances produced by the knapweeds may be partially responsible for enabling them to establish themselves at the expense of other plants.

The object of this study, therefore, was to establish the presence and location of inhibitory substances in three species of knapweed: Centaurea repens, Centaurea diffusa, and Centaurea maculosa; and if possible to identify the chemical structure of the compounds responsible.

In order to determine whether inhibitors might be present, tomatoes were grown in both infested and non infested soil. Also, dried leaf powder from the knapweed was added to greenhouse soil to observe the effects it might have on the growth of tomatoes. Leaf, stem, root and seed of the knapweed were extracted separately with different solvents: ether, water and ethanol. Germination tests, using lettuce, barley and cress as test crops, were used as methods of bioassay.

In order to isolate the inhibitory compound, ascending paper chromatography was employed using the aqueous phase of n-Butanol:acetic acid:water (4:1:5) as the solvent. The inhi-

bitor was located on the chromatographic paper by means of germination tests. Chromogenic sprays and comparisons of Rf values with different solvents were made in order to identify the inhibitor. To further characterize the compound an ultraviolet spectrum was carried out.

The findings of the soil studies indicated that knapweed infested soil, and soil containing leaf powder of knapweed, were inhibitory to the growth of tomatoes. The inhibitor was found to be present in all the three species studied, and was located mainly in the leaves. It proved to be both ether and water soluble, and non-specific, being inhibitory to all the crops tested. The inhibitor was found as a pale yellow fluorescent band at an Rf of 0.9. Chromogenic sprays indicated the inhibitor to be an indole derivative. This was confirmed by the ultraviolet absorption spectrum.

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A GROWTH INHIBITOR FOUND
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I. INTRODUCTION

The presence of knapweeds, Centaurea spp., in North America was first reported during the nineteenth century. Since then they have established themselves in many parts of the United States. Three knapweed species now present a serious problem in the dry interior parts of British Columbia. In their natural habitats these knapweeds grow in dense infestations, crowding out other plants, and some workers think that toxic plant products may be partly responsible for this condition. While some other weeds are known to produce materials which inhibit the growth of associated species, the production of toxic materials by knapweeds has not been reported so far.

The object of this study was therefore to investigate the possibility of inhibitory substances being present in Centaurea spp., to establish in which part of the plant these substances were located, and if possible to determine their nature.

II. LITERATURE REVIEW

Russian knapweed (Centaurea repens L.) is a perennial which reproduces by seeds and creeping roots. This weed, a native of southern Russia and central Asia, has reached serious proportions in North America, where it seems to have been introduced in Turkestan alfalfa seed (94). The deeply penetrating perennial root system forms dense infestations that enable Russian knapweed to suppress cereals and many other annual field crops (71). The extensive root system has been described as consisting of the original root, one to many lateral roots and their vertical extensions (27).

Diffuse knapweed (Centaurea diffusa Lam.) is a biennial or short lived perennial. This weed is at present found only in the north western United States and British Columbia. It grows well in the dry interior of this province, establishing quickly on open disturbed soil, and it is the knapweed causing the greatest local concern at the present moment (70).

Spotted knapweed (Centaurea maculosa Lam.), like the diffuse, is a biennial or short lived perennial also found in the southern interior of British Columbia. Although at present it is found only in waste places, east of the main diffuse area, it is feared that it may become a serious problem in British Columbia because it has spread rapidly within the last few years.

The knapweeds belong to a group of the Compositae which have distinctive bracts. The flower heads of Russian knapweed have entire bracts, while the bracts in the other species of knapweed have fringed or torn tips. In diffuse knapweed the bracts end in a rigid spreading spine, whereas in spotted knapweed the bracts lack these long spines and always have a terminal blackish fringe (Figure 1). The seeds in Russian knapweed have a scar at the base and the pappus is longer than the seed. In the other species the seed has a notch at one side and the pappus is much shorter than the seed. The blackish scaly roots of Russian knapweed are also distinctive (94).

Russian knapweed is known to be poisonous to sheep and horses. A worker in South Africa has reported that a full grown sheep died eighteen hours after administration of just over half a pound of dried Russian knapweed, and a Russian text claims that horses have been poisoned by this weed (94). However, animals tend to avoid all three knapweeds, where they have any choice of forage.

There is a tendency for these three Centaurea spp. to form dense infestations and to suppress the growth of other species. Based on this observation initial work on Centaurea repens in British Columbia has demonstrated that Russian knapweed infested soil has inhibitory effects on tomato plants and that water extracts of stem and leaf have a strong inhi-

Figure 1. Morphological illustrations of the three species of knapweed.

bitory effect on the germination of turnips (69).

A great deal of confusion exists in the terminology used in connection with inhibitors. Tukey (90) defines plant regulators as organic compounds, other than nutrients, which in small amounts promote, inhibit or otherwise modify any physiological process in plants. This was one of the definitions accepted by the Executive Committee of the American Society of Plant Physiologists in 1953. A minority report by Larsen (51) objected to the word "regulator", and made a counter proposal which defined plant growth substances as organic compounds which at low concentrations promote, inhibit, or qualitatively modify growth. Larsen further defined "growth inhibitors" as substances which retard growth both in shoot and root cells and have no stimulatory range of concentration. He defines anti-auxins as growth substances which competitively inhibit the action of auxins. Evenari (23) defines germination inhibitors as substances produced by plants or substances of related structure not found in plants which inhibit or delay the germination of seeds of the same or other species.

The theory that inhibitory substances may be deposited in the soil by certain plants and may influence the growth of those plants or other species was postulated as early as 1832. DeCandolle (20) showed that specific toxic substances produced by Euphorbia sp. and Cnicus sp. were inhibitory to flax and oats respectively. At that time his theory was not accepted,

as the inhibitory effects were attributed to competition for inorganic nutrients. At the turn of the century, however, interest in the possibility of toxic substances being produced by plants was reawakened. Early investigators, like Schreiner and his co-workers (73), succeeded in isolating and identifying toxic substances from certain soils. That these substances had their origin in plants was not established.

The work of Pickering (63) showed that grass exerts a directly poisonous action on apple trees. It has also been suggested that the toxin produced by brome grass was toxic to all species, and that other grasses which were sown where brome grass had been sown were pale green and lacking in vigour (60). Funke (28) stated that eighteen species of plants which were sown beside a hedge of Artemisia absinthium were severely injured and that one species, Leviticum officinale, was even killed by the chemical excretion. The presence of inhibitors in desert plants has been indicated by Bennet (7). The work of Tourneau (88) showed that extracts from twenty-four species of plants had germination reducing capacity. He also stated that extracts of various weeds and crop plants inhibit germination and growth, and crop extracts appear to be as inhibitory as extracts of weed species. It was proved by Patrick (62) that substances capable of markedly inhibiting the respiration, germination and growth of tobacco seedlings were obtained after residues from timothy, corn, rye

or tobacco had been allowed to decompose in the soil (62). Leopold (52) stated that a great many inhibitors of germination and growth have been found to be present in various plant parts. The presence of inhibitors in different plant parts has also been indicated by other workers. That inhibitors are present in leaves has been shown for peas (79), radish (80), Encelia farinosa (33), cocklebur (12), apple (56), grapes (5) and Ailanthus (26).

That inhibitory substances exist in seeds has been demonstrated for sugar beets (86), cabbage (18), red kidney beans (76), apple seeds (54), Kochia indica (75), wild oats (21), Betula sp. (11), lettuce (64), Viburnum trilobum (45), maize (39), and peach seeds (24).

That roots contain inhibitory materials or secrete substances toxic to plants has been claimed for peaches (67), brome grass (6), guayule (14), red beet (68), peas (43), and Vicia faba (49).

Inhibitors are present not only in the leaves, seeds and roots but evidence given by other workers indicates the presence of inhibitors in other plant parts as well.

Hemberg (36) found a large amount of inhibiting substances in extracts of the peripheral and central buds of Fraxinus, while Hendershot (41) extracted an inhibitory substance from peach flower buds. It was also proved by Hemberg (38) that inhibiting substances which are found in potato

tubers occurred chiefly in the peel layer.

Evenari (23) established that germination inhibitors are found in many different species in most of the different families of the plant kingdom. He also pointed out that inhibitors are not confined to seeds or fruit but occur in various parts of plants according to the species: like the fruit pulp, fruit coats, endosperm, seed coat, embryo, leaves, bulbs and roots. Bentley stated that it is now an established fact that naturally occurring inhibitors are obviously very widespread (9). In a chapter on natural plant growth inhibitors Audus (3) records that there can be little doubt from accumulated evidence that roots and underground stems of a very wide range of plant species produce specific chemical compounds that are active inhibitors of seed germination and plant root growth.

From the array of literature cited it is obvious that inhibitors are very widely occurring, existing as they do in different plant parts of many species. Evenari's review (23), moreover, states that these inhibiting effects are not restricted to phanerogamous plants. Spores and gemmae of cryptograms show an inhibited germination very similar to that of tomato seeds inside the fruit, e.g. the gemmae of *Marchantia* do not sprout inside their cupules, and spores of *Funaria hygrometrica* do not germinate within their sporangia.

It has been shown that inhibiting substances are formed by algae as well (44). In experiments with Arterionella formosa and Nitzschia palea it was found that both species formed substances which inhibited the growth of other species.

Nature and structure of inhibitors

The pioneer work in investigation of the chemical structure of toxic substances was done by Schreiner et al. (72). The toxic principles found were: picolonic acid, salicylaldehyde, vanillin and dihydroxystearic acid. Unfortunately this work did not prove that these substances had their origin in plants. Schreiner (72) conducted an extensive study with a number of compounds which occur naturally in plants and showed that most of them had inhibitory effects, especially on the roots of seedlings. The toxic effects of sugar beet seed balls has been shown to be due to free ammonia which was hydrolyzed from the organic nitrogen compounds of the seed ball during the process of germination (81).

Work done by Evenari (22) established that the inhibitor of tomato juice was a non-colloidal substance, and he suggested that it could be one of three types: essential oils, alkaloids and glucosides. Of the two substances isolated from guayule plants, one was identified to be cinnamic acid; the second was an organic acid which remained unidentified (14).

It has been stated that many of the inhibiting com-

pounds are normal plant metabolites, e.g. tryptophane and tryptamine produce certain physiological actions on plants similar to auxins (2). The structure of the toxic compound isolated from leaves of Encelia farinosa was shown to be 3-acetyl-6-methoxybenzaldehyde (34). One of the blue fluorescing inhibitory compounds in oat roots was reported to be scopoletin (32). In a review by Evenari (23) the occurrence of a large number of chemicals including: hydrogen cyanide, ammonia, ethylene, mustard oils, organic acids, unsaturated lactones, aldehydes, essential oils and alkaloids, which exhibit inhibitory properties is discussed.

Recent work on identification of inhibitory compounds

Bentley (9) thinks that little further advance can be made in the field of inhibitors until the various naturally occurring compounds have been chemically identified and made available for physiological studies. Realizing the importance of this several workers in the past decade have made useful contributions by establishing the single chemicals involved in plant inhibition.

Many investigators have attempted to identify the inhibitor of the potato tuber. It was suggested that it may be trans-cinnamic acid (17), or acid inhibitor β (40). Housley (42) thought that the inhibitions could not be associated with any particular compound but were due to a

complex mixture of aliphatic acids. He isolated azelaic acid and the coumarin, scopoletin, together with an unknown acid, probably an unsaturated polyhydroxy fatty acid.

The most active compound present as an inhibitor in Trigonella arabica was identified as coumarin (53). The compounds in Thomnosma montana were reported as furocoumarins (7), assigning them empirical formulae of $C_{16}H_{15}O_6(OCH_3)$ and $C_{11}H_4O_3(OCH_3)_2$, and an inhibitory unsaturated keto lactone Xanthinin ($C_{15}H_{18}O_3$) was isolated from the leaves of Xanthium (30).

Koves (48) identified inhibitors which he found present in twenty-four species of dry fruits. He demonstrated the presence of p-oxybenzoic acid, p-coumaric acid and ferulic acid, as well as high molecular weight tannic acids, catechuic, caffeic, chlorogenic and salicylic acids. Besides the identified compounds, the presence of some other inhibitors was also noted. These were presumed to be chemically related to the substances demonstrated. All the inhibitors identified from the dry fruits were phenolic acids.

The inhibitor in Pisum sativum roots was reported as a phenol (87), while trans cinnamic acid was found to antagonize the growth promoting effect of auxins in peas (93). A fat soluble long chain saturated keto-alcohol with forty-four carbons has been extracted from plants in the flowering stage (82). This substance inhibits the effect of indoleacetic acid (IAA) and has been referred to as an antiauxin. Organic acids were found to be present in the zone of inhibition

around peach seeds (24), while an unsaturated yellow oil was responsible for inhibition in beet seeds (46), and a saponin-like inhibitor was associated with germination inhibition in Kochia indica (75). Tourneau (89) suggests that the inhibitor of leafy spurge may be a non-alkaloidal nitrogen compound.

Luckwill (55) states that little is known about the chemical nature of inhibitors, but they probably fall into two groups: phenols, and true antiauxins, which block some essential reaction. In an outline of recent work Audus (3) notes that there is a close relationship between some inhibitors and IAA, and that the inhibitors may be either an auxin precursor or an inhibitor-auxin complex.

Properties of inhibitors

Inhibitors exhibit a variety of characteristics. A review of the germination inhibitors qualify them as being non-specific in nature (23), while Audus (3) deals with inhibitors which are specific in nature and which may therefore be useful in the field of antibiotics. The inhibitory compounds of Betula sp. was shown to be a non-fluorescent substance (11), although many inhibitory substances are known to be fluorescent. The fluorescence exhibited by coumarin and its derivatives is dealt with in detail by a number of workers (31,83).

The inhibitory substance of guayule was stated as being organic in nature (14), but at least one other review deals with several inhibitory compounds which are inorganic (23). The inhibitor in this plant as well as that formed in the potato tuber was both ether and water soluble (35). It has also been shown that there are neutral as well as acid inhibiting substances (37). An inhibitory substance in tomato juice was found to be a non colloidal. It was absorbed by animal charcoal and was volatile (22).

It is claimed that some chemical inhibitors are photodynamic. Galston (29) reported that light could lead to the formation of an inhibitor. Using lettuce seed as an indicator it has been found that coumarin is photosensitive after entering the seed and will not exhibit any inhibitive effects on germination if seeds are soaked in light or if light strikes the seed while wet. Thus the inhibitive effects of coumarin are overcome by light (6).

Siegel (76) studied the inhibitors from red kidney bean seeds and found that irradiation activated and boiling inactivated certain extracts, whereas the inhibitor of grape leaves was found to be stable to heat (5).

The diversity of inhibitors is apparent, then, as they are: both specific and non-specific in their action, both fluorescent and non-fluorescent, organic as well as inorganic, both acidic or neutral in character, ether and sometimes water soluble, formed by light as well as destroyed by it, both heat labile and heat stable compounds.

Methods of extraction and isolation

The main object of a satisfactory method is to have an extraction which would be complete. The second aim is to prevent chemical or enzymatic changes in the compounds during and following extraction. In order to reduce the extent of conversion, a number of methods to reduce enzymatic activity can be used, e.g. boiling, addition of specific inhibitors and the use of high temperatures. The experience of a number of workers has indicated however that the most practical methods of reducing conversion is by the use of low temperatures and comparatively short periods of extraction.

The main features of the extraction procedure outlined by Van Overbeek (92) are: total omission of acid, avoidance of crushing the material, one single extraction for a prolonged time (overnight), and the evaporation of the ether extract to complete dryness. Increased amounts of a growth inhibitor were extracted from the small leaves of *Xanthium* with continued extraction up to at least twelve hours at 0° C. (13). Black (93) in his work with *Betula* sp. used methanol to avoid proteinaceous material.

The different methods of extraction with their advantages and disadvantages have been reviewed by Larsen (51) in detail. He deals initially with the method of trapping and then outlines the extraction with different solvents such as: water, diethyl ether, chloroform and ethanol.

Since the crude plant extracts contain various impurities as well as possibly both growth promoting and inhibiting substances, a fractionation of the extracts is necessary for identification. Larsen (51) outlines a technique to be used: partition between solvents, chromatographic partition on columns, and paper chromatography.

Leopold (52) also deals with the chromatographic separation of auxins and discusses briefly the various types of solvents used by different workers. The technique used for auxins can also be used for inhibitors, as Powell (66) has pointed out.

A recent review by Thompson (85) deals extensively and intensively with all the aspects of partition chromatography. The thoroughness of this review makes it unnecessary to deal further with this method.

Bioassay methods

As most of the growth substances are present in very low concentrations, biological tests, which are more sensitive than chemical tests, have been adopted by workers in this field. The sensitivity of the biological assay is emphasized by Audus (3) who states that the Avena test is about 200 times more sensitive, and the root test 50,000 times more sensitive than the most delicate chemical test.

Macro methods and field assessments are still of value for making initial evaluations. Benedict (6) added dried

roots of brome grass which he suspected contained inhibitory material to test plants and measured the dry weight of the roots and shoots of his test species. Funke (28) made direct field observations of the growth of eighteen species of plants which were sown beside a hedge of Artemisia absinthium. It was found that the Artemisia absinthium severely injured the growth of these species. Gray (33) conducted both sand and solution culture tests with the leaves of Encelia farinosa, which were found to be inhibitory. A very simple and efficient test is the germination test which has been elaborated by Evenari (23).

Larsen (51) states that auxin tests based on the use of plant organs relatively rich in endogenous auxin can be used unchanged for the demonstration and assay of growth-inhibitors. He further states that the response of test organs relatively high in endogenous auxin can be standardized against pure preparations of synthetic growth inhibitors. Coumarin seems to be a suitable standard for most purposes. The various tests for auxins have been outlined by Leopold (52) which are as follows: avena test, straight growth test, pea root test, root inhibition test and the lanolin paste test. In the avena and lanolin paste test the curvature in the case of inhibitors would be positive whereas in the case of auxins there would be a negative curvature. Some workers favour other species for these tests, e.g.

Bentley (8) who recommended that wheat sections are better than oats in the bioassay of inhibitors, while some, like Patrick (62) based their bioassay tests on manometric methods and depended on the fact that inhibitory substances are capable of inhibiting respiration of the test species.

Identification

As the inhibitors which form a part of the growth substances of plants exist in very low concentrations, efforts to identify the chemical structure are extremely difficult. Attempts have been made, however, by determining Rf values, colour reactions and biological activity of unknown growth substances and comparing these with the characteristics of selected synthetic compounds.

If the substance can be isolated and crystallized, then the melting point (M.P.) can be determined. Davis, for example made a comparison of the M.P. of his unknown with that of synthetic juglone before reporting conclusively that the toxic principle of Juglans nigra was juglone.(19). Studies with the inhibitory compound found in Encelia farinosa enabled Gray (34) to determine the M.P. He also conducted a sodium fusion test and determined the elements present. By using these methods he identified the inhibitor to be 3-acetyl-6-methoxybenzaldehyde.

If the inhibitory compounds are due to precursors of

auxins or due to higher concentrations of auxins, then the compound may be an indole. Various workers (51,74,85) deal with the spray and colour reactions used to detect indole compounds. The use of chromogenic sprays and comparison of different Rf values by employing different solvents in an effort to identify coumarin and its derivatives as well as organic acids have been listed by a number of workers (31,83). The use of both ultraviolet and infrared spectroscopy help in enabling one to identify the unknown compound.

Mode of action and significance

Various theories have been postulated by different workers regarding the mode of action of inhibitors. Skoog (77) reported in his experiments with bud inhibition that inhibiting actions cannot be explained in terms of concentration alone. He stated also that IAA can inhibit growth directly in buds. Later, Skoog (78) confirmed the view that auxin acts as a co-enzyme. He presented a scheme for the action of auxins in stimulation and inhibition of growth. This scheme accounts for the ability of auxin to accelerate and inhibit growth, as a quantitative manifestation of a single function, rather than as a result of separate qualitative effects.

Audus (1) in his work with coumarin indicated that these substances must affect their inhibition by forming

loose combinations, or easily disassociated compounds with enzymes or metabolites in the plant cell. Mayer (58) reported the activity of coumarin as a germination inhibitor was due to its specific structure, that is, an unsaturated lactone linked to an unsubstituted benzene nucleus. Any substitution leads to the partial destruction of the activity as a germination inhibitor. Mayer (59) also found that seed extracts would inactivate or destroy large amounts of coumarin. He suggested that the inactivation of coumarin is effected by a step which involves its reaction with some essential metabolite which is produced enzymatically or with the enzyme producing it. The exhaustion of this metabolite or the diversion or inactivation of the enzyme leads to failure of germination.

The work of Thiamann (84) established that coumarin and protoanemonin inhibit growth by reacting with a sulfhydryl enzyme, this enzyme being normally a limiting factor in growth. This view was also supported by Elliot (21). Bentley (9) suggests that the inhibitory effects of coumarin derivatives are due to the inactivation of sulfhydryl-containing enzyme systems, on the basis of the known reaction of thiols with α , β -unsaturated lactones. This explanation of inhibitor action assumes that the primary auxin effect is exerted via a thiol compound, e.g. CoA. Such an effect would be a true competitive inhibition.

The effects of coumarin and scopoletin upon growth were studied and it was found that both these substances inhibit root growth, coumarin being about fifty times as effective as scopoletin (65). Coumarin appears to upset the polarity of growth, particularly in the region of cell elongation, but does not eliminate meristematic activity or root hair development.

In a study with inhibitory β -complex (57) it was explained that growth inhibiting concentrations of β stimulate oxygen uptake and interfere with the uptake of inorganic phosphate. It is suggested that β acts by uncoupling phosphorylation from the electron transferring systems and by this means deprives the tissue of energy (ATP) necessary for the performance of the reaction associated with growth.

It has been stated that auxin causes an irreversible plastic to an elastic tensibility of the cell walls during first phase of elongation (16). It is assumed auxin mediates a reversible dissolution of the cell wall which explains both its growth promoting and growth inhibiting action. Coumarin and daphnetin both destroy the tensibility of the wall, making it rigid. In his work with apple seeds, Luckwill (54) concluded that the inhibitory action which resulted in dormancy of the seed could be broken by the formation of growth promoting substances. It has been stated that the inhibition of growth in plants at higher auxin concentrations

could be due to a natural and predictable consequence of the two point attachment of the auxin molecule (25). At higher concentrations there is the formation of an inactive biomolecular complex.

Larsen (50) has indicated that plant growth is governed by a double system of regulators, growth accelerating and growth retarding substances. That these inhibitors have a biological function has been discussed by Evenari (23), under the following headings: prevention of premature germination, extension of the germination period over a long period, and suppression of germination and growth of other species. The significance of inhibitors in germination has also been dealt with by Koller (47) who states that inhibitory substances act as a chemical "rain gauge" in dispersal units. It is also pointed out that dormancy is a protection from the vagaries of nature. Regulated germination and prevention of viviparity in most fleshy fruits is due to the presence in them of substances that specifically inhibit germination.

It has been assumed that growth inhibiting substances are significant for the rest period in terminal buds of Fraxinus (36), and the presence of inhibiting substance in dormant flower buds of peach has been reported (41). That the dormancy in potato tubers is due to inhibitors has been suggested by many workers (10,37).

Patrick (62) points out that toxic substances may per-

form a significant role as the primary cause of inducing some root rots and in predisposing plants to attack by organisms not normally regarded as pathogenic. It has been postulated that every growing crop forms a substance which is toxic to the growth of other plants and still more so to itself, and that by oxidation this toxin loses its toxic properties and enhances the fertility of the soil (4). The work of Proebsting (67) showed that peach roots added to virgin soil inhibited growth of peach seedlings. Bonner (15) indicated the importance of toxic substances produced by certain species as being responsible for ecological phenomena, such as competition of plant communities or the sequence of a particular species in a succession. This view was supported by Francois (26), who suggested that the toxic substance found in the leaves of Ailanthus may be one of the important factors limiting natural succession in that plant.

It is now evident that although some of the naturally occurring inhibitors exert toxic effects, other may perform regulatory functions.

III. MATERIALS AND METHODS

The three species of knapweed used for study were:

Centaurea repens L. (Russian knapweed), Centaurea diffusa Lam. (diffuse knapweed) and Centaurea maculosa Lam. (spotted knapweed). In order to collect fresh plant material as well as the soil in which the three different species were growing, a trip into the interior of British Columbia was made in the summer of 1960.

Seeds and fresh plants of C. repens were collected from dense infestations found at Cawston. Soil from infested as well as from neighbouring non infested areas was collected. Fresh specimens of C. diffusa and soil from infested and non infested areas were collected from Okanagan Falls. The plants were not in seed and therefore it was not possible to get seeds of this species. Fresh specimens of C. maculosa as well as soil from infested and non infested area were collected from the outskirts of an Indian reserve at Canford. Though there were no seeds available at the time of collection, seeds of this species were obtained through the District Agriculturist at Nelson later in the season.

The leaves, stems and roots of the three species were separated and dried in an oven at a temperature of 80° C. The oven dried material as well as the seeds of C. repens and C. maculosa were then ground in a wiley mill using a 20 mesh screen. The powdered material was put in amber colored, air tight bottles and stored in the dark at room temperature.

Soil studies

Studies with the soil consisted of setting up a series of six-inch pots, three of which contained soil from an infested area of the different knapweeds. The other three, as controls, contained soil from the neighbouring non infested area in each case. In each one of the pots three tomato seedlings (var.Firesteel) which were thirty days old were transplanted. The average height of the three test plants in each pot was recorded periodically. At the end of ninety days the dry weights of both the tops and roots were recorded.

In a second experiment four six-inch pots were filled with greenhouse soil. In three of the pots five, ten and fifteen grams of C. repens air-dried leaf powder were added. A pot to which nothing was added served as a control..Into each pot two thirty day old tomato seedlings (Firesteel) were transplanted. The height of the plants was measured periodically. At the end of sixty days the dry weights of the shoots and roots were recorded and the root-top ratio calculated.

Proximate analysis

A proximate chemical analysis of the powdered leaves, stems and roots of the three species was conducted by the usual methods recommended by the Association of Official Agricultural Chemists (1960). All the analyses was done in duplicate and the results expressed on dry weight basis.

Extraction

Five grams each of the powdered leaf, stem, seed and root of the different varieties was extracted by means of a Soxhlet apparatus using ether as a solvent. The final volume in each case was made up to 100 ml. thereby giving a 5% extract. The temperature maintained during extraction was approximately 65° C.

Five grams each of the powdered leaf, stem, seed and root was put into 200 ml. Erlenmeyer flasks. To each was added 100 ml. of distilled water and the flask then placed on a shaker for twelve hours. The extract was filtered through a Buchner funnel and the final volume brought up to 100 ml. thereby giving a 5% extract.

In order to determine the solubility of the inhibitor, the ether extract was partitioned with an equal volume of water. Each of the two fractions thus obtained was used for germination studies.

Another attempt was made to extract the inhibitor with ethanol. The procedure was similar to that of Soxhlet ether extraction except that a higher temperature around 95° C. had to be employed.

Bioassays

Germination tests were used as a method of bioassay to determine the presence of inhibitors. The indicator plants used in this study were: lettuce (var. New York), barley

(var. Vantage) and cress (var. Pepper). These crops were chosen because they represented different families and included both large and small seeds. Lettuce and cress give a more sensitive response than does barley.

Petri dishes, 9 cm. in diameter, were thoroughly cleansed and repeatedly rinsed with hot water. The washed dishes were then sterilized in an autoclave at a temperature of 125° C. and a pressure of 15 pounds for 15 minutes. In each petri dish an extra thick Whatman filter paper was placed.

In the case of the ether extract, 5 ml. of the ether extract were added to each dish. Then 10 minutes were allowed to elapse so that the ether could evaporate, to avoid inhibiting effects because of ether contamination. Then 5 ml. of distilled water were added. In these tests there were two controls. One with distilled water alone, and one with distilled water added to a dish where ether had been allowed to evaporate.

With the water extract 5 ml. of the different extracts were added to each dish and 25 seeds each of the kinds mentioned above were equally spaced on the filter paper. One dish with only distilled water served as a control.

In the germination studies with the ethanol extracts, 5 ml. of the different extracts were added to the filter paper. The petri dishes were then gently heated on a hot

plate until the filter papers were dry and the ethanol had evaporated. Then 5 ml. of distilled water was added and 25 seeds of the different varieties mentioned were placed in position. Again these two controls; one with distilled water and one with distilled water added to the ethanol dried filter paper, were used.

In all the germination studies the petri dishes were covered and placed in a Mangelsdorf germinator. The temperature of the germinator ranged between 25° C. to 27° C. After a period ranging from 5 to 8 days the number of seeds germinated was determined and the average height of both the shoots and roots of 10 seedlings picked at random was measured. Those seeds where the radicle had emerged to more than 1 mm. were considered as having germinated.

The germination per cent, particularly in C. repens, was determined. A few pre-treatments of the seeds were employed prior to germination, which consisted of either soaking the seeds for 6 hours in water at room temperature, or chilling the dry seeds for 6 hours at 5° C. in a refrigerator, or planting the seeds directly (without pre-treatment) in the petri dishes containing either the filter paper or greenhouse soil.

Isolation

The common procedure of fractionating the crude extract into acidic and basic parts was not attempted, because it

might have led to the addition of other substances, or created conditions which might have interfered with the germination tests.

In order to isolate the inhibitor, paper chromatography was used. Since it was initially determined that the inhibitor was mainly concentrated in the leaves of the three species of knapweeds all further work of isolating the inhibitor was done only with the crude ether extract of the leaf powder.

The crude leaf extract of the three different species consisted of 5 grams of powdered leaves in 100 ml. of ether extracted by the Soxhlet procedure. The 100 ml. volume was initially reduced at 40° C. to 15 ml. in a flash evaporator. The concentrated extract was then centrifuged at a high speed of 10,000 r.p.m., this done in order to get rid of impurities which might usually cause overloading of chromatographic paper. Whatman No. 3 filter paper was used and 5 ml. of the concentrated extract was applied as a band about 5 mm. wide and two inches away from the edge of the paper. The band was applied with the aid of a capillary tube, one end of which had a slight curvature to facilitate application. The following solvents were used:

1. Aqueous phase	4. Isopropanol:ammonia:water
Butanol:acetic:water	10 : 1 : 1
4 : 1 : 5	
2. Organic phase	5. 70% ethanol
Butanol:acetic:water	6. Butanol:ammonia:water
4 : 1 : 5	100 : 3 : 18
3. Water	7. Butanol:ethanol:water
	4 : 1 : 1

Descending chromatography did not give satisfactory resolution, so all chromatographic work was done by employing the ascending method. After application of the band the paper was made into a cylinder and both the edges were stapled. The solvent was poured into a tall cylindrical glass jar and the jar was covered with a wax-coated lid. The solvent was allowed to diffuse into the atmosphere of the glass jar overnight before the Whatman paper cylinder was placed in it.

Of the various solvents mentioned the one which was most satisfactory was the lower aqueous phase of n-butanol:glacial acetic acid:water in the ratio of 4:1:5. The solvent was obtained by mixing the three ingredients and allowing the mixed solvent to stand in a separatory funnel overnight. The lower phase was drained off and used. Any organic phase present interfered with the resolution.

The solvent was allowed to rise until the solvent front reached a distance of 20 cm. which occurred in approximately sixteen hours. After this the paper was taken out, the staples removed and the paper was air dried. The air dried paper was viewed under ultraviolet light (long wave 3660 Å) and the different bands as well as the solvent front were marked and the colour noted.

Each band was cut out as a long strip, folded at one end and placed in a trough containing water as the eluting solvent. The other end of each band was allowed to just dip into a beaker. After a period of eighteen hours, about

15 ml. of the eluate was obtained from each strip.

Germination tests with eluates

To the different petri dishes, which contained extra-thick filter paper, 5 ml. of the eluate from each band was added. For the germination tests, 25 seeds of lettuce were used. The area on the Whatman paper immediately preceding the band or spot of application of the extract served as a control for any inhibition due to the solvent.

To confirm the Rf values of the inhibitory substance, the chromatographic paper which had been banded and developed was cut out into equal parts representing successive Rf values. On each of the strips so obtained, usual germination trials were conducted using lettuce seeds.

Crystallization

The volume of eluates from the inhibitory bands was reduced at its boiling point to an optimal level for crystallization. The Ehrlenmeyer flask containing this eluate was then placed in the refrigerator. As the results were unsatisfactory, another method was also used. In this case, methanol was added to hot and concentrated eluate and then rapidly cooled.

Identification of the inhibitor

The following methods were attempted in an effort to identify the inhibitor:

1. the Rf values of the inhibitor were recorded in different solvent systems
2. the colour under visible light as well as under ultraviolet light was recorded
3. the inhibitory band was exposed to ammonia and its reaction noted, both under visible and ultraviolet light
4. spray tests - the inhibitory band was sprayed with the following reagents and the reaction recorded:

(a) 2 N-NaOH	(f) 1% aqueous FeCl_3
(b) 2 N-HCl	(g) $\text{FeCl}_3 - \text{HClO}_4$ (Salkowski)
(c) 1% ethanolic $\text{Al Cl}_3 - \text{NH}_3$	(h) 1% P-dimethylamino- benzaldehyde (Ehrlich)
(d) 1% aqueous KMnO_4	(i) $\text{KNO}_2 - \text{HNO}_3$
(e) 1% ethanolic FeCl_3	(j) cinnamaldehyde
5. one milligram of the crude crystallized product was dissolved in 2 ml. of water and 3 ml. of ethanol to determine the ultraviolet absorption spectra

IV. RESULTS

A comparative study of the knapweed infested and non infested soil gave results as shown in Table 1. The infested soil of the Russian and spotted knapweed produced marked inhibition both to tomatoes and barley. The height of the shoot was reduced and there was a reduction of the dry weight of both shoots and roots. In the case of the diffuse knapweed the results were the opposite, possibly due to the fact that the non infested soil was collected from an old railway siding, of poor growing characteristics. The inhibition of growth with two of the species is shown in Figure 2.

Addition of 5, 10 and 15 grams of air dried leaf powder to green house soil produced inhibition to the growth of tomatoes. There was a consistent decrease of height as well as a decrease in the dry weight of shoots and roots (Table 2). The difference is graphically represented in Figure 3.

Results of the chemical analyses of the root, stem and leaf of the three varieties are represented in Table 3. The nitrogen content and consequently the amount of protein in the leaves of C. repens was considerably higher than in the other two species.

Preliminary efforts to detect the presence of inhibitors were accomplished through growth and germination studies. The results obtained with the ether extract of the different

SOIL STUDIES

Table 1. Comparative study of growth of tomatoes and barley on infested and non infested soil.

(a) Averages of 3 tomato plants after 90 days.

Type of Soil	Height of Shoot (cms.)	Dry Weight of Shoot (gms.)	Dry Weight of Root (gms.)
Russian	19.5	1.10	0.38
Non Russian	35.2	1.79	0.61
Diffuse	33.6	1.59	0.49
Non Diffuse	29.5	0.85	0.19
Spotted	18.2	0.68	0.20
Non Spotted	49.1	2.44	0.73

(b) Averages of 25 barley seedlings after 25 days.

Type of Soil	Height of Shoot (cms.)	Dry Weight of Shoot (gms.)	Dry Weight of Root (gms.)
Russian	24.0	1.14	1.76
Non Russian	26.5	1.56	2.40
Diffuse	24.0	1.20	1.94
Non Diffuse	22.5	1.10	1.58
Spotted	18.2	0.68	0.20
Non Spotted	21.0	1.10	1.52

Figure 2. Comparative study of growth of tomato seedlings in knapweed infested and non infested soils.

Table 2. Effect of C. repens leaves on the growth of tomato plants.

Averages of two tomato plants.

C. repens leaves (gms.)	Height of Shoot (cms.)							Dry Wt. of Shoot (gms.)	Dry Wt. of Root (gms.)	Root Top
	1st day	10th day	20th day	30th day	40th day	50th day	60th day			
Control	8.5	10.9	14.1	17.3	21.4	25.2	35.1	2.11	0.41	0.19
5	8.7	10.8	13.5	16.5	20.5	24.1	33.2	2.02	0.32	0.16
10	8.6	10.6	12.8	15.1	18.5	22.9	30.1	1.63	0.25	0.15
15	8.0	10.3	11.9	14.1	17.3	21.8	28.5	1.52	0.22	0.14

Figure 3. Effect of the addition of powdered leaves of *C. repens* on the growth of tomato plants in soil.

Table 3. Chemical analysis of 3 Centaurea spp.*

(a) <u>C. repens</u>	Root	Stem	Leaf
Moisture	7.22	4.60	4.12
Ash	8.06	7.30	11.10
Fat	0.80	1.49	2.33
Crude fibre	50.60	42.80	15.65
Nitrogen	0.83	1.46	3.39
Protein	5.20	9.14	21.20
Nitrogen free extract	35.34	39.27	49.72
(b) <u>C. diffusa</u>	Root	Stem	Leaf
Moisture	3.51	4.52	2.43
Ash	13.73	4.07	15.62
Fat	1.19	0.80	2.34
Crude fibre	29.40	41.80	21.75
Nitrogen	0.59	0.43	1.33
Protein	3.71	2.74	8.31
Nitrogen free extract	51.97	50.59	51.98
(c) <u>C. maculosa</u>	Root	Stem	Leaf
Moisture	1.37	2.86	3.73
Ash	43.60	6.53	16.18
Fat	0.78	1.23	2.87
Crude fibre	31.10	43.55	16.85
Nitrogen	0.42	0.87	0.92
Protein	2.62	5.40	5.68
Nitrogen free extract		43.29	46.42

* The values obtained above are expressed in per cent on dry weight basis.

parts of the three knapweeds are presented in Tables 4, 5 and 6, and Figure 4. It was found that an inhibitor was present in all three species. The inhibiting substance was found mostly in the leaves and to a lesser extent in the seeds. The inhibitor was non-specific, being inhibitory to all three test crops (barley, lettuce, cress). There was both germination and seedling growth inhibition; and the growth of roots was more inhibited than that of the shoots.

In order to determine whether the inhibitor was water soluble, both growth and germination studies were again conducted with the water extract. The results are represented in Tables 7, 8 and 9, and Figure 5. The inhibitor was found to be water soluble, and was again present to the greatest extent in the leaf extract. It was inhibitory to both the test plants used (barley and lettuce), exhibiting germination as well as growth inhibition.

An experiment to establish whether the inhibitor was more water or ether soluble proved the latter to be the case. It was also found, however, that the inhibitor was not completely taken up by the ether fraction. Inhibitory activity of a lesser degree was also found in the water fraction, (Table 10).

An ethanol extraction was attempted but it did not prove satisfactory. It was found very difficult to remove

Table 4. Effect of C. repens ether extracts on crop seedlings.*

Test Crop	Knapweed Test Extract	Germination %	Shoots (cms.)	Roots (cms.)
BARLEY	Control water	96	14.2	13.5
	Control ether	96	13.5	12.5
	Leaf	40	2.8	1.4
	Stem	80	15.4	12.8
	Root	80	12.4	4.1
	Seed	96	13.3	10.9
LETTUCE	Control water	100	3.5	3.2
	Control ether	96	3.5	3.3
	Leaf	72	1.5	0.6
	Stem	92	3.0	2.8
	Root	96	2.3	3.1
	Seed	84	2.6	2.2
CRESS	Control water	96	3.1	12.2
	Control ether	96	2.9	10.3
	Leaf	8	1.3	1.0
	Stem	--	--	--
	Root	84	2.3	7.4
	Seed	--	--	--

* Measured 8 days after sowing.

Table 5. Effect of C. diffusa ether extracts on crop seedlings.*

Test Crop	Knapweed Test Extract	Germination %	Shoots (cms.)	Roots (cms.)
BARLEY	Control water	92	9.5	11.1
	Control ether	76	10.1	11.2
	Leaf	68	3.1	3.2
	Stem	88	9.2	11.4
	Root	88	7.3	7.2
	Seed	--	--	--
LETTUCE	Control water	96	3.6	3.6
	Control ether	96	3.2	3.5
	Leaf	80	1.8	0.8
	Stem	96	3.0	3.1
	Root	92	2.5	3.0
	Seed	--	--	--
GRESS	Control water	96	3.1	12.2
	Control ether	96	2.9	10.3
	Leaf	16	1.6	1.4
	Stem	--	--	--
	Root	96	2.4	8.2
	Seed	--	--	--

* Measured 7 days after sowing.

Table 6. Effect of C. maculosa ether extracts on crop seedlings.*

Test Crop	Knapweed Test Extracts	Germination %	Shoots (cms.)	Roots (cms.)
BARLEY	Control water	92	11.5	10.0
	Control ether	92	10.6	9.1
	Leaf	80	4.0	5.3
	Stem	84	10.5	9.4
	Root	84	10.5	7.5
	Seed	80	10.2	7.1
LETTUCE	Control water	100	3.7	3.5
	Control ether	100	3.4	3.3
	Leaf	84	1.8	0.9
	Stem	96	3.1	3.2
	Root	88	2.9	3.0
	Seed	88	2.8	2.4
CRESS	Control water	96	3.1	12.2
	Control ether	96	2.9	10.3
	Leaf	16	1.6	1.5
	Stem	--	--	--
	Root	96	2.7	9.5
	Seed	--	--	--

* Measured 7 days after sowing.

Figure 4. Response of lettuce seedlings to the ether extract of the three knapweeds. The length of histogram above the line indicates the length of shoot, and below the line, the length of root.

Table 7. Effect of C. repens water extracts on crop seedlings.*

Test Crop	Knapweed Test Extracts	Germination %	Shoots (cms.)	Roots (cms.)
LETTUCE	Control water	96	3.4	3.5
	Leaf	64	2.5	0.9
	Seed	92	2.6	2.0
	Root	96	3.6	3.2
BARLEY	Control water	84	12.6	10.2
	Leaf	76	7.5	5.9
	Seed	76	3.5	5.0
	Root	80	11.1	9.4

*Measured 7 days after sowing.

Table 8. Effect of C. diffusa water extract on crop seedlings.*

Test Crop	Knapweed Test Extract	Germination %	Shoots (cms.)	Roots (cms.)
LETTUCE	Control water	96	3.4	3.5
	Leaf	84	2.6	1.0
	Seed	--	--	--
	Root	84	3.1	3.5
BARLEY	Control water	84	12.6	10.2
	Leaf	72	7.7	6.4
	Seed	--	--	--
	Root	80	11.4	9.1

* Measured 7 days after sowing.

Table 9. Effect of C. maculosa water extracts on crop seedlings.*

Test Crop	Knapweed Test Extract	Germination %	Shoots (cms.)	Roots (cms.)
LETTUCE	Control water	96	3.4	3.5
	Leaf	80	2.6	1.5
	Seed	80	2.0	1.9
	Root	92	3.2	3.2
BARLEY	Control water	84	12.6	10.2
	Leaf	72	7.8	6.5
	Seed	84	3.5	3.6
	Root	84	12.1	9.8

* Measured 7 days after sowing.

Centaurea diffusa

Centaurea maculosa

Figure 5. Response of lettuce seedlings to the water extract of the three knapweeds. The length of histogram above the line indicates the length of shoot and below the line, the length of root.

Table 10. Fractionation of the ether extract of leaves of
C. repens.

Lettuce measured after 5 days.

<u>C. repens</u> leaf fraction	Germination %	Shoots (cms.)	Roots (cms.)
Control water	96	2.4	2.5
Control ether fraction	92	2.2	2.4
Control water fraction	96	2.4	2.2
Leaf ether fraction	60	0.6	0.3
Leaf water fraction	82	1.2	0.6

the inhibition caused by the solvent itself. The results are presented in Table 11.

In a comparative study of the leaf extracts of the three different knapweeds it was found that the inhibitor in the leaves of C. repens was more toxic than that from C. diffusa which was again more toxic than that from C. maculosa (Table 12).

As the inhibition was also found to be present in small amounts in the seeds of C. repens and C. maculosa a study was made to obtain the germination percentage of C. repens seeds which were one year old. Results are given in Table 13. The germination percentages after 15 days in the germinator were very low and it was only after a period of a month that the seeds showed maximum germination. Because of the slowness of germination and the low germination percentage it was decided to forgo any further experiments of this type.

Of the various solvents tried the one which gave the best resolution was the lower aqueous phase of n-butanol:acetic acid:water in the ratio of 4:1:5. The ascending method of chromatography proved more suitable than the descending one. In all other solvents, and with the descending method, there was poor resolution due mainly to trailing of associated plant materials.

A diagrammatic representation of the various compounds visible under ultraviolet light (long wave) is presented in Figure 6. A pale yellow fluorescent compound at an Rf of

Table 11. Effect of ethanol extract of C. repens leaves on growth of 2 crop seedlings.*

Test Crop	Knapweed Test Extract	Germination %	Shoots (cms.)	Roots (cms.)
BARLEY	Control water	84	8.8	8.4
	Control ethanol	50	3.8	4.2
	Leaf	48	2.8	2.7
LETTUCE	Control water	92	3.0	3.1
	Control ethanol	60	1.8	1.7
	Leaf	56	1.3	0.9

* Measured 6 days after sowing.

Table 12. Comparative study of the toxic effect of the ether extract of 3 knapweeds.*

Test Crop	Leaf Extracts	Germination %	Shoots (cms.)	Roots (cms.)
BARLEY	Control water	100	7.8	8.1
	Control ether	96	7.7	8.1
	<u>C. repens</u>	40	3.0	1.5
	<u>C. diffusa</u>	68	3.1	2.2
	<u>C. maculosa</u>	68	4.0	2.9
LETTUCE	Control water	92	3.0	3.1
	Control ether	84	3.0	3.2
	<u>C. repens</u>	10	0.2	0.2
	<u>C. diffusa</u>	40	0.5	0.3
	<u>C. maculosa</u>	52	2.0	1.0

* Measured 5 days after sowing.

Table 13. Germination percentage of C. repens seeds.

Treatment	Germination %	
	After 15 days	After 30 days
Untreated	35	85
Soaked	40	85
Chilled	30	56
Soil	30	60

Figure 6. Chromatographic resolution of crude ether extract of leaves of the three species of knapweed as observed under ultra-violet light.

0.9 was common to all three species. A dark absorbing compound was found at an Rf of 0.5 in C. diffusa and C. maculosa. Another light absorbing compound was present at an Rf of 0.6 in C. maculosa. Two blue fluorescent compounds were present only in C. repens.

In locating the inhibitory compound on the chromatographic paper, germination studies with the eluates of the different bands yielded positive results. These findings are presented in Tables 14, 15, and 16. In all three varieties studied the eluate from the pale yellow fluorescent band at an Rf of 0.9 produced severe inhibition to the growth of shoots and roots of lettuce seedlings. There was also a depression of the germination percentage. Growth stimulation, especially of the shoots was observed in the eluates obtained from the solvent front.

A confirmatory method of locating the inhibitor proved to be simpler and more efficient than the elution studies. This was done by means of the spot germination tests, results of which are presented in Tables 17, 18 and 19. Again, severe germination and growth inhibition were encountered at the pale yellow fluorescent band (Rf 0.9). A little inhibition was also found at an Rf of 0.8, the band immediately preceding the inhibitory band. This was considered to be due to trailing. In these studies again a stimulation of the shoots was found at the solvent front. In all three of the knapweeds there was a greater inhibition of the lettuce roots as compared with the shoots. Figure 7 represents the length of

Table 14. Location of the inhibitor on the chromatogram.

Method I. By elution and lettuce germination.

C. repens

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	96	3.6	3.4
Control solvent	Band before spot	92	3.1	3.3
0.0	Spot	92	3.0	3.1
0.1	--	96	3.5	3.7
0.2	Blue	96	3.5	3.4
0.3	--	92	3.4	2.3
0.4	--	96	3.6	3.2
0.5	Light absorbing	92	3.2	3.3
0.6	--	96	3.3	3.2
0.7	--	96	3.1	3.0
0.8	Blue	80	2.4	2.1
0.9	Yellow	64	1.1	0.6
1.0	Solvent front	96	5.8	3.9

Table 15. Location of the inhibitor on the chromatogram.

Method I. By elution and lettuce germination.

C. diffusa

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	92	3.8	3.2
Control solvent	Band before spot	92	3.5	3.3
0.0	Spot	88	3.3	3.2
0.1	--	84	3.7	3.0
0.2	--	88	3.7	3.2
0.3	Dark absorption	92	3.5	3.1
0.4	Dark absorption	92	3.4	3.3
0.5	Dark absorption	88	3.5	3.1
0.6	--	84	3.4	3.3
0.7	--	88	3.2	3.0
0.8	--	80	2.0	1.6
0.9	Yellow	68	1.2	0.8
1.0	Solvent front	96	4.5	3.2

Table 16. Location of the inhibitor on the chromatogram.

Method I. By elution and lettuce germination.

C. maculosa

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	92	3.3	3.8
Control solvent	Band before spot	88	3.3	3.6
0.0	Spot	85	3.2	3.6
0.1	--	92	3.3	3.5
0.2	--	88	3.2	3.4
0.3	--	80	3.1	3.4
0.4	Dark absorption	84	3.4	3.5
0.5	Dark absorption	88	3.2	3.3
0.6	Light absorption	92	3.0	3.2
0.7	--	84	3.1	3.4
0.8	--	80	3.4	3.0
0.9	Yellow	76	1.4	1.0
1.0	Solvent front	88	4.0	4.0

Table 17. Location of the inhibitor on the chromatogram.

Method II. By Spot germination

C. repens

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	100	3.6	3.5
Control solvent	Band before spot	95	2.9	2.8
0.0	Spot	85	2.8	2.8
0.1	--	80	3.0	2.8
0.2	Blue	85	2.8	2.9
0.3	--	90	2.9	2.8
0.4	--	85	2.7	2.8
0.5	Light absorption	90	2.9	2.8
0.6	--	100	2.6	2.7
0.7	--	95	2.7	2.6
0.8	Blue	80	1.5	0.9
0.9	Yellow	25	0.5	0.1
1.0	Solvent front	95	3.9	2.8

Table 18. Location of the inhibitor on the chromatogram.

Method II. By Spot germination

C. diffusa

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	95	3.6	3.5
Control solvent	Band before spot	95	3.0	2.7
0.0	Spot	90	2.9	2.7
0.1	--	100	2.5	2.5
0.2	--	85	2.7	2.4
0.3	Dark absorption	90	2.8	2.0
0.4	Dark absorption	85	2.9	2.6
0.5	Dark absorption	80	2.9	2.6
0.6	--	90	2.6	2.4
0.7	--	85	2.0	2.1
0.8	--	80	1.0	0.4
0.9	Yellow	35	0.6	0.2
1.0	Solvent front	95	4.0	2.9

Table 19. Location of the inhibitor on the chromatogram.

Method II. By Spot germination

C. maculosa

Rf	Nature of Band	Germination %	Shoots (cms.)	Roots (cms.)
Control water	--	100	3.6	3.5
Control solvent	Band before spot	95	3.2	3.0
0.0	Spot	90	3.0	3.0
0.1	--	95	2.9	3.1
0.2	--	100	3.2	3.5
0.3	--	90	3.1	3.4
0.4	Dark absorption	85	3.4	3.4
0.5	Dark absorption	90	3.5	2.9
0.6	Light absorption	90	3.4	3.2
0.7	--	80	3.2	3.0
0.8	--	65	2.0	1.9
0.9	Yellow	40	0.8	0.2
1.0	Solvent front	95	4.9	2.9

Figure 7. Spot germination tests conducted directly on the successive Rf regions of the developed chromatogram using lettuce seeds.

the roots in the different Rf's. The greatest inhibition was again at an Rf of 0.9.

The eluates of the inhibitory bands were concentrated by reduction under vacuo and stored at 5° C. in an attempt to crystallize the compound. After a period of one month the results were negative. As there was no crystallization, methanol was added and the solution then heated. This method produced an immediate precipitation which was considered to be due to the inhibitory compound.

The inhibitory compound on the chromatogram was tested with various sprays outlined for coumarins and their derivatives. In addition, sprays for aromatic acids were used. The results of all these trials proved negative; the compound remaining yellow (Table 20).

The spray tests outlined for indole and its derivatives yielded positive results. The compound was reactive to both the Salkowski and the Ehrlich reagents, turning pink and pinkish brown respectively. The results obtained with the chromogenic sprays for indoles are presented in Table 20.

In order to confirm the presence of an indole nucleus, an ultraviolet absorption spectrum was run (Figure 8). The absorption spectrum is similar to that expected of compounds with an indole nucleus, having a maximum at 2250 Å and secondary maxima at 2700 Å and 2790 Å (91).

In order to characterize the unknown inhibitory compound further, studies of the Rf's obtained with different

Table 20. The inhibitory band -
color reactions with chromogenic sprays.

Treatment	<u>C. repens</u>	<u>C. diffusa</u>	<u>C. maculosa</u>
Visible light	faint yellow	faint yellow	faint yellow
Ultra-violet (short wave)	greenish yellow	greenish yellow	greenish yellow
Ultra-violet (long wave)	yellow*	yellow*	yellow*
NH ₃ vapour	--	--	--
<u>SPRAYS FOR COUMARIN</u>			
2 N-NaOH	--	--	--
2 N-HCl	--	--	--
1% ethanolic Al Cl ₃ - NH ₃	--	--	--
1% aqueous KMnO ₄	--	--	--
1% ethanolic FeCl ₃	--	--	--
1% aqueous FeCl ₃	--	--	--
<u>SPRAYS FOR INDOLE COMPOUNDS</u>			
FeCl ₃ - HClO ₄	pink	pink	pink
1% P-dimethylamino- benzaldehyde	pinkish mauve	pinkish mauve	pinkish mauve
KNO ₂ - HNO ₃	yellow brown	yellow brown	yellow brown
Cinnamaldehyde	yellow brown	yellow brown	yellow brown

*fluorescence

Figure 8. Ultraviolet absorption spectrum of the crude inhibitory compound isolated from *C. repens*. The sample shows absorption maximum at 2250 Å and additionally two maxima between 2700 Å and 2790 Å.

solvents were made (Table 21). It was observed that the compound moved rapidly with aqueous solvents and very slowly, being almost immobile, in organic solvents. The Rf's obtained with synthetic indole acetamide and tryptophane are also presented, as the inhibitory compound was suspected of being one of these. The Rf's for both compounds were run. Those for indole acetamide were not available in the literature. The comparative study indicated that the inhibitor was neither tryptophane nor indole acetamide and Rf's did not coincide with those of the known indoles.

Table 21. Rf values of the inhibitory compounds of Centaurea spp. in different solvents, compared with the Rf values of indole acetamide and tryptophane.

Rf values			
Solvent	<u>Centaurea</u> spp. inhibitor	Indole acetamide	Trypto- phane*
Aqueous phase			
Butanol:acetic:water 4 : 1 : 5	0.90	0.74	0.76
Organic phase			
Butanol:acetic:water 4 : 1 : 5	0.24	--	--
Water	0.82	0.64	0.63
Isopropanol:ammonia:water 10 : 1 : 1	0.16	0.78	0.19
70% ethanol	0.60	0.76	0.40
Butanol:ammonia:water 100 : 3 : 18	0.10	--	--
Butanol:ethanol:water 4 : 1 : 1	0.12	0.80	0.26

* Some of the Rf values for tryptophane are recorded from literature. (74).

V. DISCUSSION

The presence of naturally occurring inhibitors in plants is now an established fact. These inhibitors occur not only in a wide variety of plants but also in different parts of the plant. It was also observed that the knapweeds grew in dense infestations and outcompeted other species. The rapidity with which they have established themselves, and their tendency to exclude other species leads to the belief that the knapweeds could be making the soil toxic to other species and may contain some inhibitory substances.

The present work with the leaves, stems, seeds and roots of the three different knapweeds: Russian, spotted and diffuse, has confirmed the presence of an inhibitory substance in all three of the species studied. The inhibitory substance was found to be located mainly in the leaves and in smaller amounts in the seed. No inhibitory substance was found either in the stem or the roots.

It was also found that the knapweed infested soil was inhibitory to the growth of tomatoes and barley. This leads one to believe that the toxicity found in the knapweed soil is due to the inhibitor of the leaves becoming incorporated into the soil, either by rain washing it out of the leaves, or because the leaves are broken down in the soil when they fall. Though the inhibitor was not found in the roots it would be wrong to assume that it may not be excreted as a

root exudate. At the time of collection which was in summer the inhibitor was found to be located mainly in the leaves. In another season it is quite possible that the inhibitor could also be translocated to the roots.

Though the presence of inhibitors in a number of species of plants has been established, yet the exact nature of the inhibitory substance has been shown only in a very few cases. In the present study, the inhibitor was found to be both ether and water soluble, being more soluble in ether.

By chromogenic sprays as well as by means of an ultra-violet absorption spectrum it was found that the inhibitory compound was an indole. Specific colour reactions with the inhibitory compound indicated that it might be indole acetamide or tryptophane. However, comparison of Rf's with the synthetic compound's did not confirm either of these. It could be concluded, therefore, only that the inhibitor found in the leaves of the knapweeds studied was an indole derivative, the exact nature of which was not known since the color reactions and Rf's did not coincide with any other known compound.

The inhibitory compound having been identified as an indole, provides further evidence that precursors of auxin, or auxin itself in higher concentrations, could be an inhibitor.

The function of these inhibitors can only be speculated upon. The inhibitors may perform a part in the dual regu-

lation of growth. In the form of a precursor they might be inhibitors, thereby causing such phenomena as dormancy. When converted to IAA they then could act as an auxin, promoting growth. They could also be responsible for the production of toxic materials which are sometimes poisonous to the parent plant. Usually, however, the inhibitor is toxic to enzymes systems of other species, enabling the species producing it to outcompete and exclude other forms. This may partially explain why the knapweeds are rapidly establishing themselves at the expense of other plants.

VI. CONCLUSION

This study conducted on the presence, location, nature and role of growth inhibiting substances in Centaurea spp. gave evidence of the following:

1. a growth inhibiting fraction is present in all three species of Centaureas studied
2. soil on which different knapweeds were growing was found to be inhibitory to the growth of tomatoes
3. addition of air dried leaf powder of C. repens to greenhouse soil also produced inhibitory effects on the growth of tomatoes
4. the inhibitor is mostly located in the leaves of the three species tested, but is present in smaller amounts in the seed of C. repens and C. maculosa
5. studies with the stems and roots did not indicate the presence of any inhibiting substance
6. the inhibitor was found to be both ether and water soluble, though the inhibitor in the leaves was more ether soluble, and that of the seeds was more water soluble
7. the inhibitor was located as a pale yellow fluorescent band at an Rf of 0.9
8. as the inhibitor was water soluble the inhibitory band could be eluted using water as the eluting solvent
9. attempts to crystallize the water eluate of the inhibitor yielded negative results, however, addition of

methanol and evaporation resulted in precipitation

10. chromogenic sprays and comparison of Rf's indicated that the unknown inhibitor was not coumarin or one of its derivatives

11. the sprays for indole compounds gave positive tests thereby indicating that the inhibitory compound is an indole derivative

12. the ultraviolet spectrum was also typical of compounds having an indole nucleus

13. a comparison of the various spray tests showed similarities between the unknown and both indole acetamide and tryptophane but Rf values in various solvents would indicate that it is neither.

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