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PHYTOTOXIC EXUDATES FROM VELVETLEAF
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GLANDULAR TRICHOMES
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Tracy M. Sterling

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PHYTOTOXIC EXUDATES FROM VELVETLEAF (*Abutilon theophrasti* Medic.) GLANDULAR TRICHOMES

By

Tracy M. Sterling

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ABSTRACT

PHYTOTOXIC EXUDATES FROM VELVETLEAF (Abutilon theophrasti Medic.) GLANDULAR TRICHOMES

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Glandular trichomes, one of four types present on velvetleaf stems and petioles, exude water soluble globules which were efficiently collected by wiping with cotton swabs. Aqueous extracts of the exudate (4 mg/2 ml H_2O) inhibited root growth of several weed and crop species in petri plate bioassays. Cress (Lepidium sativum L.) was the most sensitive species. The quantity and phytotoxicity of the exudates varied when velvetleaf was grown under different temperature and water regimes, densities, and between two velvetleaf accessions. Both accessions interfered with soybean growth similarly in the field. Trichome exudate did not appear to contribute to the interference velvetleaf imposed on neighboring species in field and greenhouse studies. The extract from trichomes was quickly deactivated in non-autoclaved soil except at the highest dosage. Partitioning with solvents and chromatography (column, thin layer, and high performance liquid) were employed to separate the phytotoxic components.

DEDICATION

To my parents, Ruth and Richard

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Velvetleaf (Abutilon theophrasti Medic.) was introduced into the United States by the early colonists who produced it as a fiber crop (Spencer,1984). It has since become widely distributed across the eastern and midwestern United States and is now a major broadleaf weed problem in corn (Zea mays), cotton (Gossypium hirsutum) and soybeans (Glycine max). The economic loss due to velvetleaf in corn and soybeans is estimated to be $343 million annually (Spencer,1984). Yield losses due to the presence of velvetleaf have been attributed to its interference with crop growth. Interference may be mediated through competition for limited resources (light, nutrients, and water) and allelopathy, the chemical effects of one plant on the growth and development of another. Several investigators have suggested that velvetleaf interference is due to allelopathy (Bhowmik and Doll, 1982; Colton and Einhellig, 1980; Dekker et al., 1983; Gressel and Holm, 1964; Houtz et al., 1984). Houtz et al. (1984) observed that the numerous glandular trichomes on velvetleaf stems and petioles exude liquid globules. An aqueous extract of these globules severely inhibits radicle elongation of cress (Lepidium sativum) seeds. It was hypothesized that these trichome exudates may play a role in the

interference that velvetleaf imposes on other plants.

The purpose of this thesis is to assess the allelopathic potential of exudates from the glandular trichomes on velvetleaf stems and petioles. Although other routes of allelopathy may be involved, this research evaluates only the chemical effects of the exudate while excluding other factors of interference. Benefits of such research might include a better understanding of allelopathic mechanisms, their importance in agriculture and potential usefulness as naturally occurring herbicides.

COMPONENTS OF PLANT INTERFERENCE

Interactions among plants have long been the object of much study and controversy. Many of the ideas of population dynamics, community structure, adaptation and demography can be attributed to animal ecologists (Harper, 1961). However, plants are more stationary organisms, usually unable to move away from undesirable locations or to move to sites where resources are more plentiful. Therefore, they have developed a variety of physical and chemical strategies which may aid survival or even dominance in a plant community. Interference among plants "includes all forms of reaction by one plant that prove deleterious to another" (Muller, 1969). Interference encompasses all the interactions among species in mixtures which lead to reduced growth (plasticity) or increased mortality in one or both species (Harper, 1961). Unfavorable effects that neighboring plants impose on one another's growth may involve both competition and allelopathy (Fuerst and Putnam, 1983; Harper, 1977; Muller, 1969; Rice, 1984). A third

source of interference may be the indirect effect of environmental and physical factors, such as temperature, disturbance, or herbivory, which alter the growth of neighboring species (Fuerst and Putnam, 1983; Halligan, 1976; Harper, 1977). Experiments designed by ecologists and agricultural scientists have evaluated how species respond when grown in mixtures. Studies such as replacement series, additive and substitutive arrangements, diallel designs and neighborhood modeling evaluate the effects neighbors have on one another but contribute very little to an understanding of the mechanism (Dekker et al., 1983; Fennimore et al., 1984; Harper, 1977; Hill and Shimamoto, 1973; Weiner, 1982).

Competition

Competition is one mechanism of plant interference. It is defined as the depletion of one or more resources, e.g. light, carbon dioxide, water or mineral nutrient, from the environment which limits the growth and development of a neighboring plant (Grime, 1977; Harper, 1977; Hill and Shimamoto, 1973; Rice, 1984). Many "competition" studies have been conducted which actually evaluate the overall effect (interference) of weeds on crops. Yield reductions have been related to the duration of weed presence and to weed density. Many investigators have attributed the yield losses caused by weeds to competition without additional experiments to test which factors of interference were actually responsible (Rice, 1984). Some investigators have attempted to evaluate the effects of a limiting resource (light, nutrients, and water) as to their influence in total interference (Harper, 1977; Rice, 1984; Zimdahl, 1980). Because these

factors are closely interrelated, ie. water and nutrient uptake, it is difficult to study any one factor without affecting another (Zimdahl, 1980). Aldrich (1985) has proposed categorizing the relative importance of each of these factors on soybean yield. He hypothesizes that the chronology of factors responsible for soybean yield loss was shading compounded later in the season by water competition, then allelopathy, and finally by competition for phosphorous and potassium.

Allelopathy

Another component of interference is the biochemical interactions among plants (including microorganisms) termed allelopathy. Molisch and Rice have both used the term to encompass both the stimulatory and inhibitory effects that a chemical produced by one plant will have on another (Rice, 1984). This mechanism of interference can be separated from others because the deleterious effect results from the release of a chemical by the interfering plant. Allelopathy appears to alter plant distribution in both natural and agroecosystems (Grummer, 1961; Lovett, 1982; Muller, 1969; Newman, 1978; Putnam and Duke, 1978; Whittaker, 1969; Whittaker and Feeny, 1971). Allelopathy may alter community dynamics by influencing ecological processes (Muller, 1966). Wilson and Rice (1968) have found a correlation between the sensitivity of the associated species to Helianthus annuus extracts and the pattern of those species around H. annuus in the field. However, no such correlation was found for plant distribution in an Illinois old-field (Stowe, 1979). For many plant communities, laboratory bioassays may have no ecological meaning.

Allelochemical Release: An essential component of allelopathy is the movement of the allelochemicals into the environment (Rice, 1984; Whittaker, 1969). This may involve volatilization (Halligan, 1975; Heisey and Delwiche, 1984; Muller and Muller, 1964), root exudation (Rovira, 1969; Tang and Young, 1982), residue decomposition (Behmer and McCalla, 1963; Elliot et al., 1978) and exudation or leaching by rain (Grummer and Beyer, 1960; delMoral and Muller, 1969). The nature of the compounds released include phenolics, terpenoids, alkaloids, nitriles and lactones (Rice, 1984; Whittaker, 1969). These have been called secondary compounds, since they have no obvious metabolic function (Swain, 1977).

Many techniques have been implemented to study allelopathy (Putnam and Duke, 1978). However, when attempting to identify the toxins responsible for the alleged allelopathic effect, manipulation and masceration of the plant material are common practices. This method of analysis may allow for the release of compounds normally compartmentalized or conjugated within the plant cell. These compounds may be different from allelochemicals that occur naturally in the plant or its environment. Evaluation of intact plants or plant organs for allelopathic potential may more accurately identify the allelochemical responsible for the toxic activity. One such method is the study of plant leachates or exudates.

Leaching: Morgan (1963) defines leaching as the removal of metabolites from plant parts by aqueous solutions. Organic compounds (carbohydrates, amino acids, organic acids) and inorganic compounds can be leached from many plants by rain, dew, or mist (Tukey, 1966). Fog drip from under Eucalyptus globulus contained chlorogenic acid, p-

coumarylquinone, and gentisic acid which were inhibitory to Bromus rigidus (delMoral and Muller, 1969). Various acids were identified in aqueous leachates from the foliar branches and leaf litter of Arctostaphylos glandulosa and were toxic to the growth of annual grasses (Chou and Muller, 1972). The artificial and natural leachates of intact Adenostema fasciculatum crowns inhibit Bromus rigidus seedling growth (McPherson and Muller, 1969). Leachates from intact leaves of A. fasciculatum were also toxic and contained nine phenolic compounds and one non-phenolic substance (McPherson et al., 1971). Five of the phenolic toxins and the non-phenolic toxin were also identified from the soil beneath the shrub canopy indicating the toxins may influence the patterning of surrounding vegetation through soil activity. Grummer and Beyer (1960) reported up to 40% reductions in flax (Linum usitatissimum) yield when artificial rain washed a substance from false-flax weed, Camelina alyssum, onto the flax plants. Several phenolic compounds including p-hydroxybenzoic acid and vanillic acid were identified and found toxic when assayed against Lepidium root growth (Grummer and Beyer, 1960). They speculated that these phenolic compounds comprise less than 10% of the toxic compounds leached from the leaves by rain. Therefore, a variety of chemicals may combine to elicit an allelopathic response. Lovett and Sagar (1978) found that bacteria present on the leaves of false-flax (Camelina sativa) break down a chemical exuded by its leaves. The components produced are less complex and include benzylamine, which can disrupt the functioning of cell membranes and also may modify soil structure resulting in increased soil moisture with enhanced benzylamine concentrations (Lovett, 1982). Therefore, Lovett speculates this allelochemical may

act directly on the flax plant or indirectly by altering the soil where flax is grown.

Release from Trichomes: Exudation or secretion from trichomes is another possible method for allelochemical release. Trichomes are highly variable appendages derived from the epidermis, including glandular (or secretory) and non-glandular hairs (Uphof, 1962). Trichomes often have unicellular or multicellular heads composed of cells which produce secretions and which can be located on a stalk of non-glandular cells (Esau, 1977). They vary widely in structure and sometimes can be used for taxonomic purposes (Behnke, 1984; Luttge, 1971; Payne, 1978). To help clarify the often obscure function of plant trichomes, a recent symposium focused on their structure, development, chemistry, and ecological significance (Rodriguez et al., 1984). Trichomes may have anti-herbivore and water loss functions, but no conclusive data are available to resolve these possibilities (Ehleringer, 1984). Although pubescence increases the boundary layer on the leaf surface, the effect on overall transpiration rate is small (Ehleringer, 1984). Since light reflectance is increased when trichomes are present, leaf temperature and photosynthesis both decrease (Ehleringer, 1984). Trichomes may play a role in insulating the mesophyll from excessive heat (Black, 1954).

Trichomes may also provide physical and chemical defense against insects (Johnson, 1975; Levin, 1973). In some species, trichome density is negatively correlated with insect feeding and oviposition and with the nutrition of the larvae (Hoxie et al., 1975; Johnson, 1975; Levin, 1973). Hooked trichomes may impale and kill adult insects and their larvae (Levin, 1973). Glandular trichome exudates from mite-

resistant geraniums (Pelargonium hortorum) were found to contain two anacardic acid derivatives, o-pentadecenylsalicylic acid and o-heptadecenylsalicylic acid (Gerhold et al., 1984). These compounds were moderately toxic when bioassayed with the two-spotted spider mite, Tetranychus urticae. Gibson and Pickett (1983) found the aphid alarm pheromone, (E)-β-farnesene, present in substantial quantities in air around wild potato (Solanum berhaultii) foliage. This chemical, which secretes from leaf trichomes, also induced rapid dispersal of settled aphid colonies. Green peach aphids (Myzus persicae) were killed by contact with secretions from the trichomes of several Nicotiana species (Thurston et al., 1966). These secretions contain alkaloids, of which nicotine is the major constituent.

A variety of secondary compounds are localized in or secreted from plant hairs. These include flavonoids (Wollenweber, 1984), phenols (Beckman et al., 1972), sesquiterpene lactones(Kelsey and Shafizadeh, 1980; Henderson et al., 1970), and monoterpenes (Venkatachalam et al., 1984). Isoprenoids (mono-, sesqui-, and diterpenes) are the most common metabolites found in the trichomes of flowering plants (Kelsey et al., 1984). These secondary products of the plant system may have numerous ecological roles.

Secretions from trichomes may also be involved in allelopathy (Lovett, 1982; Seigler, 1977; Swain, 1977; Whittaker and Feeny, 1971). For example, the glandular hairs of Primula obonica secrete a phytotoxic quinone (Schildknecht et al., 1967). Funke (1943) observed that leaves of Artemisia absinthium bear glandular hairs which excrete ethereal oils and the alkaloid, absinthin. His studies indicated that plants in close proximity to A. absinthium had reduced vigor, and he

attributed this primarily to absinthin, which washed off Artemsia plants. By releasing phytotoxic chemicals into its environment, the plants may interfere with the growth of neighbors and ultimately dominate the community.

Factors Influencing Secondary Product Formation: Factors such as, light quality and intensity, mineral deficiencies, age of the plant, pathogens and predators, water stress, temperature, and genetics affect the production of allelochemicals (Rice, 1984). The plasticity of secondary product formation in response to stress may aid in plant adaptation and survival particularly when resources are limiting.

Tso et al. (1970) found significantly higher levels of alkaloids in tobacco (Nicotiana tabacum) plants which were receiving red rather than far red light. In contrast, there was a higher concentration of soluble phenols in tobacco plants receiving far red light when compared to those receiving red light. Increasing the intensity of U.V. light enhanced scopolin concentration in tobacco stems and leaves and in sunflower (Helianthus annuus) leaves (Koeppe et al., 1969). In this same study, low intensity U.V. provided increased levels of chlorogenic acid in tobacco stems, leaves, and roots and reduced levels in sunflower leaves. Ultraviolet light increased the concentration of phenolic compounds in sunflower tissue when nitrogen was abundant but decreased the concentrations when nitrogen was deficient. When Phalaris aquatica plants were shaded to 30-50% of full sun, alkaloids increased but were not affected in environmental chambers (Ball and Hoveland, 1978). The alkaloid concentrations were greatest in the upper one-half of the leaves, less in the leaf sheaths and least in the stems. Hanson et al. (1983) also found varying amounts of secondary

products depending on the plant part tested. There was a high level of the indole alkaloid, gramine, in the first leaf of barley (Hordeum vulgare) with lower concentrations in successive leaves. The gramine concentration decreased sharply as the barley plant aged. Grummer and Beyer (1960) found the production of the alkaloid, absinthin, increased as false-flax weed (Camelina) aged but this may have been because of increased plant weight.

Temperature may also have an effect on secondary product formation. Flavonol biosynthesis by the fern, Asplenium trichomanes, increased when grown at a low temperature (12C) (Lebreton, 1972). However, leucoanthocyanin, also produced by the fern, was not sensitive to this environmental parameter. Gramine content in barley increased when plants were transferred from a 21/16C environment to a 30/25C environment (Hanson et al., 1983).

Water is the single environmental resource most commonly limiting plant growth (Kramer, 1980). Water deficiencies change plant metabolism and therefore, secondary product formation (Gershenzon, 1984). For example, monoterpene concentrations in loblolly pine (Pinus taeda) xylem changed when under water stress. In stressed trees, α-pinene concentrations increased and β-pinene, myrecene, and limonene concentrations decreased (Gilmore, 1977). Ball and Hoveland (1978) found greater levels of alkaloids in water stressed Phalaris aquatica plants when grown in field and greenhouse studies. When studying the alkaloid content of threadleaf groundsel (Senecio longilobus), Briske and Camp (1982) monitored water stress by measuring its potential. Severe water stress of -20 bars for a 32 days increased total plant alkaloid concentration 4.6 times in comparison to less water stressed

plants (-10 bars).

Genetics may also play a role in the quantity and quality of secondary compounds produced by plants. Putnam and Duke (1974) screened 526 cucumber (Cucumis sativa) accessions and found several to be inhibitory to the growth of two weed species. Soybean cultivars vary in their ability to inhibit the growth of velvetleaf and foxtail millet (Setaria italica) chemically (Rose et al., 1984). Several lines of oats (Avena fatua) have also been found allelopathic (Fay and Duke, 1977). The genotypes of Trifolium repens less favored by predators contained higher amounts of cyanogenic compounds (Daday, 1965). However, these genotypes also had reduced low-temperature tolerance. Hanover (1966) found the monoterpene content of western white pine (Pinus monoticola) to be under rigid genetic control. There was no difference in monoterpene levels when genotypically identical trees were grown under diverse environmental conditions.

VELVETLEAF

History and Distribution

The source of origin for the malvaceous weed, velvetleaf, is thought to be China where it was an important fiber plant. It was introduced to the United States as a fiber crop before 1750 to help fulfill the cordage needs of the maritime industry and the American colonies. Commercially, this venture was unsuccessful. Velvetleaf could not compete successfully with the established hemp industry due to a lack of proper equipment for adequately processing the fiber. Also, it was documented that velvetleaf was becoming a problem weed in

Illinois and there were conflicting assessments as to its merit as a rope source (Spencer, 1984). Velvetleaf now grows throughout most of the Corn Belt. Andersen et al. (1985) found that velvetleaf will produce seed as far south as Weslaco, TX (26° 09'N) and as far north as Rosemount, MN (44° 43'). Its north-south range appears to be increasing and recently a heavy infestation was sighted near Wolverton, MN (46° 34'N).

Growth and Development

Kheider and Roeth (1981) found 51 million viable velvetleaf seeds/ha in the top 20 cm of soil. Velvetleaf can produce 17000 seeds/plant during a growing season. It emerges best from about 2.0 cm below the soil surface, but is capable of coming up from a depth of 7.6 cm (Chandler and Dale, 1974). It develops a strong, well-developed taproot and can grow to a height of 3-4 m within 10 weeks after it emerges. Velvetleaf grows rapidly during its sixth through tenth week.

This plant bears large heart shaped leaves born alternately on long petioles attached to a simple erect stem (Luckan, 1917; Spencer, 1984). Both leaf surfaces are densely covered with four types of trichomes (Luckan, 1917). The non-glandular types are the unbranched single-celled form or the stellate form. The two glandular types are either long, made up of 12 to 15 cells, or short, with only four to five cells. The cells of the longer trichomes have a thin cuticle except the cell just above the basal cell which has its cell walls cutinized throughout. The apex of the 12 to 15-celled trichome "is rounded out, and here drops of oil collect as it is secreted" (Luckan, 1917). The smaller glandular trichomes occur along the leaf midrib and

larger veins. These glandular and nonglandular trichomes are also present on the stem.

Flowers are yellow with 5 petals and produce a 12-15 chambered fruit (carpels), known as a capsule (Winter, 1960). Normally three seeds are produced in each carpel or a total of 36-45 seeds for each capsule (Winter, 1960).

Interference by Velvetleaf

Interference studies are conducted to establish the minimum weed densities and durations allowable for maximum crop production. This helps to establish weed thresholds whereby appropriate management strategies can be implemented to minimize crop loss. The deleterious effects of velvetleaf are well documented, however, rarely have the components of interference been evaluated. Schweizer and Bridge (1982) found that 6, 12, 18 and 24 velvetleaf plants/30 m of row reduced sugarbeet root yield by 14, 17, 25 and 30%, respectively. Seed cotton yields were significantly reduced when velvetleaf was present at 1.3 plants/m of crop row for the entire season (Chandler, 1977). Delay of velvetleaf emergence from 4 to 6 weeks did not reduce seed cotton yields. Velvetleaf densities ranging from 67 to 114 plants/m^2 significantly reduced the stem diameter and yield of field corn (Weaver, 1983). Corn grain yield, number of seeds/ear, and soil moisture content were significantly reduced when velvetleaf was present (DeFelice, 1984). There was no yield reduction when velvetleaf emergence was delayed six weeks.

Hagood et al. (1980) concluded that velvetleaf densities ranging from 5 to 40 plants/m^2 caused significant reductions in soybean

vegetative and reproductive growth. Soybean seed yield was reduced by 27 to 56%. Soybean yield losses at high velvetleaf densities decreased under high moisture conditions. Eaton et al. (1976) demonstrated soybean seed yield losses as high as 720 kg/ha (23% reduction) from velvetleaf interference. Pods per plant was the major component of seed yield that was reduced. Densities of 1.6 and 3.3 velvetleaf plants/m^2 reduced soybean yield 13 and 27% when soybeans were planted in mid May and 11 and 13% when soybeans were planted in late June (Oliver, 1979). This difference in yield loss was attributed to the short-day photoperiodic response of velvetleaf. Velvetleaf flowered 6 weeks after the late planting date which was 2 weeks sooner than it had flowered following the early planting date. This caused cessation in vegetative growth and probably reduced competitive interference. Staniforth (1965) found that 3.3 velvetleaf plants/m of row when present for the entire season reduced soybean seed yield by 31% when compared to weed-free checks. However, in another study, there was no significant soybean yield loss when velvetleaf was present at 9.8 and 19.7 plants/m of row (Staniforth and Weber, 1956). There were significant reductions in soybean dry matter, flowering nodes and seed yield when velvetleaf densities ranged from 2.4 to 4.7 plants/m^2 (Dekker and Meggitt, 1983a). Dekker and Meggitt (1983b) also investigated the population dynamics between these two species and concluded that velvetleaf has an adaptive ability for differential mortality. The mortality rate of velvetleaf will change in the presence of soybeans, whereas, the mortality rate of soybeans will remain constant with varying densities of velvetleaf. Because soybeans lack differential mortality, the individual plants are less productive

and yield is reduced.

Many investigators have speculated that the yield losses due to velvetleaf involve competition for water (DeFelice, 1984; Dekker and Meggitt, 1983a; Hagood, 1980; Oliver, 1979), light (Chandler, 1977; Oliver, 1979; Staniforth, 1965), and nutrients (Oliver, 1979), and/or allelopathy (Dekker and Meggitt, 1983a), however, none of these possibilities was adequately tested. Aldrich (1985) attributed soybean yield loss to a "compounding of effects" when weeds are present. He suggests that reduction in soybean yield due to early weed presence is the direct effect of shading. As the duration of weed presence continues into the season, yield loss increases and this is due to shading, allelopathy, and competition for light, then water. Full season weed interference involves shading, allelopathy, and competition (for light, water, phosphorous and potassium in that order). In contrast, Dekker and Meggitt (1983a) found severe reductions in the number of soybean flowering nodes early in the season. At this time velvetleaf had not yet canopied over the soybeans. This provides evidence against competition for light as the main cause of interference at this stage of soybean development. Using simulated shade, Stoller and Woolley (1985) investigated what portion of total interference in soybeans was due to competition for light. Velvetleaf at 1 to 2 plants/m^2, forms a full canopy which intercepts 44 to 56% of sunlight. This amount of shade was simulated using black polyethylene in 5-cm-wide strips attached to poultry netting. Soybean yield reductions due to shade without weeds were 19 to 26%. Velvetleaf at 0.7 to 2.5 plants/m^2 has been reported to reduce soybean yields 12 to 31%. Stoller and Woolley conclude that most of the interference

velvetleaf imposes on soybean is due to competition for light. This study is important in that it implemented the first 3 of the 4 criteria set by Fuerst and Putnam (1983) necessary to separate the various components of interference.

Other factors such as cultural practices, soil conditions and temperature may affect how plants interfere with one another. Flint et al. (1983) found that temperature can modify the competitiveness of a species. Using a modification of deWit's replacement series design, the relative yield of cotton when grown in monoculture with velvetleaf was calculated. The relative yield for cotton was greater than 1.0 when it was grown with velvetleaf at 32/23C. The value was approximately 1.0 when grown at 26/17C. A relative yield value equal to 1.0 indicates that cotton performs the same when grown with velvetleaf as when grown with itself at the same density. When the relative yield value is greater than 1.0, the yield per cotton plant is greater when grown with velvetleaf than when grown with itself. Therefore, cotton interferes more with velvetleaf at 32/23C in comparison to 26/17C. This is substantiated by the results of Chandler (1977). In 1973, four velvetleaf plants/12 m significantly reduced cotton while in 1974, 32 velvetleaf plants/12 m were needed to achieve the same yield reduction. Chandler attributed this to the warmer, more favorable growing conditions in 1974.

Velvetleaf as an Alleged Allelopathic Plant

Several investigators have suggested that a component of velvetleaf interference is allelopathy. Research has indicated the existence of phytotoxic compounds in velvetleaf tissues. In a

greenhouse study, Bhowmick and Doll (1982) incorporated velvetleaf plant residues into the soil and determined the effect on soybean growth. Soybean shoot weight and root growth were decreased when velvetleaf residue was present. This was substantiated in a field study where the incorporation of velvetleaf residue into the soil caused a 14% reduction in soybean yield. Colton and Einhellig (1980) demonstrated that phytotoxins in extracts from fresh leaves depressed germination of radish seed. Growth and chlorophyll production of soybean seedlings in nutrient cultures containing fresh leaf extracts were also inhibited. They characterized the compounds only as water-soluble phytotoxins. It was concluded that these compounds reduce germination and growth and inhibit several plant functions.

Velvetleaf seeds have been shown to affect plant growth adversely. Gressel and Holm (1964) found that velvetleaf seeds placed near other crop or weed seeds inhibited germination and growth. An extract prepared from ground velvetleaf seeds also inhibited germination. The authors implied that inhibition from velvetleaf seeds was due to amino acids, but did not identify the specific components. In a similar study, Elmore (1980b) reported that velvetleaf seed imbibed for two days inhibited radicle growth of turnip seed by 17%. Velvetleaf seed extracts severely inhibited germination of the turnip seed. When fractionated by ion-exchange chromatography, the extracts contained amino acids and plant phenolics. Elmore (1980a) summarized the free amino acid profile of dry velvetleaf seed. Several of these are known to inhibit germination and radicle elongation (Wilson and Bell, 1978). Lysine and arginine reduced radicle elongation 70 and 55%, respectively (Elmore, 1980b). Retig et al. (1972) demonstrated that germinating

velvetleaf seeds have growth regulatory compounds that induce enlargement of parenchyma cells in adjacent cabbage roots. Extracts prepared from the leaching of velvetleaf seed coats also inhibited germination of velvetleaf seed (LaCroix and Staniforth, 1964). The water extract contained tannins, one or more amino acids, and nitrogenous compounds. They hypothesized that this self-inhibition of seed germination may be an important mechanism for delaying germination a few days or weeks, but would not be important in controlling long term dormancy.

Kremer et al. (1984) evaluated the relationships between microorganisms and velvetleaf seed to understand velvetleaf seed resistance to deterioration. They found that the dense palisade layer in the seed coat provided a physical barrier to microorganisms. Also, localized within the seed coat, there are compounds inhibitory to various external microorganisms. This phenomenon along with seed dormancy may increase the longevity of velvetleaf seed in the soil.

The evaluation of an intact plant or plant organ for its allelopathic potential may more accurately identify the allelochemical responsible for the toxic activity. Houtz et al. (1984) have observed that the numerous glandular trichomes on velvetleaf stems and petioles exude liquid globules. These globules have been collected with cotton swabs or by a water mist from the intact velvetleaf plant. The water soluble material extracted from the globules has been shown to severely inhibit radicle elongation of cress (Lepidium sativum) seeds. Characterization of exudate movement from velvetleaf into the soil and study of its fate and subsequent activity in the soil, will establish whether these compounds lend allelopathic activity.

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CHAPTER 2

FACTORS INFLUENCING TRICHOME EXUDATION BY VELVETLEAF (*Abutilon theophrasti* Medic.).

ABSTRACT

Exudates from velvetleaf glandular trichomes inhibited root and shoot growth of several weed and crop species in petri plate bioassays. Cress (*Lepidium sativum* L.) was the most sensitive indicator species. Scanning electron microscopy showed four types of trichomes present on the stem surface. The water soluble globules present on the 12 to 15-celled glandular trichomes recurred within seven days after removal with cotton swabs.

Both the quantity and phytotoxicity of the exudates from velvetleaf plants grown under varying environmental conditions and densities, and from different velvetleaf accessions were compared. A velvetleaf accession from Rosemount, Minnesota (MN) produced 1.4X more exudate/stem dry weight than an accession from Stoneville, Mississippi (MS). However, the MS exudate was about 1.35X more toxic to cress root growth when compared to the MN exudate. Although greenhouse grown plants had approximately 3.3X more exudate/g stem than field grown plants, the field exudate was 1.6X more toxic. Velvetleaf grown at a density of 3 plants/m^2 produced 1.3 and 2.0X more exudate than those

grown at densities of 12 and 36/m^2, respectively. However, as velvetleaf densities increased, their exudates became more toxic. While total exudate production was not affected at 3 different temperatures (16, 24, and 36 C), the exudates from plants grown at the two higher temperatures were almost twice as toxic as the exudate collected from plants grown at the lower temperature. Although water stress decreased the amount of exudate collected, it increased its activity by approximately the same factor.

INTRODUCTION

Secondary plant products are diverse in structure and distribution throughout the plant kingdom and have no obvious function in primary metabolism (26). Implicated in the defensive and protective mechanisms of plants, these secondary metabolites may have numerous ecological roles (18, 24, 26). The compounds are released through volatilization, exudation, leaching or decomposition of plant residues. Some may act as allelochemicals which help plants defend against herbivores, insects, or neighboring plants. Factors such as temperature, light quality and intensity, mineral deficiencies, age of plant, pathogens and predators, water stress, and genetics have all been shown to affect the production of secondary compounds (21, 26). The plasticity of secondary product formation in reponse to stress may aid in plant adaptation and survival.

A variety of secondary compounds with biological activity are localized in or secreted from specialized plant hairs called trichomes. Glandular trichome-exudates from mite-resistant geraniums (Pelargonium

hortorum) contained two anacardic acid derivatives, o-pentadecenylsalicylic acid and o-heptadecenylsalicylic acid (8). These compounds were moderately toxic when bioassayed with the two-spotted spider mite, Tetranychus urticae. The aphid alarm pheromone, (E)-β-farnesene, was present in substantial quantities in air around wild potato (Solanum berhaultii) foliage (9). It was demonstrated that this chemical which secretes from leaf trichomes, induced rapid dispersal of settled aphid colonies. Green peach aphids (Myzus persicae) are killed by contact with secretions from the trichomes of several Nicotiana species (27). These secretions contain alkaloids, of which nicotine is the major constituent. Isoprenoids (mono-, sesqui-, and diterpenes) are also metabolites commonly found in the trichomes of flowering plants (15).

The secondary compounds released from trichomes have also been implicated in allelopathy (18, 24, 26), the stimulatory or inhibitory effects that chemicals produced by one plant may exert on another (21). For example, the leaves of Artemisia absinthium bear glandular hairs which excrete ethereal oils and the alkaloid, absinthin (7). Plants in close proximity to A. absinthium have reduced vigor which is attributed primarily to absinthin, which washes off the Artemisia plants onto the neighboring species. The glandular hairs of Primula obonica secrete a phytotoxic quinone (23).

When the exudates from the glandular trichomes on velvetleaf stems and petioles were collected, their aqueous extracts severely inhibited radicle elongation of cress (Lepidium sativum) seeds in petri plate bioassays (14). The inhibitor was not present on the leaf surface. It was hypothesized that these trichome exudates from velvetleaf stems and

petioles may have allelochemical properties. However, the possible ecological role of these exudates is unclear and the phytotoxic components remain to be identified.

The primary objective of this research was to quantify the exudates collected from the trichomes on velvetleaf stems and petioles in relation to several variables. Both the quantity and quality (phytotoxicity) of the exudates collected from the stems and petioles of velvetleaf grown under varying environmental conditions and densities and from different velvetleaf accessions were determined. Other objectives included, determining a sensitive indicator species in petri plate bioassays and obtaining ultrastructural detail of trichomes with scanning electron microscopy.

MATERIALS AND METHODS

Indicator species screen. Trichome exudate was removed from greenhouse grown velvetleaf by wiping the stems and petioles with cotton swabs every seven days from twenty to thirty days after planting until capsule development. The exudate was thoroughly extracted from the cotton swabs with distilled water (dH_2O) and vacuum filtered through Whatman #1, 4 and 42 filter papers, consecutively. The extract was lyophilized and weighed.

The extract from velvetleaf trichomes (4 mg/2 ml dH_2O) was applied to a Whatman #1 filter paper in 60 by 15 mm petri plates. The control treatment contained 2 ml dH_2O. Indicator species included: cress (*Lepidium sativum* 'Curly'), cucumber (*Cucumis sativus* 'Green Star'), Japanese millet (*Echinochloa crus-galli*), radish (*Raphanus sativus*

'Cherry Belle'), soybean (Glycine max 'Corsoy'), sweet corn (Zea mays 'Calico King'), tomato (Lycopersicon esculentum 'UC-82'), and velvetleaf. Ten seeds from each indicator species (5 for soybean and sweet corn) were placed in the petri plates and incubated for 72 h at 27 C. Root and shoot growth were measured and the average of the number of seeds per plate was considered the experimental unit. Root and shoot growth as percent of controls were calculated. The experiment was placed in a randomized complete block design with four replications and was repeated. The data was submitted to analysis of variance and the means were compared by the LSD test.

Electron micrographs. Scanning electron micrographs of the velvetleaf stem surface were obtained to evaluate trichome ultrastructure and the effects of exudate removal with cotton swabs. Exudate was removed from one set of velvetleaf plants by wiping the stems and petioles with cotton swabs and not removed from another. Micrographs were used to compare the stem surfaces immediately and seven days after initiation of treatments.

The stem surfaces of two velvetleaf accessions were also compared. The velvetleaf accessions[1] were single plant collections from Stoneville, Mississippi (MN) and Rosemount, Minnesota (MN) which had been grown at Rosemount, Minnesota in 1981 for seed production.

Stem samples between the sixth and seventh nodes of thirty-day-old greenhouse grown velvetleaf plants were cut approximately 1.0 cm long by 0.5 cm wide by 0.5 cm deep. Samples were placed on moist filter paper in glass petri plates containing small watch glasses filled with

[1]Supplied by R. N. Andersen, USDA, ARS, St. Paul, MN 55108

2% OsO_4 solution. The petri plates were enclosed with parafilm and the samples fixed by the OsO_4 fumes. After 48 h, the samples were dried at 65 C for 15 min, mounted on stubs, and gold coated with a sputter coater. Samples were viewed and micrographs taken with a JEOL JSM-35C scanning electron microscope.

The glandular trichomes within four replicate 3 mm^2 areas were counted. The number of trichomes/mm^2 was calculated. Data for each accession were analyzed as a completely randomized design.

Stem and petiole surface area to dry weight correlation. Since the exudates appear to occur only on the stems and petioles, and surface areas of these structures are difficult to measure, it was necessary to develop a system to rapidly estimate these surface areas.

To obtain stem and petiole surface area and dry weight measurements, 90 field and greenhouse velvetleaf plants were harvested intermittently throughout their growing seasons. Each plant was separated into leaves and stems and petioles and surface areas were measured using a Licor area meter. To measure stem and petiole surface area, the outer phloem and epidermal tissue was peeled away from the pith and its surface area measured. Dry weights were then obtained. Linear regression of stem and petiole surface areas (y) as a function of dry weight (x) was calculated.

Accessions. Field experiments were conducted on the Michigan State University Horticultural Research farm in East Lansing, Michigan on a Marlette sandy loam (Glossoboric Hapludalf) with 2.5% organic matter and 6.0 pH. The factorial experiment (accession by days after planting) was placed in a randomized block design with four replications. Velvetleaf seeds were treated with sulfuric acid (25) to

enhance germination. The seeds of the two velvetleaf accessions were planted on 6/11/84 and 6/19/85 with a Nibex precision seeder and alachlor (3.4 kg/ha) was applied preemergence for weed control. Plots were 10.8 m^2 and the plants were thinned to 22 plants/m^2 ten days after emergence. Two 1.0 m^2 subsamples were harvested from each accession at 44, 66, and 77 days after planting. At harvest, exudate was removed from velvetleaf stems and petioles by wiping with cotton swabs and immediately frozen for later weight determinations and bioassays. Plants were separated into leaves, stems and petioles, and capsules for dry weight measurement. Data presented are the average for 1984 and 1985.

A greenhouse study was also conducted to compare quantities and toxicities of the exudates from MN and MS accessions. Velvetleaf seeds were planted into 9 L, 23 cm diameter plastic pots containing a soil mixture of 50% sandy loam, 30% peat, and 20% Torpedo sand. The mixture contained 3.5% organic matter and a pH of 6.8. After emergence the plants were thinned to two plants per pot. The plants were grown under metal halide lights (470 $\mu E \cdot m^{-2} \cdot s^{-1}$) with a 16 h photoperiod at 25/20 $\pm$ 5 C day/night temperatures and treated with soluble fertilizer (1.0 g/L of 20% N, 8.8% P, and 16.6% K) every seven days. One pot (two velvetleaf plants) of each accession was harvested at 44, 66, and 77 days after planting following the same procedures as in the field study. The factorial experiment was designed as a randomized complete block. Treatments were replicated five times and blocked for plant size and for location under the supplemental lighting.

Density. Velvetleaf densities of 3, 12, and 36 plants/m^2 were established on 6/11/84 using the location and methods described in the

accession field study. The velvetleaf seed had been collected from the Crop Science farm near East Lansing, Michigan in 1982. All data compilation and analyses were also the same as those in the accession field study.

Temperature. Growth chambers maintained at 16/14, 24/22, and 32/30 C day/night temperatures were used. The experiment was a randomized complete block design with six replications. Treatments were blocked over time and for each replication, each growth chamber was randomly assigned one of the three temperatures. When the experiment was initiated, exudate was removed by wiping the stems and petioles with cotton swabs. Two pots (two plants per pot) of greenhouse grown 40-day-old velvetleaf plants were placed in each growth chamber under fluorescent and incandescent lights (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) with a 16 h photoperiod. Pots were irrigated twice daily to avoid water stress.

Water potential of leaf petioles was measured with a pressure chamber.[1] Three measurements of petiole water potential were taken every other day. For measurement, petioles were cut near the stem and placed immediately in the chamber. Pressure was raised at a rate of .2 to .3 bar/second. After seven days, the plants were removed from the growth chambers. Exudate was wiped from the stems and petioles with cotton swabs and frozen for later weight and bioassay measurements. Plants were harvested to obtain leaf and stem and petiole dry weights.

Water. Forty-day-old greenhouse grown plants were used to evaluate the effect of water stress on velvetleaf trichome exudation. Immediately prior to initiating the treatments, exudate was removed

[2]Soilmoisture Equipment Corp., Model 3000, Santa Barbara, CA.

from stems and petioles using cotton swabs. Each experimental unit consisted of two pots (two plants per pot) of velvetleaf. Two treatments were established based on a wide range of water potential levels. 'Non-stressed' treatments were watered daily and maintained near full turgor. The soil in pots with the 'stressed' treatments was allowed to dry, maintaining the velvetleaf plants at less than full turgor. Some wilting of plants occurred. As with the previous experiment, petiole water potentials were measured on every other day. After seven days, the plants were harvested. The exudate was removed and frozen for later weight and bioassay measurements. The experiment was placed in a randomized complete block design with five replications and was repeated.

Extract preparation. Upon completion of each experiment, the exudate was extracted from the cotton swab samples four times with 50 ml distilled water and vacuum filtered through Whatman #1, 4, and 42 filter papers, consecutively. Each sample was concentrated with a rotary evaporator maintained at 45 C and then suction filtered through a millipore 0.45 μ metricel membrane. Samples were then lyophilized and weighed. Subsample values from the field accession and density studies were averaged. To allow for comparison between greenhouse and field grown velvetleaf plants and to account for differences in plant number and plant biomass accumulation, mg exudate/plant and mg exudate/g of stem were calculated. Analyses of variance for these parameters were conducted.

Bioassay. Petri plate bioassays were conducted to compare the phytotoxicity of exudate extracts. Amounts of velvetleaf extract applied to 60 by 15 mm petri plates in 2 ml distilled water were 0, 2, 4, and 8 mg. Ten cress (Lepidium sativum L.) seeds were placed on a

Whatman #1 filter paper in the petri plates and incubated in the dark for 72 h at 27 C. Cress root growth was measured and the average of 10 seeds per plate was considered the experimental unit. Cress root growth as percent of control was calculated and subsample values from the field accession and density studies were averaged. I_{50} values (mg of exudate required for 50% cress radicle growth inhibition) were estimated from the dose response curves.

RESULTS AND DISCUSSION

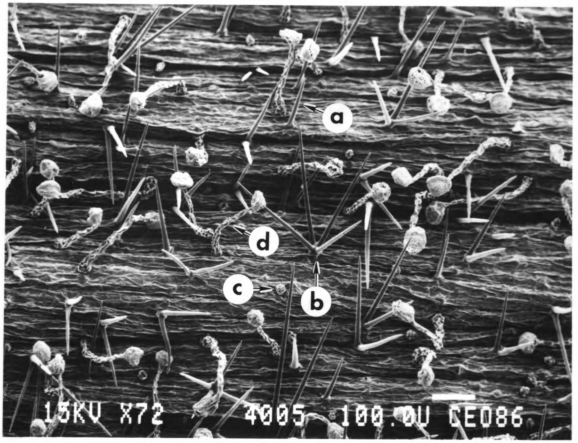
Indicator species screen. Velvetleaf aqueous extract inhibited the root growth of the indicator species by 30 to 96% and the shoot growth by 0 to 86% (Table 1). Velvetleaf root and shoot growth were inhibited by 60 and 45%, respectively, indicating the extract may be autoinhibitory. Because cress was the most sensitive species tested and because it germinates and emerges uniformly, it was chosen as the primary indicator of velvetleaf aqueous extract activity. Since root and shoot growth were inhibited similarly, root growth was selected as the standard parameter measured in all petri plate bioassays.

Electron micrographs. Velvetleaf stem, petiole and leaf surfaces are densely covered with four types of trichomes (19). Luckan (19) used standard histological methods to describe the morphological characteristics of these trichomes. Similar trichomes on the velvetleaf stem surface were viewed with the scanning electron microscope (Figure 1). The non-glandular types are the unbranched single-celled form (Figure 1a) or the stellate form (Figure 1b). The

Table 1: Effect of velvetleaf aqueous extract on root and shoot growth of several species.

	% of Control	
Species	Root	Shoot
Cress	3.7	14.0
Cucumber	47.8	72.3
Japanese millet	57.5	103.6
Radish	21.6	55.5
Soybean	60.2	-
Sweet corn	70.7	-
Tomato	48.4	83.2
Velvetleaf	39.1	55.2
LSD .05	19.4	41.1
.01	26.4	57.0

Figure 1. Scanning electron micrograph of the trichomes present on velvetleaf stems (72X); scale = 100μ: a) unbranched single-celled type, b) stellate type, c) long glandular type with 12 to 15 cells, d) short glandular type with 4 to 5 cells.


a
d
c
b
15KV X72 4005 100.0U CE086

two glandular types are either long, made up of 12 to 15 cells containing living protoplasts (Figure 1c), or short, with only four to five cells (Figure 1d). The cells of the longer trichomes have a thin cuticle except the cell just above the basal cell which has its cell walls cutinized throughout. The apex of the 12 to 15-celled trichome "is rounded out, and here drops of oil collect as it is secreted" (19). Similar trichomes were viewed with the scanning electron microscope (Figure 2).

Before the exudate was removed (Figure 2a and 2c), several globules were present at the apex of the 12 to 15-celled trichomes. When the stems were wiped with cotton swabs, the globules were removed and the trichomes remained intact (Figure 2b and 2d). Based on stem surface area counts from the scanning electron micrographs, approximately 7 glandular trichomes/mm^2 were present on the stem surface before and after exudate was removed. Seven days after exudate removal, the globules recurred (Figure 2f) and appeared similar to those where the exudate had not been removed (Figure 2e). Houtz et al. (14) found that globules with similar phytotoxicity returned within eight days of exudate removal.

Stem and petiole surface area to dry weight correlation. Stem and petiole surface areas were closely related to stem and petiole dry weights (Figure 3). The coefficient of determination (r^2) indicated that more than 99% of the variation was due to to the relationship between surface area and dry weight. Since dry weight is the more convenient parameter to measure, it was used to quantify the amount of stem material from which exudate was obtained.

Figure 2. Scanning electron micrographs of the trichomes present on velvetleaf stems. **Day 0:** a) exudate not removed (180X); b) exudate removed with cotton swabs (180X); c) exudate not removed (700X); d) exudate removed with cotton swabs (1500X); **Day 7:** e) exudate not removed (180X); f) exudate removed (180X).

Scale = 100μ in upper and lower micrographs and 10μ in center micrographs.

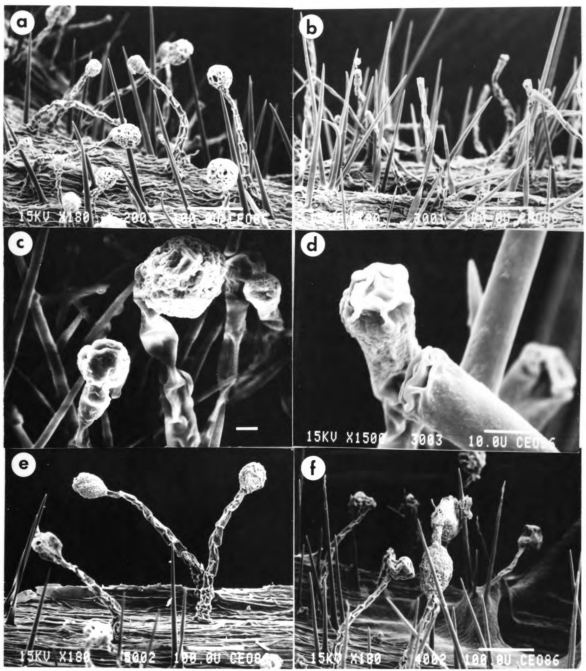
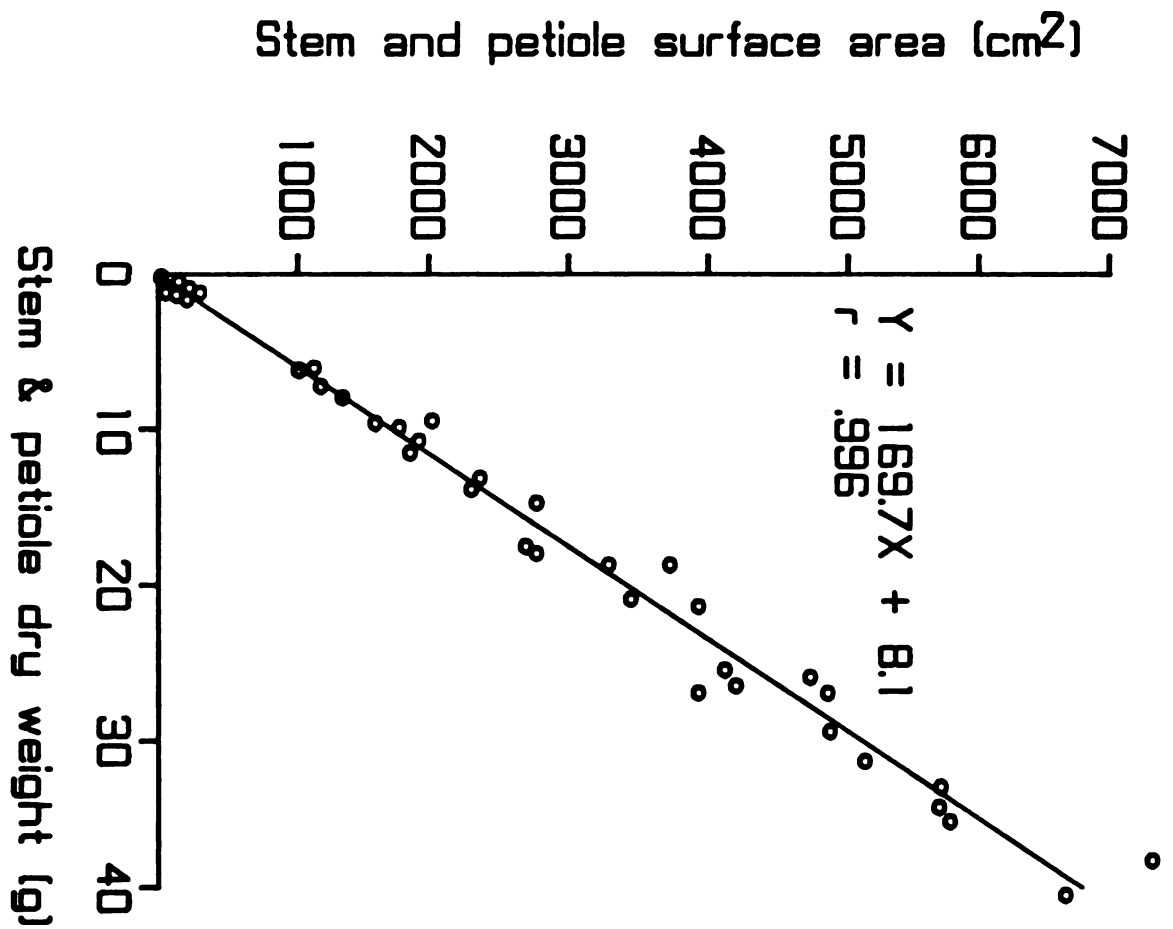

a
b
c
d
e
f
15KV X150
3003
10.0U CE086

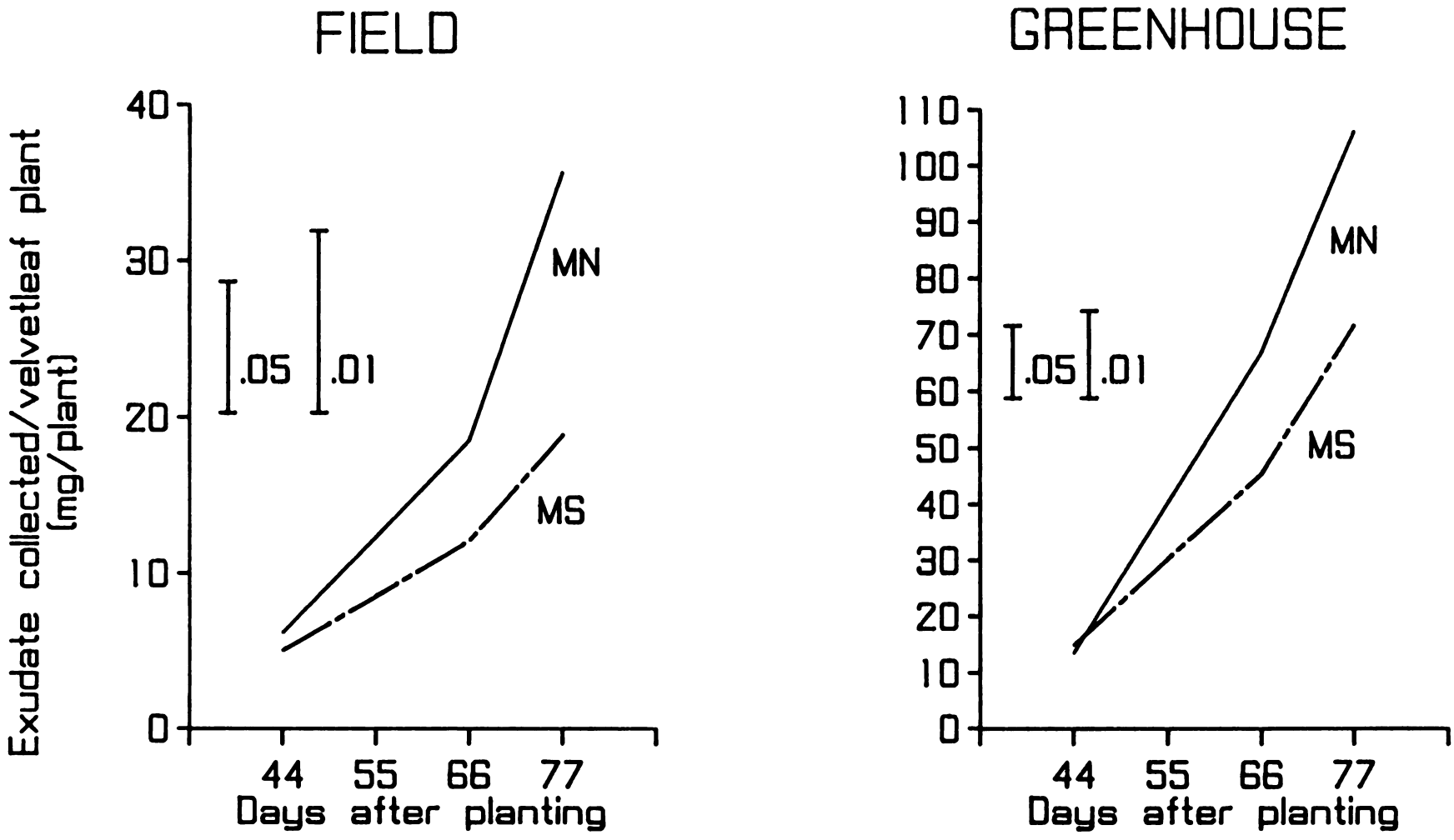
Figure 3. Regression analysis of velvetleaf stem and petiole surface area against velvetleaf stem and petiole dry weight (n = 90).


Stem and petiole surface area (cm2)
1000
2000
3000
4000
5000
6000
7000
Stem & petiole dry weight (g)
0
10
20
30
40
Y = 169.7X + 8.1
r = .996

Accessions. Several investigators have found genotypes from the same species to vary in their abilities to chemically inhibit other species (6, 20, 22). For example, the genotypes of Trifolium repens less favored by predators had greater amounts of cyanogenic compounds (4). Hanover (12) found the monoterpene content of western white pines (Pinus monoticola) to be under rigid genetic control. There were no differences in monoterpene levels among genotypically identical trees grown under diverse environmental conditions. The two velvetleaf accessions, MN and MS, differ in the number of days to maturity and the amount of seed capsules produced (1). It was speculated that secondary product formation, exudation or exudate composition might also differ between these accessions.

Although both accessions initially produced similar quantities of exudate, the MN accession consistantly produced more at 66 and 77 days after planting (DAP) (Figure 4). This result was not related to the number of trichomes present on the stem surface. Based on stem surface area counts from the scanning electron micrographs, the MN accession had 7.1 glandular trichomes/mm^2 and the MS accession had 7.3/mm^2. More exudate/plant was collected with time for both accessions perhaps due to increased stem and petiole surface area and trichome numbers. When averaged over time, there was 1.7X and 1.4X more exudate collected from MN when compared to MS in the field and greenhouse, respectively. Greenhouse grown plants had approximately 30 mg/plant more exudate than field grown plants when averaged over accessions and time. Factors such as, UV light or microbial decomposition, chemical degradation, wind, or leaching by rain may have reduced the quantity of exudate collected in the field.

Figure 4. Amount of exudate (mg) collected/velvetleaf plant from two velvetleaf accessions (MN = Rosemount, Minnesota; MS = Stoneville, Mississippi). The F values for the interaction between accessions and days after planting were significant at the 1% level for both the field and greenhouse studies. Field values are the average of four replications from two years of data. Greenhouse values are the average of five replications.


FIELD
Exudate collected/velvetleaf plant (mg/plant)
40
30
20
10
0
.05
.01
MN
MS
44
55
66
77
Days after planting
GREENHOUSE
110
100
90
80
70
60
50
40
30
20
10
0
.05
.01
MN
MS
44
55
66
77
Days after planting

In both field and greenhouse studies, more exudate was collected/stem dry weight from the MN accession at 66 and 77 DAP than from the MS accession (Figure 5). In the field as the DAP increased, the amount of exudate/g stem increased for the MN accession but decreased for the MS accession. However, in the greenhouse as DAP increased, the amount of exudate/g stem increased for the MN accession but did not change for the MS accession. More exudate was collected on a stem weight basis from the greenhouse grown accessions than from field grown accessions.

When the I_{50} values (mg exudate for 50% cress radicle growth inhibition) were analyzed, the accessions responded similarly regardless of the days after planting. The MS exudate was about 1.35X more toxic than the MN exudate to cress root growth (Table 2). However, the amount of exudate collected/g stem from the MN accession was 1.4 times greater than that collected from the MS accession. Possibly, the active component is more concentrated in the exudate from the MS accession.

The exudates from field grown velvetleaf were approximately 1.6X more toxic when compared to greenhouse grown velvetleaf. In the field and greenhouse studies, toxicity increased over time for field grown exudates and decreased over time for greenhouse grown exudates. Overall, the exudate from field grown plants was more toxic than the exudate from greenhouse grown plants.

Density. The amount of exudate collected increased at 66 and 77 DAP when plants were grown at the low density (3 plants/m^2) (Figure 6a). There were no increases in the amounts of exudate collected/plant over time at the higher densities (12 or 36·plants/m^2). When expressed

Figure 5. Amount of exudate (mg) collected/g of velvetleaf stem and petiole dry weight from two velvetleaf accessions (MN = Rosemount, Minnesota; MS = Stoneville, Mississippi). The F values for the interaction between accessions and days after planting were significant at the 1% level for both the field and greenhouse studies. Field values are the average of four replications from two years of data. Greenhouse values are the average of five replications.

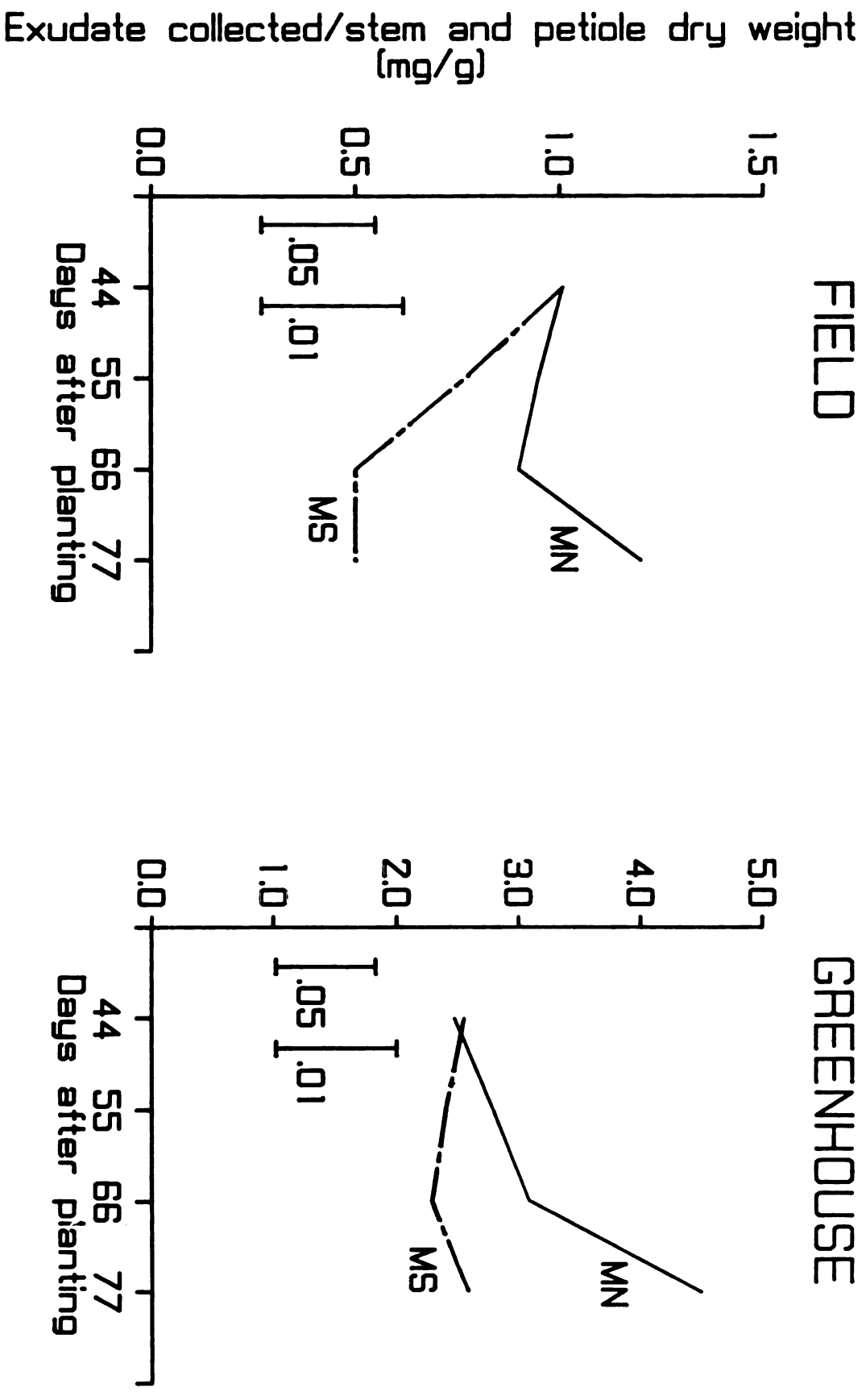

Exudate collected/stem and petiole dry weight (mg/g)
0.0
0.5
1.0
1.5
.05
.01
MS
MN
FIELD
Days after planting
44
55
66
77
0.0
1.0
2.0
3.0
4.0
5.0
.05
.01
MS
MN
GREENHOUSE
Days after planting
44
55
66
77

Table 2: The response of cress to extracts from different accessions of velvetleaf and different days after planting (DAP) (I_{50} = mg exudate for 50% inhibition of cress root growth).

Accession[1]	Field[a]	Greenhouse[b]	DAP[2]	Field[a]	Greenhouse[b]
	-------I_{50}---------			----------I_{50}----------	
Rosemount, MN	4.57	7.04	44	4.74	5.05
Stoneville, MS	3.30	5.48	66	3.81	5.76
			77	3.25	7.98
LSD 0.05	0.62	0.93		0.76	1.13
0.01	0.86	1.27		1.01	1.54

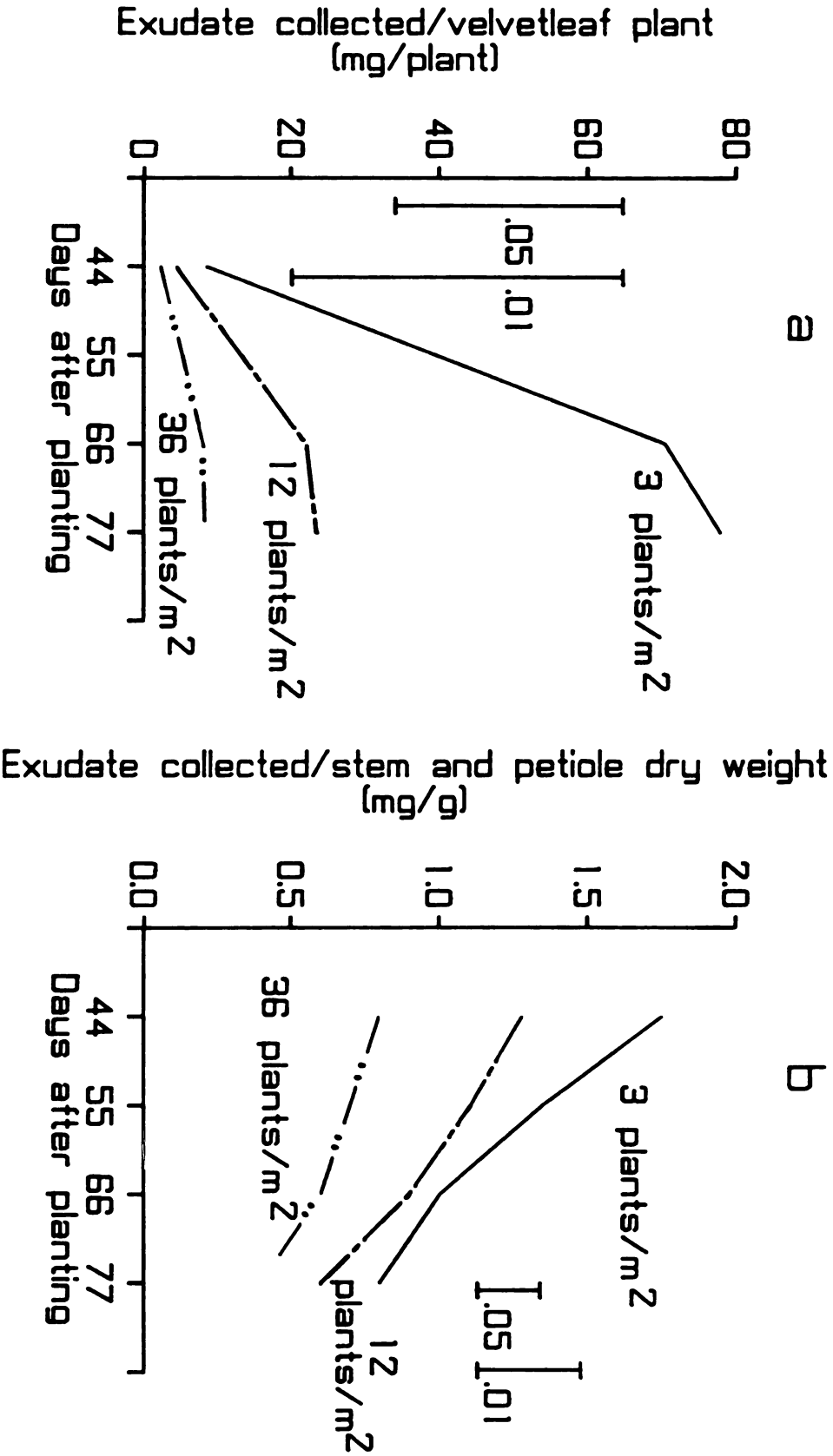
[1]Values are averaged across days after planting (n = 12).

[2]Values are averaged across accessions (n = 8).

[a]Average of data from two years (1984 and 1985).

[b]Average of data from five replications.

Figure 6. a.) Amount of exudate (mg) collected/velvetleaf plant and b.) amount of exudate (mg) collected/g of velvetleaf stem and petiole dry weight from velvetleaf plants grown at three densities (3, 12, and 36 plants/m^2). The F values for the interactions between densities and days after planting were significant at the 1% level for exudate/plant and exudate/g stem. Values are the average of four replications.


Exudate collected/velvetleaf plant
(mg/plant)
0
20
40
60
80
.05
.01
Days after planting
44
55
66
77
36 plants/m2
12 plants/m2
3 plants/m2
a
Exudate collected/stem and petiole dry weight
(mg/g)
0.0
0.5
1.0
1.5
2.0
.05
.01
36 plants/m2
12 plants/m2
3 plants/m2
b

as mg exudate/dry weight of stem, there was a decrease over time for all of the densities (Figure 6b). At 44 DAP, exudate production was inversely related to density.

As velvetleaf densities increased, their exudates became more toxic (Table 3). Exudate collected from plants grown at 36 plants/m^2 was more phytotoxic than the exudate from plants grown at either 3 or 12 plants/m^2. This may have been a response to stress induced by resource limitation at the higher densities.

Table 3: The response of cress to extracts from velvetleaf grown at different densities and at different days after planting (I_{50} = mg exudate for 50% inhibition of cress root growth).

Density[1] (plants/m^2)	I_{50}	Days After[2] planting	I_{50}
3	7.26	44	7.33
12	6.42	66	5.13
36	4.63	77	5.86
LSD 0.05	1.39		1.39
0.01	1.88		N.S.

[1]Values are averaged across days after planting (n = 12).

[2]Values are averaged across densities (n = 12).

Approximately 43% of the variation in the age main effect was due to the linear trend and 57% to the quadratic. Exudates collected from

older plants (66 and 77 DAP) were more toxic than exudate collected at 44 DAP. This was the same pattern found over time for the exudates collected from field grown MN and MS accessions.

Temperature. Temperature has been shown to affect secondary product formation in several plant species. For example, content of the indole alkaloid, gramine, in barley (Hordeum vulgare) increased when plants were transferred from a 21/16 C to a 30/25 C environment (13). The amount of divaricatic acid and depsidone salazanic acid in the Lichen, Ramalina subbreviuscula, was greater when grown in habitats with higher temperatures (11). In contrast, flavonol biosynthesis by the fern, Asplenium trichomanes, increased when grown at low temperatures (12 C) (17). Koeppe et al. (16) found increased concentrations of the allelochemicals, chlorogenic acid and scopolin in tobacco (Nicotiana tabacum) leaves when placed under cold conditions.

More exudate per plant was collected from velvetleaf plants grown at the higher temperatures (Table 4). The quadratic trend as temperature increased accounted for 67% of the total variation for treatments. Although more exudate/plant was collected when velvetleaf was grown at 24 C, followed by 32 C and then 16 C, the amount of exudate/dry weight of stem did not differ. Therefore, differences in the amount of exudate/plant could have been due to greater biomass accumulation at the higher temperatures and not because temperature affected the amount of exudate/stem surface area. Maximum velvetleaf dry weight accumulation also occurred at 24 C.

Table 4: The effect of temperature on velvetleaf water potential, exudation, and exudate activity (I_{50} = mg exudate for 50% inhibition of cress root growth).

Temperature[1] (C)	Velvetleaf exudate (mg/plant)	Velvetleaf exudate (mg/g stem)	I_{50}	Water potential (bars)
16	8.6	1.03	3.63	6.8
24	15.9	1.26	2.02	7.8
32	12.7	1.30	2.19	6.9
LSD 0.05	5.7	N.S.	1.15	N.S.

[1]All values are the average of six replications.

More importantly, the exudates collected from the velvetleaf grown at higher temperatures were almost twice as toxic as the exudate collected from the plants grown at the lower temperature. The I_{50} values for exudate from plants grown at 24 and 32 C were 55 to 60% smaller than for exudates from plants grown at 16 C. Because there were no differences among the leaf petiole water potential values (bars) at the three temperatures, water stress was not considered to be influential in the exudate differences observed in this experiment. Higher temperatures may induce qualitative changes in the exudates as indicated by the greater amounts of the active components when velvetleaf was grown at 24 or 32 C.

Water. Water deficiencies change plant metabolism and therefore, may alter secondary product formation. Increased quantities of terpenes (10), alkaloids (2, 3), and phenolics (5) have been measured

in plants tissue grown under drought conditions.

The water potentials of 'stressed' and 'non-stressed' velvetleaf plants in this experiment were 14.3 and 6.8 bars, respectively (Table 5). On a per plant basis, there was more exudate collected from plants which were not water stressed. However, the amount of exudate collected/stem dry weight (or surface area) did not differ. The increased amounts of exudates collected from non-stressed plants appear to be due to increased growth of the velvetleaf plants under non-stressed conditions.

Table 5: The effect of water stress on velvetleaf water potential, exudation, and exudate activity (I_{50} = mg exudate for 50% inhibition of cress root growth).

Treatment[1]	Velvetleaf exudate (mg/plant)	Velvetleaf exudate (mg/g stem)	I_{50}	Water potential (bars)
Stressed	7.8	0.78	1.49	14.3
Non-stressed	12.8	0.94	2.53	6.8
F value	**	N.S.	**	*

[1]All values are the average of five replications.

Exudate collected from the stressed plants was however, more toxic. The I_{50} value for non-stressed plants was 1.7X greater than the I_{50} value for stressed plants. Although water stress decreased the amount of exudate collected, it increased its activity by approximately the same factor. It is speculated that the concentration of the active

compound(s) in the exudate increases under water stress, however, the overall amount produced may be similar whether or not the plants are stressed.

The quantity and activity of exudate collected from velvetleaf trichomes varies depending on the environment in which it is grown. This knowledge will help maximize the amount of exudate that can be collected for the purpose of purification, isolation and identification of the phytotoxic compound(s).

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CHAPTER 3

POSSIBLE ROLE OF TRICHOME EXUDATES IN INTERFERENCE BY VELVETLEAF (*Abutilon theophrasti* Medic.)

ABSTRACT

Because velvetleaf trichome exudate is water soluble, rain may facilitate its movement from velvetleaf stems and petioles into the surrounding soil environment where it may act as an allelochemical. In field and greenhouse studies, an attempt was made to separate the influence of exudates from other mechanisms of interference. Exudates were removed from one set of velvetleaf plants and not from others. In the control treatment, no velvetleaf was present, therefore interference by velvetleaf was totally eliminated. Velvetleaf trichome exudate did not reduce the growth of the associated indicator species in either field or greenhouse studies. The interference imposed by two accessions of velvetleaf known to have different exudate quantities and toxicities and grown at 3 and 12 plants/m^2 reduced soybean growth similarly in the field.

A cress (*Lepidium sativum*) bioassay was used to monitor velvetleaf exudate activity in autoclaved and non-autoclaved soil. Although active in autoclaved soil, extract activity was lost at all but the highest dosage when the soil was not autoclaved. Microorganisms appear

to rapidly detoxify the velvetleaf extract and may reduce or eliminate its impact in the field.

INTRODUCTION

Velvetleaf interferes with the growth of crops including corn (Zea mays L.) (6), cotton (Gossypium hirsutum L.) (4), soybeans [Glycine max (L.) Merr.] (7, 8, 12, 17, 21, 25) and sugarbeets (Beta vulgaris L.) (20). Interference is the result of interactions among species in mixtures which lead to reduced growth (plasticity) or increased mortality in one or both species (13). Unfavorable effects from neighboring plants may involve both allelospoly (depletion of one or more resources needed for plant growth) and allelopathy (presence of chemicals which alter plant growth) (10, 13, 19). Although numerous density and duration studies have quantified crop growth reduction by velvetleaf, few have separated the individual mechanisms of interference.

Several investigators have suggested that velvetleaf interference is due to allelopathy. Incorporation of velvetleaf plant residues into soil resulted in a 14% reduction in soybean yield (3). In addition extracts from fresh leaves were toxic to radish seedlings and the growth and chlorophyll production of soybean seedlings (5). Velvetleaf seeds have also been shown to adversely affect germination and seedling growth (9, 11, 18, 21).

Houtz et al. (15) have observed that the numerous glandular trichomes on velvetleaf stems and petioles exude liquid globules. The globules were collected with cotton swabs or by a water mist from the

intact velvetleaf plant. The water soluble material extracted from these globules severely inhibited radicle elongation of cress (Lepidium sativum L.) seeds. Globules with similar activity returned within seven days after removal from the trichomes. It was hypothesized that trichome exudates contribute to the interference that velvetleaf imposes on other plants.

Because the exudate is water soluble, rain may facilitate its movement from velvetleaf stems and petioles into the surrounding soil environment where it may act as an allelochemical. The primary objective of this research was to ascertain what, if any, portion of the interference can be attributed to exudates from its glandular trichomes. It was hypothesized that other mechanisms of interference could be separated from the exudate effect by simply removing the exudate. Other secondary objectives included, comparing the interference imposed by two accessions of velvetleaf known to have different exudate quantities and toxicities (24), and assessing the effect of microorganisms on exudate activity in soil.

MATERIALS AND METHODS

Separation of trichome exudate influences from other components of interference. Field experiments were established in 1984 and 1985 on the Michigan State University Horticultural Research farm in East Lansing, Michigan. Experiments were conducted on a Marlette sandy loam (Glossoboric Hapludalf) with 2.5% organic matter and a pH of 6.0. The experimental design was a split plot with seven replications, with velvetleaf treatments as main plots and distance of indicator species

from the donor velvetleaf plant as subplots. Each main plot was 1.2 m^2 with one velvetleaf plant established in the center on 6/11/84 and 6/12/85.

To test the effect that trichome exudates may have on the associated indicator species, three treatments (main plots) were imposed on the center donor velvetleaf. Interference by velvetleaf was totally eliminated by removing the center velvetleaf nine days after emergence. This treatment served as the no-velvetleaf control. The second treatment removed the potential allelopathic effect of exudates by wiping the exudate from the stems and petioles with cotton swabs on the dates indicated in Table 1. In the third treatment, the exudate was not removed, allowing for total interference. The experiment was irrigated following exudate removal to facilitate exudate movement into the soil and to reduce competition for water. The irrigation schedule and rainfall data is presented in Table 1.

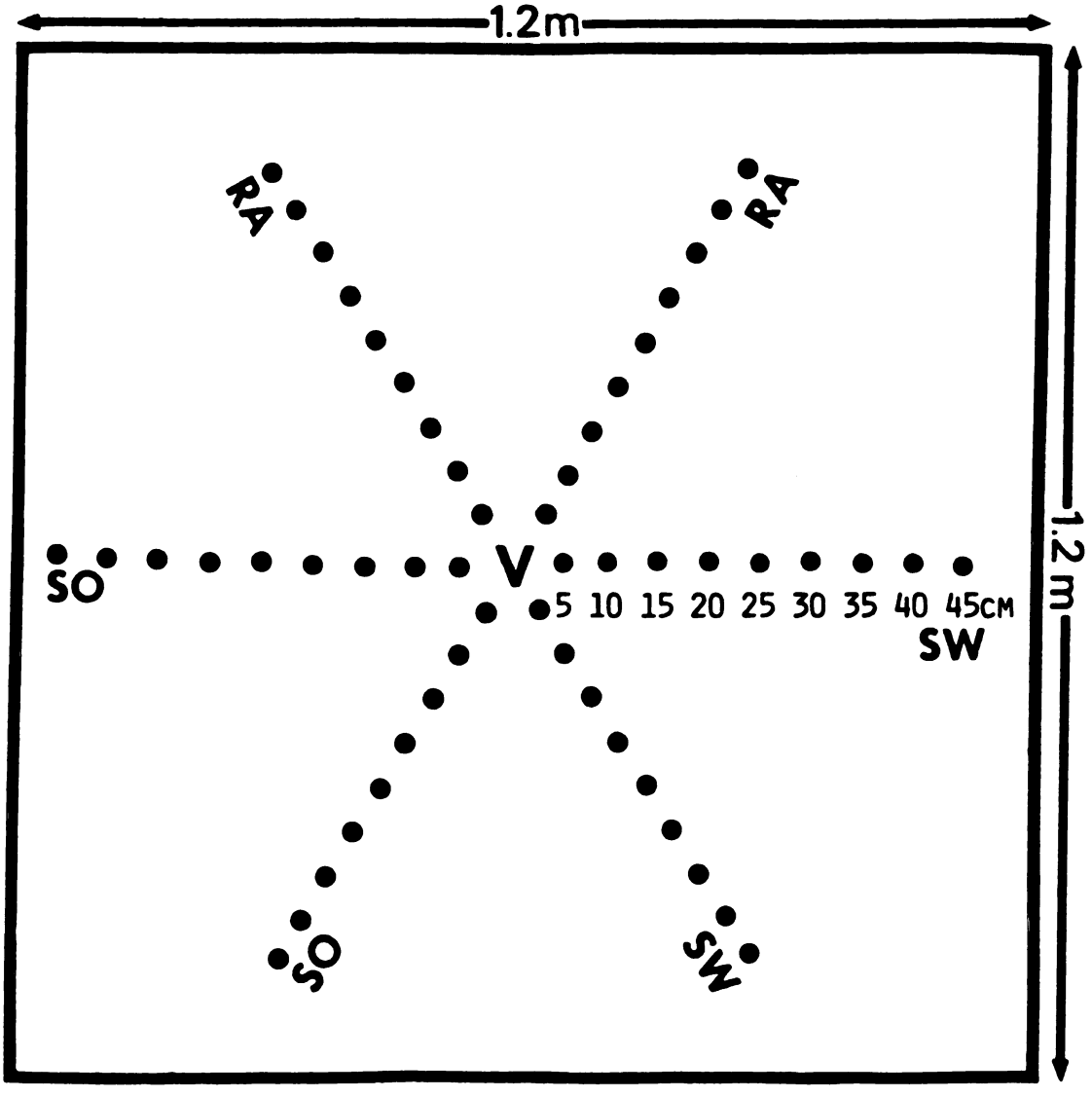
Associated indicator species were planted as subplots to test the different treatments imposed on the velvetleaf in the main plots (Figure 1). Two rows of each indicator species were planted in 5 cm increments radiating 5 to 45 cm from the established velvetleaf plants on 7/24 and 8/21 in 1984 and 7/8 and 8/15 in 1985. Because more exudate is produced as velvetleaf accumulates stem and petiole area (24), the early and late plantings were established to compare this difference. The indicator species were 'Corsoy' soybean, 'Calico King' sweet corn and 'Cherry Belle' radish. Three days after emergence, each was thinned to one plant per location. Because the same planting design was used within each main plot, interference between the indicators remained constant. After 30 days, indicators were harvested

Table 1: Exudate collection, irrigation, and rainfall for 1984 and 1985 velvetleaf interference study.

1984					1985				
Exudate removal date		Irrigation[1] (cm)	Rainfall date	cm	Exudate removal date		Irrigation[1] (cm)	Rainfall date	cm
			June 11 to July 8	1.1				June 12 to July 12	5.2
July	9	-	July 10	1.0	July	13	-	July 16	0.8
			11	2.3		19	-	20	1.6
			12	0.2		24	-	26	0.7
	14	1.4	15	0.2		29	1.4	31	0.1
	20	-	22	0.2	Aug.			Aug. 1	0.2
	24	-	25	0.5		2	1.4	5	0.9
			28	0.1				6	1.1
	30	1.4						7	0.1
Aug.	4	-	Aug. 4	2.0		8	-	8	0.2
	7	-	8	3.1		12	1.4	15	2.7
			9	0.4				16	0.2
	13	1.4	10	0.4		18	-	18	0.1
	19	1.4	19	T				19	1.3
	25	1.4	28	T		24	-	24	0.2
	29	1.4	30	0.7				25	2.4
Sept.			Sept. 1	0.1				27	0.3
			2	0.1		29	-	30	0.7
	3	-	4	0.2	Sept.			Sept. 2	T
			5	0.3		3	1.4	5	0.2
			7	0.4				6	1.4
	8	-	8	0.2				7	0.9
			9	0.3				8	1.8
			10	0.3		9	-	9	1.4
			11	1.1				10	0.2
	13	-	14	0.8		13	-		
			15	0.6					
	17	-							

[1]Irrigation followed exudate removal on the corresponding dates. Amounts applied were approximately 1.4 cm.

Figure 1. Planting design used to evaluate the influence of velvetleaf (V) exudate presence on sweet corn (SW), soybean (SO), and radish (RA) growth at increasing distances (cm) from the velvetleaf stem. No velvetleaf was present in the center of the control plot.


1.2m
RA
RA
SO
V
5 10 15 20 25 30 35 40 45CM
SW
SO
SW
1.2m

and shoot dry weights (two plants per location) were obtained. The corresponding velvetleaf plants were also harvested and dry weights of leaves, stems, petioles, and capsules were measured.

Separate analyses of variance were performed on dry weight data for each species and planting date. Trend analysis and regression analysis were also conducted to characterize any relationship across the distances.

Greenhouse studies were also conducted to test the potential influence of exudates. Velvetleaf seeds were treated with sulfuric acid (23) to enhance germination and were planted into 9 L, 26 cm diameter plastic pots containing a soil mix consisting of 50% sandy loam, 30% peat, and 20% Torpedo sand. The mix had 3.5% organic matter and pH of 6.8. Plants were thinned to two plants per pot after emergence. The plants were grown under metal halide lights (470 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 16 h photoperiod at 25/20 $\pm$ 5 C day/night temperature and treated with a soluble fertilizer (1.0 g/L of 20.0% N, 8.8% P, 16.6% K) every seven days. 'Corsoy' soybean, 'Calico King' sweet corn, and 'Cherry Belle' radish were planted in three separate rows (3.5 cm apart) in 13 by 18 cm flats into 1200 g Spinks loamy sand with 1% organic matter. Each species was thinned three days after emergence to 10 plants per row. The flats were subirrigated with equal amounts of water as needed and with half strength Hoaglund's solution (14) every three days.

To separate the potential allelopathic effect that the exudate might have on the indicator species, exudate was removed from one set of velvetleaf plants but not from another by wiping the stems and petioles with cotton swabs. Rainfall of 0.64 cm was simulated by

passing a two-nozzle (8008 LP) boom repeatedly over the velvetleaf plants at a constant speed. The leachate (ca. 400 ml) was collected in a plastic basin and broadcast over the indicator species using a misting bottle. An additional control was the same quantity of collected simulated rainfall applied to the indicators. Treatments were initiated 40 days after velvetleaf was planted and three days after emergence of indicator species. Indicators were treated four times at seven day intervals and were harvested seven days after the final treatment.

Treatments were arranged in a randomized complete block design with four replications and repeated twice. Shoot dry weights were obtained and analyses of variance for dry weight as percent of no velvetleaf control were performed for each indicator species.

Interference by different accessions of velvetleaf. This field study was established at Kellogg Biological Station, Hickory Corners, Michigan on a Kalamazoo sandy loam soil (Typic Hapludalf) with 3.0% organic matter and pH 7.0. 'Corsoy' soybeans were no-till planted[1] May 21, 1984 into an area previously treated with glyphosate (May 11, 1984). Each plot consisted of four rows of soybean 6.1 m long and 0.7 m apart. Velvetleaf seeds (acid treated) were hand planted directly into the soybean row where weeds would most likely escape cultivation. The velvetleaf accessions were single plant collections from Stoneville, MS and Rosemount, MN which had been grown at Rosemount, MN in 1981 for seed production.[2] Two weeks after emergence, the

[1] Moore Uni-Drill Ltd., Newhill House, 33 Kirk Road, Ballymoney BT53 6PP, Co. Antrim, N. Ireland

[2] Supplied by R. N. Andersen, USDA, ARS, St. Paul, MN 55108

velvetleaf was thinned to three or 12 plants/m^2. Plots were maintained weed-free throughout the growing season.

To estimate biomass accumulation, four harvests (35, 58, 75, and 89 days after emergence) of two representative velvetleaf plants and their closest soybean neighbors were harvested from each plot. Leaf, stem and petiole, and capsule or pod dry weights were measured. At 100 days after soybean emergence, plants were harvested from the center two rows. The leaves, stems and petioles, and capsule dry weights of the velvetleaf were also measured. At maturity, soybean pods were collected and the seed separated using a stationary thrasher. Total dry weight from 9 m of row and dry weight per 100 seeds were measured. The experiment was designed and analyzed as a randomized complete block design with four replications.

Extract activity in autoclaved and non-autoclaved soils. Once phytotoxins are produced and move into the environment, decomposition by microorganisms or other chemical action can occur to alter their activity (19). Petri plate bioassays were conducted to evaluate the activity of velvetleaf exudates in autoclaved and non-autoclaved soil. Seven g of Spinks loamy sand (1% organic matter) were placed in a 60 by 15 mm glass petri plate with Whatman #1 filter paper placed over the soil. Sterile treatments were autoclaved (121 C; 23 psi) four times for 30 minutes at 24 h intervals. Cress seeds were surface sterilized with a 1% solution of Sigma (Antibiotic-Antimycotic) cell culture reagent (Penicillin, 10000 U/ml; Streptomycin, 10 mg/ml; Amphotericin B, 25 ug/ml; in 0.9% NaCl). Germination tests showed that cress seed germination and emergence were unaffected by this solution.

Velvetleaf exudate was collected by wiping greenhouse grown velvetleaf stems and petioles every seven days. The swabs were extracted with distilled water (dH_2O) and vacuum filtered through Whatman # 1, 4, and 42 filter papers, consecutively. The extract was then lyophilized and weighed. Distilled water was added to the dried material and the solution was vacuum filtered with a Nalgene 115-ml sterilization filter unit through a 0.2 micron triton-free cellulose nitrate membrane. Concentrations of velvetleaf extract applied to the soil in 4 ml sterile dH_2O solutions were 0, 1.25, 2.5, 5.0, 10.0, and 20.0 mg. Ten cress seeds were placed on the filter paper after the extract was applied. The treatments were then incubated in the dark at 28 C for 72 h. Cress root growth was measured and the average of 10 seeds per plate was considered the experimental unit. Cress root growth as percent control in autoclaved or non-autoclaved soils was calculated.

The factorial experiment was designed as a randomized complete block with three replications and was repeated twice. Analysis of variance for percent control values (average of two experiments) was performed and LSD values at 0.05 and 0.01 were calculated.

RESULTS AND DISCUSSION

Separation of trichome exudate influences from other components of interference. In 1985, there was no significant difference in dry weight accumulation of soybean, sweet corn or radish when grown under velvetleaf with the exudate present or with the exudate removed. Removal of the exudate had no effect on velvetleaf biomass

accumulation, therefore all other mechanisms of interference imposed by velvetleaf on the indicator species were assumed to be equal. Field results in 1984 were similar except for sweet corn at the early planting date. Sweet corn dry weight was 25 and 44% greater at 40 and 45 cm, respectively when grown under velvetleaf where the exudate had been removed. This indicated that the trichome exudate could be inhibiting the growth of sweet corn. However, this was not repeated in the 1985 field trials or in greenhouse studies. In these experiments, soybean growth was also unaffected by exudate presence. Because results from the radish data were similar to soybean results, only the soybean data will be discussed.

Although the exudate did not influence the growth of the associated indicator species, there was a significant distance effect in both the 1984 and 1985 field trials. Therefore, the percent control values for exudate treatments were combined and analyzed for each species and planting date. Sweet corn and soybean growth were reduced more as the distance from velvetleaf was reduced. Regression analysis further characterized the distance effect on the two species at the two planting dates (Figures 2 and 3). The further the indicator species were from the velvetleaf plant, the greater the dry weight accumulation when compared to the control plot where no velvetleaf was present. At the early planting date, sweet corn 5 cm from the velvetleaf was inhibited 51% while that at 45 cm away was not inhibited (Figure 2). At the later planting, when velvetleaf had accumulated more biomass, sweet corn was severely inhibited by 91% at 5 cm and 74% at 45 cm. Soybean growth was reduced by 19% at 5 cm and was not affected at 45 cm for the early planting date (Figure 3). At the later planting date,

Figure 2. Regression of the shoot dry weight(g) as percent control of sweet corn against distance from the velvetleaf plant. Early sweet corn planting date (—) (6/28/85) and late sweet corn planting date (--) (8/12/85) are significantly different at the 1.0% level. Each value is the average of seven replications.

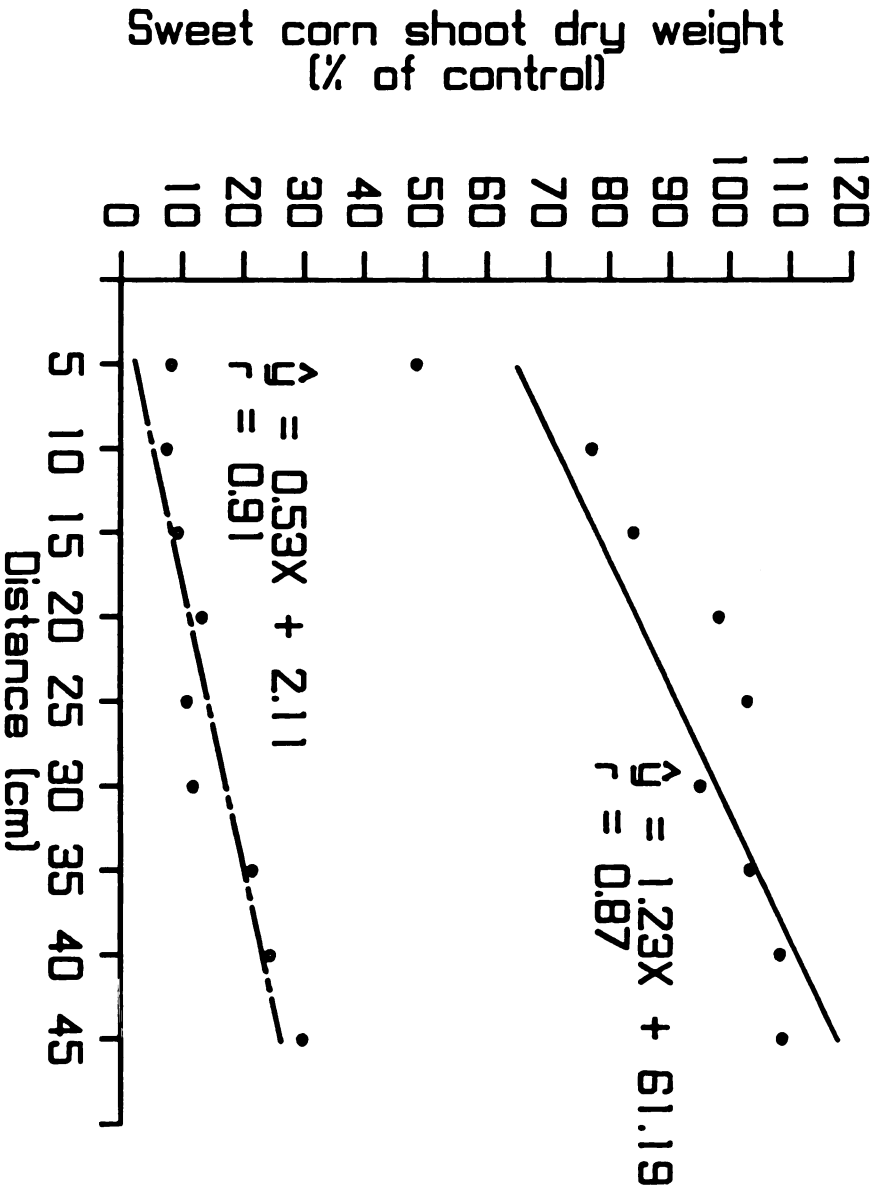
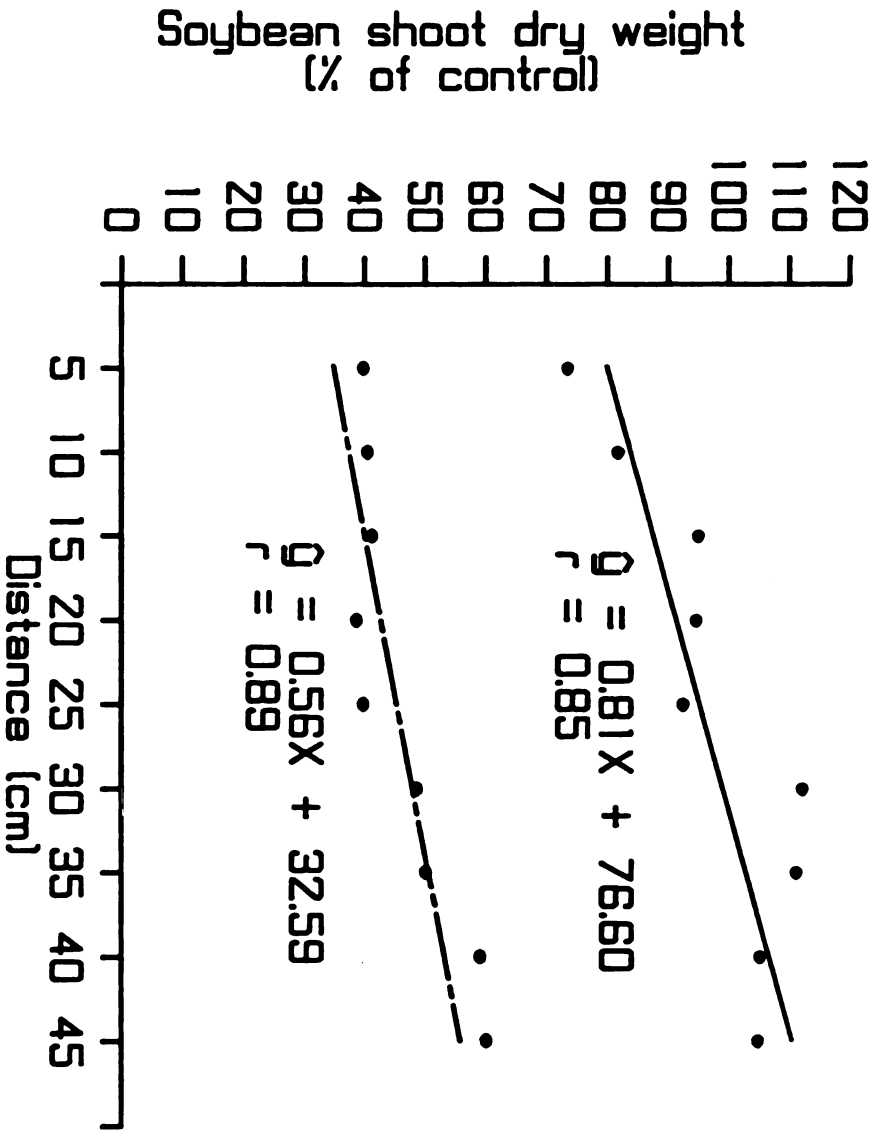

Sweet corn shoot dry weight
(% of control)
0
10
20
30
40
50
60
70
80
90
100
110
120
5 10 15 20 25 30 35 40 45
Distance (cm)
y = 0.53x + 2.11
r = 0.91
y = 1.23x + 61.19
r = 0.87

Figure 3. Regression of the shoot dry weight(g) as percent control of soybean against distance from the velvetleaf plant. Early soybean planting date (—) (6/28/85) and late soybean planting date (--) (8/12/85) are significantly different at the 1.0% level. Each value is the average of seven replications.


Soybean shoot dry weight
(% of control)
0
10
20
30
40
50
60
70
80
90
100
110
120
5
10
15
20
25
30
35
40
45
Distance (cm)
ŷ = 0.56X + 32.59
r = 0.89
ŷ = 0.81X + 76.60
r = 0.85

soybean was inhibited by 65% at 5 cm and 42% at 45 cm.

As the distance of the associated species from the velvetleaf plant increases, the interference imposed on them decreases. This interference may involve competition for light, nutrients, or water or a source of allelopathy other than the exudates from trichomes. In these experiments, superoptimal nutrients and water were supplied indicating that light was the component responsible for most of the inhibition measured across the distances. Light appeared to be decreased in the late planting where velvetleaf biomass was 730 g/plant in comparison to a biomass of 42 g/plant at the early planting. As the velvetleaf biomass increased from the early to late planting date, the percent dry weight accumulated for the indicator species decreased (Figure 2 and Figure 3). Stoller and Woolley (25) state that a full canopy of velvetleaf leaves will intercept 44 to 56% of sunlight resulting in a 12 to 31% decrease of soybean yield. Velvetleaf at 125% of soybean height reduced soybean yield by 64% (1). Whereas, there was only a 7% and 16% reduction in soybean yield when velvetleaf was allowed to attain 25% and 100% of soybean height, respectively.

Interference by different accessions of velvetleaf. The yield was similar for the two accessions regardless of density (Table 2). Three and 12 velvetleaf plants/m^2 reduced soybean seed yield by 37 and 72% respectively in comparison to the weed free control (Table 2). In other studies, Hagood et al. (12) found that velvetleaf densities ranging from 5 to 40 plants/m^2 reduced soybean seed yield by 27 and 56%, respectively. Densities of 1.6 and 3.3 plants/m^2 reduced soybean yield 13 and 26% respectively when soybeans were planted in mid May and 11 and 13% when soybeans were planted in late June (17). Soybean dry

Table 2: Effect of velvetleaf accessions and densities on soybean seed yield.

Velvetleaf accession	Density	Soybean Seed Yield (dry weight)	
	(plants/m^2)	(g/9 m of row)	(g/100 seeds)
None	0	552	16.8
Rosemount, MN	3	318	12.7
Rosemount, MN	12	127	12.9
Stoneville, MS	3	378	12.9
Stoneville, MS	12	178	13.1
LSD 0.05		162	N.S.
0.01		227	

matter, flowering nodes, and yield were reduced with 2.4 to 4.7 velvetleaf plants/m^2 (7). In another study, 3.3 velvetleaf plants/m of row reduced soybean seed yield by 31% when compared to the weed free control (21). However, in another study, there was no soybean yield loss when velvetleaf was present at 10 and 20 plants/m^2 (22).

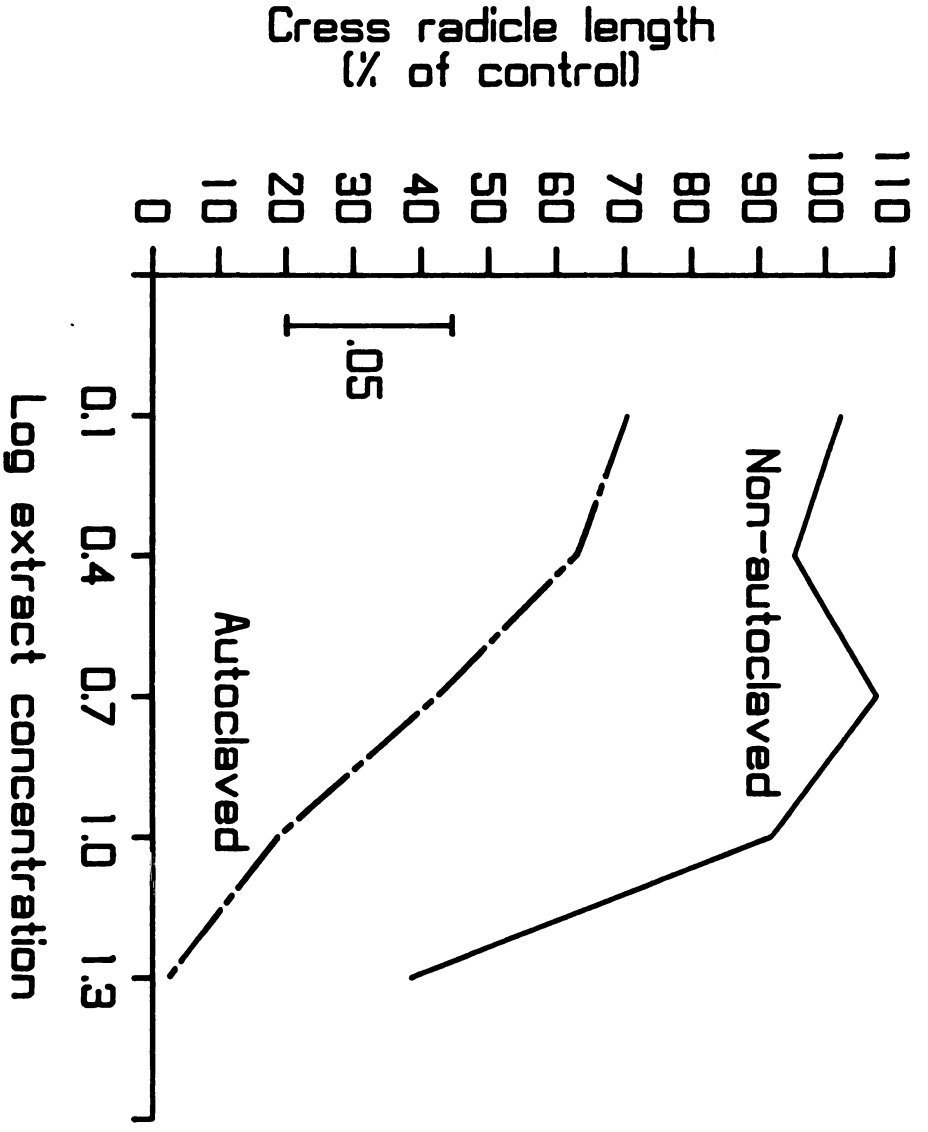
Both accessions accumulated similar biomass throughout the growing season except at the final harvest where the accession from Stoneville, Mississippi (MS) had more biomass than the accession from Rosemount, Minnesota (MN). It appeared that MN matured and started senescing sooner. When previously grown at Rosemount, Minnesota the heights of the MN and MS accessions had not differed except once during the season when MN was significantly taller than MS (2). MN produced more mature seed capsules earlier in the growing season than MS (2). However, MN had reached maximum height and ceased flowering two weeks before frost while MS was still growing and flowering (2). Because the biomass accumulation for the two accessions did not differ except at the final harvest, it might be expected their competitive abilities were similar.

There were no differences in soybean yield losses when comparing the MN or MS accessions (Table 2). In previous studies, the MN accession produced more exudate than the MS accession. However, the exudate from the MS accession was more toxic to cress root growth when compared to the MN exudate on an equal weight basis (24). Because MN and MS reduced soybean yield similarly, either the exudate plays no role in the interference that velvetleaf imposes on soybean or the increased quantities of exudates from MN balances out qualitative differences between the two.

Extract activity in autoclaved and non-autoclaved soils. The activity of an allelochemical in the soil depends on the rate at which it is released and the rate of microbial or chemical decomposition or deactivation. Velvetleaf aqueous extracts reduced cress root length 30 to 95% in autoclaved soil (Figure 4). Velvetleaf extract activity was lost at all but the highest dosage when the soil was not autoclaved. Microorganisms appear to rapidly detoxify the velvetleaf extract and may play a role in decreasing its impact in the field.

Although the exudates from velvetleaf glandular trichomes are phytotoxic to growth of several species in petri plates (15, 24), they do not appear to play a role in the interference that velvetleaf imposes on crops in the field. Nonetheless, velvetleaf grown at 22 plants/m^2 may produce up to 9 kg/ha of this exudate during a growing season (24). To ascertain its ecological significance, studies implicating the exudate in possible insect, herbivore, or pathogen interactions should be conducted.

Figure 4. Cress radicle length as percent control in autoclaved or non-autoclaved soil at increasing velvetleaf aqueous extract log concentrations. Concentrations used were 1.25, 2.5, 5.0, 10.0, and 20.0 mg/4 ml distilled water in 7 g sandy loam soil. Values are the average of 3 replications.


Cress radicle length
(% of control)
0
10
20
30
40
50
60
70
80
90
100
110
.05
Log extract concentration
0.1
0.4
0.7
1.0
1.3
Autoclaved
Non-autoclaved

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CHAPTER 4

SEPARATION OF THE PHYTOTOXIC COMPONENTS IN THE TRICHOME EXUDATE FROM VELVETLEAF (*Abutilon theophrasti* Medic.).

ABSTRACT

Although velvetleaf trichome exudates did not appear to play a major role in velvetleaf interference, they were phytotoxic to several crop and weed species in petri plate bioassays. The phytotoxic components of the exudates were further studied in relation to their potential novelty and usefulness as naturally occurring herbicides. Solvent partitioning and chromatographic methods were used in an attempt to isolate and purify the phytotoxic compound(s). During separation, bioassays of cress root growth were used to monitor toxicity. The water soluble exudate was removed from the stems and petioles of greenhouse grown velvetleaf plants with cotton swabs and was extracted from the swabs with distilled water. Sequential solvent partitioning (hexane, chloroform, diethyl ether, dichloromethane, ethyl acetate, and butanol) of the water extract resulted in major toxicity in the diethyl ether fraction. There was a 20 fold enrichment of activity when compared to the aqueous extract. This fraction was further separated using a silica column under pressure. Gradient elution with dichloromethane : methanol produced the greatest toxicity

in the 90 : 10 fraction. Bioassay of a thin layer chromatograph of this fraction indicated one major band of toxicity at Rf zones .37 to .50. High performance liquid chromatography of this fraction indicated the more polar components to be responsible for the phytotoxicity. With further purification, the phytotoxic compound(s) may eventually be identified.

INTRODUCTION

The glandular trichomes on velvetleaf stems and petioles exude water-soluble liquid globules which can be collected with cotton swabs or by water misting (4). Aqueous extracts of these exudates severely inhibited radicle elongation of cress (Lepidium sativum) seeds, however, did not affect germination (4). After removal from the stems and petioles, the liquid globules recurred and were at their original potency within eight days. These non-volatile inhibitor(s) were not present on the leaf surface.

It was speculated that these trichome exudates may play a role in the interference, mediated through allelopathy, that velvetleaf imposes on other plants (4). Allelopathy encompasses both the stimulatory and inhibitory effects that chemicals produced by one plant have upon others (6). An important step in proving allelopathy is the isolation and identification of the chemical(s) responsible for phytotoxicity (1). Ideally, after identification, the suspected allelochemical(s) should be reapplied to the plant system where interference was observed to assess their relative importance to other mechanisms of interference, such as competition. If the original phytotoxic symptoms

and selectivity characteristics are similar to those imposed by the suspected allelochemical, it lends credence that this allelochemical is involved in interference. Nonetheless, compounds exuded from plants may still be of interest even if they are not proven to be allelochemicals.

Although the exudates did not appear to play a major role in the interference that velvetleaf imposes on sweetcorn, soybean, or radish in soil (Chapter 2), the phytotoxicity was of sufficient interest to explore the potential usefulness of its components as naturally occurring herbicides. The purpose of this research was to isolate and purify the phytotoxic compound or compounds using solvent partitioning and chromatographic methods of separation.

MATERIALS AND METHODS

General. Velvetleaf (acid treated) seeds were planted into 9 L, 23 cm diameter plastic pots with a soil mix of 50% sandy loam, 30% peat, and 20% Torpedo sand. This mix had 3.5% organic matter content and a pH of 6.8. Plants were thinned to two plants per pot after emergence. The plants were greenhouse grown under metal halide lights (470 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 16 h photoperiod at 25/20 $\pm$ 5 C day/night temperatures and treated with soluble fertilizer (1.0 g/L of 20% N, 8.8% P, and 16.6% K) every seven days.

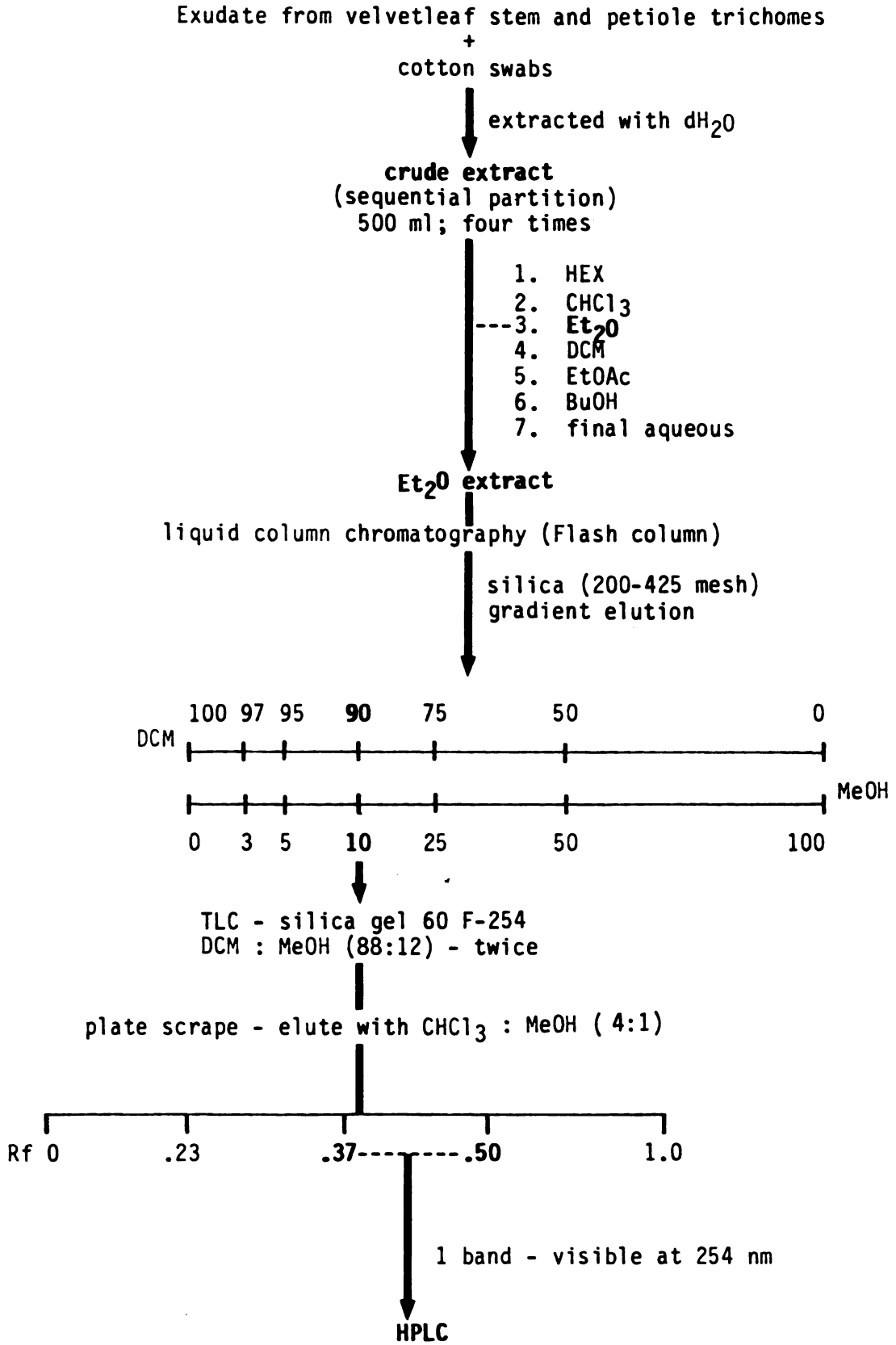
Twenty to 30 days after planting and until capsule development, trichome exudates were removed from velvetleaf stems and petioles by wiping with cotton swabs every seven days. These swabs were stored at -16 C prior to extraction.

Solvent partitioning. The exudate collected from velvetleaf stems and petioles with cotton swabs was thoroughly extracted from the cotton swabs with 1 L distilled water (dH_2O) and vacuum filtered through Whatman #1, 4, and 42 filter papers, consecutively. Solvents (500 ml; 4 times) of increasing polarity [hexane (HEX), chloroform ($CHCl_3$), diethyl ether (Et_2O), dichloromethane (DCM), ethyl acetate (EtOAc), and water-saturated butanol (BuOH)] were sequentially partitioned against the crude extract (Figure 1). All solvents were analytical or HPLC grade. Fractions were concentrated and bioassayed using the cress seed assay described on page 96. After finding the major portion of activity in the Et_2O fraction, the extraction procedure was simplified. Five hundred ml volumes of hexane then diethyl ether were partitioned against one liter of crude extract four to five times. Emulsions which formed between immiscible layers were collected and bioassayed separately.

Each fraction was concentrated with a rotary evaporator at 50 C and suction filtered through a Millipore 0.45 μ metricel membrane. Small volumes were then dried by evaporation with nitrogen gas while heating the sample in a 50 C water bath. All fractions were weighed to calculate % extracted and for bioassay on an equal weight basis. Samples were stored at -16 C.

Liquid column chromatography. The Et_2O extract was separated using a silica (200-425 mesh; Type 60A, Fischer) 'Baker' Flash chromatography column (25 cm length; 2 cm diameter) under air line pressure. The Et_2O extract was gradient eluted with volumes of DCM : Methanol (MeOH) (100:0, 99:1, 97:3, 95:5, 75:25, 50:50, 0:100). All fractions were concentrated, evaluated by thin layer chromatography (TLC), weighed, and bioassayed.

Figure 1: The separation scheme used for purification of the active components in the trichome exudate from velvetleaf stems and petioles.


Exudate from velvetleaf stem and petiole trichomes
+
cotton swabs
extracted with dH_2O
crude extract
(sequential partition)
500 ml; four times
1. HEX
2. $CHCl_3$
3. Et_2O
4. DCM
5. EtOAc
6. BuOH
7. final aqueous
Et_2O extract
liquid column chromatography (Flash column)
silica (200-425 mesh)
gradient elution
100 97 95 90 75 50 0
DCM
MeOH
0 3 5 10 25 50 100
TLC - silica gel 60 F-254
DCM : MeOH (88:12) - twice
plate scrape - elute with $CHCl_3$: MeOH (4:1)
Rf 0 .23 .37 .50 1.0
1 band - visible at 254 nm
HPLC

Thin layer chromatography. For further isolation, the active fraction from the silica Flash column was separated using TLC. Merck silica gel 60 F-254 TLC plates were prewashed with DCM:MeOH (88:12). The active fraction from the Flash column was applied in DCM to the plates and developed twice in DCM:MeOH (88:12) (16 cm). Bands were visualized with ultraviolet light (UV) (254 nm) absorbance or 5% vanillin in H_2SO_4 (using the outer edges of the plate and heating at 100 C for 5 min). Rf zones (0-.23; .23-.37; .37-.50; .50-1.0) were scraped from the plates, eluted with 300 ml $CHCl_3$:MeOH (4:1), filtered, weighed and bioassayed. Control plates were also developed and the same zones eluted for bioassay to control against any possible toxicity from the silica plates.

High performance liquid chromatography (HPLC). HPLC was employed to separate the compound or compounds responsible for the phytotoxicity. The mobile phase of MeOH:H_2O (90:10) eluted the fraction through a Radial-PAK liquid chromatography C_{18} cartridge[1] (8 mm by 10 cm) at 2 ml/min. The column was connected in series to an UV detector (230 nm). Samples were collected based on peaks obtained by UV detection, and were concentrated, weighed, and bioassayed. After each sample injection, the system was eluted with 100% MeOH to flush out the nonpolar components remaining on the column.

Bioassay. Petri plate bioassays were conducted to monitor the activity of the fractions throughout the separation scheme. Only the active fractions were further separated (Figure 1). Fractions were applied to Whatman #1 filter paper in 60 by 15 mm petri plates.

[1]Waters Associates, Maple Street, Milford, MA 01757

Organic soluble fractions were applied to the filter paper in 0.5 ml solvent solutions. After the solvent evaporated, 2 ml dH_2O was added. Equivalent concentrations of aqueous fractions were applied in 2 ml dH_2O solutions. Control plates received either 0.5 ml solvent and 2 ml dH_2O or 2 ml dH_2O. Curly cress (Lepidium sativum) was the indicator species. Cress germinates and emerges uniformly and was the most sensitive indicator to velvetleaf aqueous extracts (Chapter 2). Twenty cress seeds were added to each petri plate and incubated in the dark at 27 C. After 72 h, cress radicle length was measured and the average of 20 seeds per plate was considered one replication. When dose response bioassays were performed, I_{50} values (mg required for 50% cress radicle inhibition) were estimated from the response curve.

Cress bioassays from each solvent partitioning and Flash column fraction were replicated two or three times. These separations were repeated and bioassayed three times after which the resulting values were averaged. Because of limited material, TLC fractions were unreplicated but the experiments were repeated three times and the values were averaged. Bioassay of HPLC fractions were replicated twice.

RESULTS AND DISCUSSION

Solvent partitioning. Diethyl ether (Et_2O) removed the most active compounds (Table 1). The I_{50} value for Et_2O extract was 0.3 mg in contrast to 6.2 mg for the original crude. This was an approximate 20 fold enrichment of activity. I_{50} values for the other fractions were greater than that for the original crude. Therefore, the Et_2O

extract was the only fraction subjected to further separation.

Table 1: Sequential solvent partitioning of velvetleaf trichome crude extract.

Fraction	% of Crude[1]	I_{50}[2]
crude	----	6.2
hexane	0.3	9.8
diethyl ether	0.5	0.3
aqueous	93.1	14.5
emulsion	6.1	16.0

[1]% of total crude recovered.

[2]mg for 50% inhibition of cress root growth obtained by extrapolation from dose response curves.

After solvent partitioning, 93% of the crude extract weight remained in the aqueous fraction, indicating that most of the compounds in the crude extract were relatively polar (Table 1). Only 0.5% of the crude weight was partitioned into the Et_2O, a solvent of intermediate polarity. Therefore, one gram of velvetleaf trichome exudate extracted from cotton swabs yielded only about 5 mg of the phytotoxic Et_2O extract.

Liquid column chromatography. After gradient elution through a silica Flash column, the major activity in the Et_2O extract separated into fraction 4 which was eluted with DCM : MeOH (90:10) (Table 2). Essentially all of the applied Et_2O extract was recovered when the

Table 2: Liquid column chromatography[1] separation of diethyl ether (Et_2O) extract.

Fraction	Gradient elution DCM : MeOH		% of Et_2O extract	Cress Root Growth[2] (% of Control)
1	100	0		
	99	1	20.6	69.3
2	97	3	13.8	91.2
3	95	5	2.8	67.0
4	90	10	13.3	4.6
5	75	25	15.9	83.8
6	50	50	10.1	77.3
7	0	100	7.6	64.2

[1]Silica flash column under air line pressure.

[2]Obtained with 0.5 mg in 2 ml dH_2O; Average of three replications.

column was eluted. Because of limited material, dose responses of cress were not tested and therefore I_{50} values were not calculated. Results were based on % of control radicle growth when fractions were applied at 0.5 mg/2 ml dH_2O. Fraction 4 reduced cress radicle growth by about 95%. Since other fractions only reduced radicle growth a maximum of 36%, they were not considered active enough to warrant further separation. Only 13% of the weight of Et_2O extract applied was eluted into the phytotoxic fraction 4. One gram of velvetleaf trichome exudate extracted from cotton swabs yielded only about 0.67 mg of the phytotoxic fraction. At this point in the separation scheme, less than 1% of the crude extract from velvetleaf trichomes contained the most active components.

Thin layer chromatography. When the phytotoxic fraction 4 was developed on silica TLC plates, five major bands were visible with ultraviolet light at 254 nm. After elution and bioassay of the scraped plate zones, high phytotoxic activity was present at the Rf zone .37 to .50 (Table 3). At 0.1 mg/2 ml dH_2O, this fraction reduced cress radicle growth by 96%. This zone consisted of one visible band which absorbed at UV 254 and turned brown in color with 5% vanillin in H_2SO_4 before and after heating.

Only 40% of the Flash column fraction 4 was recovered from the eluted zones when this TLC method was used for separation (Table 3). Although inefficient, this method of separation was reproducible and successful in maintaining and enriching the activity measured in the original column fraction.

HPLC. When the active TLC zone was eluted through the reversed phase HPLC column, several peaks were detected at 230 nm (Figure 2).

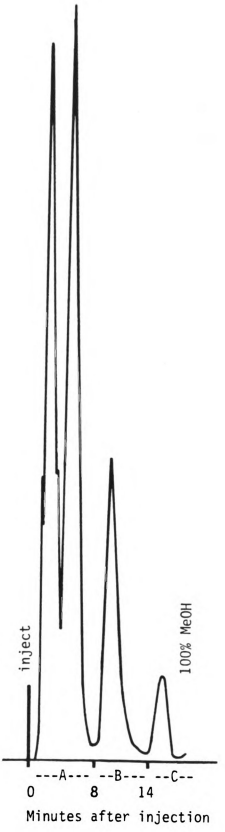
Table 3: Thin layer chromatography and bioassay of silica flash column phytotoxic fraction 4.

Rf[1]	% Recovered	Cress Root Growth (% of Control)[2]
.50 - 1.0	45.9	129
.37 - .50	25.3	3.9
.23 - .37	16.0	76.6
.00 - .23	12.6	125

[1]Merck silica gel 60 F-254 eluted twice with DCM : MeOH (88:12).

[2]Based on 0.1 mg in 2 ml dH_2O application rate; Average of three replications.

Figure 2: HPLC chromatograph of the active TLC plate scrape zone. Three fractions (A, B, C) were collected based on UV detection (Y axis) and bioassayed.


inject
100% MeOH
---A--- --B--- --C--
0
8
14
Minutes after injection

Fraction collection was based on the three major peak areas. Fraction A (eluted 0-8 min after injection) had four observable peaks and fraction B (8-14 min) and C (14-MeOH flush) each had one peak. The more polar fraction A reduced cress radicle growth by 99% when applied at 0.1 mg/2 ml dH_2O (Table 4). Fractions B and C were inactive. Fifty-five percent of the active TLC fraction recovered from the HPLC separation was eluted from the column in the first 8 min.

Table 4: HPLC separation and bioassay of the active Rf zone (0.37 - 0.50) from TLC.

Fraction	Min from Injection	% Recovered	Cress Root Growth (% of Control)[1]
A	0 - 8	55.3	1.2
B	8 - 14	12.0	98.3
C	14 - MeOH	33.0	97.1

[1] Based on 0.1 mg/2 ml dH_2O application rate; Average of two replications.

For further separation and to determine if there are in fact, four or more compounds in the active polar fraction, an HPLC solvent system of increased polarity should be eluted through the column. With further purification, the phytotoxic compound(s) may eventually be identified. This may prove difficult because less than 1% of the exudate from velvetleaf trichomes is responsible for the inhibition of

cress radicle elongation. Collection of an ample quantity for purification and characterization may be a major limitation.

Identification of the phytotoxic compound(s) in the exudate from the glandular trichomes on velvetleaf stems and petioles could increase knowledge regarding the types of secondary compounds exuded by plants and may identify new compound(s) with potential usefulness as herbicides. Since several biologically active secondary compounds have been found in the trichome exudates from several plant species (2, 3, 5, 7), exudates from velvetleaf trichomes may have some ecological role. Although these exudates do not appear to exert allelopathic interference on neighboring crop plants (Chapter 2), these exudates could influence other organisms in the plant community such as insects, nematodes or pathogens.

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