ALLELOPATHIC ACTION OF QUACKGRASS

(*Agropyron repens* (L.) Beauv.)

By

Leslie A. Weston

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1985

ABSTRACT

ALLELOPATHIC ACTION OF QUACKGRASS

(*Agropyron repens* (L.) Beauv.)

By

Leslie A. Weston

Previous studies indicated that quackgrass (*Agropyron repens* (L.) Beauv.) has allelopathic potential. Legumes grown in the greenhouse and the field in living quackgrass or its residues exhibited decreased nodule numbers, nodule fresh weights and nitrogen fixation when compared to legumes grown under similar conditions in screened quackgrass soil or a control soil. Shoot and root dry weights were significantly decreased in field and greenhouse experiments when legumes were grown in the presence of quackgrass tissues. Decreases in legume growth and nodulation in the presence of quackgrass residues were attributed to allelochemical effects. Aqueous extracts of quackgrass shoots and rhizomes inhibited seed germination and root growth at concentrations of less than 2.5 mg dried extract/ml. Root and shoot dry weights of snapbeans (*Phaseolus vulgaris* L.) grown aseptically in agar were reduced by aqueous extracts of shoots. Root systems were stunted, necrotic and lacked root hairs when observed by scanning electron microscopy. Extracts of quackgrass shoots were

generally more inhibitory to plant growth than were extracts of rhizomes. However, the growth of Rhizobium species was not influenced by the presence of extracts of shoots or rhizomes. Soil microorganisms were not neccessary for the development of quackgrass toxicity in soil or agar. Upon sequential partitioning of aqueous extracts of quackgrass shoots, ether extracts possessed the greatest activity, and caused 50% reductions in radicle elongation of eight species at concentrations of less than 1.0 mg/ml. Root and shoot dry weights of snapbeans grown aseptically in agar were reduced two to three fold by ether extracts (100 ug/ml) but ether extracts had no inhibitory effect on the growth of Rhizobium species on agar or in broth culture. Phytotoxins present in quackgrass shoots may inhibit the legume Rhizobium symbiosis indirectly by reducing root growth and root hair formation. The ether extracts of quackgrass shoots were separated using TLC and HPLC. Two related phytotoxins were purified and subjected to ^{1}H-NMR and mass spectroscopy. One was identified as the methoxyflavone, tricin, and the other appeared closely related. These compounds may contribute to the allelopathic interference exhibited by quackgrass and its residues.

DEDICATION

I would like to dedicate this Thesis to the memory of my grandfather and his great love of horticulture.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Alan R. Putnam for his encouragement, guidance, and support during my Ph.D. program. His enthusiasm and energy helped me through some rough times, and made my three years at the Pesticide Research Center my most memorable.

I would also like to thank the members of my committee for their excellent courses, and their helpful suggestions for my research (Dr. F. Dazzo, Dr. S. Ries, Dr. I. Widders and Dr. M. Zabik).

Thanks go to my colleagues and student workers at 105 Pesticide Research Center, for their help, encouragement, and friendship. I am also grateful to Jackie Schartzer for her typing expertise, manuscript preparation and sense of humor. I will miss you all.

Finally, a tribute to my husband Paul, for his patience, understanding, and encouragement during the past six years.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Crop losses caused by all weeds are estimated to exceed 10 billion dollars annually in the United States (14). Quackgrass (Agropyron repens (L.) Beauv.) may be the most serious perennial weed problem of the cooler northern temperate zones (21). Quackgrass is commonly found as a strong competitor in grains, forages, vegetable crops and fruits, as well as forestry plantations, lawns, nurseries and industrial sites (5). Past studies have shown that the presence of perennial grass weeds, such as quackgrass, in legume crops leads to serious yield losses (5, 68). It is a particular problem in soybeans (Glycine max (L.) Merr.), navybeans (Phaseolus vulgaris L.) and alfalfa (Medicago sativa L.) where few herbicides are available for effective control (68, 69).

Crop losses due to weed interference may occur as the result of weed-crop competition or the allelopathic effects of a weed upon crop growth. The ability of quackgrass to maintain high growth rates through cooler periods of the year, its prolific vegetative reproduction, and high consumption of available nutrients contribute to its strong competitive ability (64). Quackgrass competes with crop plants for available light, water and nutrients (14). Considerable evidence has also been accumulated to suggest that quackgrass, either in its living

or dead form, may have allelopathic potential. The objective of this research was to further investigate the alleged allelopathy in quackgrass with emphasis on effects of its residues in no-tillage systems.

ALLELOPATHY: A CHEMICAL MECHANISM OF PLANT INTERFERENCE

For centuries it has been hypothesized that the detrimental effects of certain plants on other plants may be due to the production of chemical inhibitors. This concept is referred to as allelopathy (33), and can be more thoroughly defined as the detrimental effects of a donor plant (higher plant or single celled plant species) on the seed germination, growth or development of a receptor plant. Allelopathy can be separated from other mechanisms of plant interference in that the detrimental effect is exerted through the release of a chemical by the donor (45, 55, 67). Many higher plants are known to contain substantial amounts of toxic chemicals. These toxins, together with a wide variety of other chemicals with no apparent function in the basic metabolism of plants, are referred to as secondary plant products. Secondary metabolites include alkaloids, flavonoids, phenolics, terpenoids, lactones, tannins, cyanogenic glycosides, quinones, polyacetylenes, coumarins, sulfides and water soluble organic acids and alcohols (66, 67).

Allelopathy may serve as an important form of self-defense in higher plants. This concept has not been investigated until recently. Since plant species are immobile and cannot escape pests, predators, or competitors, it is not surprising that allelochemicals might serve as

an important means of self-defense against many organisms (28). However, it appears that the allelopathic potential of many crop species may have been reduced when compared to weed members of the same family as a result of concentrated plant breeding efforts for other characters (29).

Discharge of secondary substances into the environment occurs by exudation of volatile chemicals from living plant parts (34, 35, 36), by leaching of water-soluble toxins from above ground parts in response to the action of rain, fog or dew (3, 10, 61), by exudation of water soluble toxins from below ground parts (42, 58, 61) or by the release of toxins from non-living plant parts through leaching or from litter decomposition (17, 18, 27). Under some chemical, physical and biological conditions in the field, toxins can accumulate to such an extent that plant growth is reduced. The duration of activity of allelochemicals and the accumulation of toxins in the soil vary with the plant and its growth habit (30).

Allelochemicals released into soil bind to organic matter or undergo microbial or chemical decomposition. Depending upon the type of allelochemical, they may become more or less active with decomposition (46). Phytotoxic substances produced by soil microorganisms living in the presence of plant residues have been implicated as factors involved in reduced yields in stubble-mulched agriculture. For example, patulin is a toxin produced by the soil microorganism Penicillium urticae in soil amended with wheat straw. Patulin inhibits the growth of wheat seedlings and other molds at concentrations of less than 20 ppm, and soil concentrations of 75 ppm have been reported under wheat straw residues (31).

Allelopathic compounds can act through a variety of mechanisms including inhibition of cell division, cell growth, photosynthesis, respiration, protein synthesis and specific enzymes; or alterations of membrane permeability, mineral uptake, and stomatal opening (49). For example, flavone, flavonol and isoflavone compounds have been shown to inhibit root and shoot growth in wheat through a striking inhibition of ion uptake in plant roots. Other flavonoids inhibit seedling growth by the inhibition of phosphorylation in mitochondria at extremely low concentrations (32). However, the primary mode of action of most allelopathic agents has not been determined, owing in large part to the difficulty in separating secondary from primary effects. The main criteria used in determining the effectiveness of allelopathic compounds are the measured changes in seed germination or growth of test organisms used in a bioassay.

Allelopathic compounds may interact synergistically, but few researchers have investigated this phenomenon. Einhellig (49) has studied the synergistic activities of various phenolic compounds released from allelopathic plant species. Combinations of cinnamic, ferulic, caffeic, p-coumaric, and vanillic acids exerted synergistic action and resulted in reduced growth of seedling indicators (49). These phenolic compounds and benzoic acid derivatives have been isolated from many plant species, and are commonly reported as inhibitors of seedling radicle elongation (31).

QUACKGRASS

Morphology and Distribution. Quackgrass (Agropyron repens (L.) Beauv.) is an erect, rhizomatous perennial grass. At least 8 ecotypes

of quackgrass are now recognized, based on the floral structure, plant color, leaf pubescence, leaf width and growth habit (65). Variation also exists in date of flowering and ability to produce seed (48). Leaf sheaths are generally hairy on the lower leaves, with smooth sheaths above and clasping auricles present. The leaf blade is soft and relatively flat, with a whitish bloom in drier conditions and dark green appearance in humid conditions. The inflorescence consists of dense spikes, with four to six compressed flowered spikelets. The brownish caryopsis is 4 to 5 mm long, pointed at the base and surrounded by a ring of hairs. Quackgrass can also be recognized by its distinctive pale yellow rhizomes, with a dark brown sheath at each joint, giving a scaly appearance to the rhizome (14).

Quackgrass is indigenous to Europe, and was introduced to the United States during colonization. It was apparently introduced to the New England States as a food source for cattle (64). Agropyron repens (L.) Beauv.) is now present in all major agricultural areas of the northern temperate zone and New Guinea. It is generally found on arable land, lightly-grazed pastures and in waste areas. It is associated with many soil types but performs best on fine-textured soils. Quackgrass cannot tolerate low soil pH or exposed rock faces. Its rhizomes are susceptible to freezing conditions if located on the soil surface. If undisturbed, quackgrass tends to be quite drought-tolerant (14).

Quackgrass colonizes a new area by first appearing in clumps, secondly forming larger patches and finally, if undisturbed, goes on to form pure stands (14). It may make up greater than 90% of the biomass in an abandoned field for several years, and exclude colonization by

herbaceous dicotyledonous species (64).

Growth and Vegetative Reproduction. Both quackgrass rhizomes and tillers are initiated from axillary buds on the parent shoot. The rhizomes are generally produced from the basal buds, whereas tillers arise from buds at higher nodes on the stem. Quackgrass seedlings will produce rhizomes and tillers after three to four true leaves are formed. Coleoptile buds usually produce rhizomes whereas buds from lower leaf axils can form either tillers or rhizomes, depending on environmental factors. High nitrogen levels, high temperatures, short day length and low light intensity are environmental factors which promote tiller formation and inhibit rhizome formation (57).

In the fall, the tip of each rhizome develops into an aerial shoot which begins active growth early in the spring. The buds at the base of each shoot begin to form either tillers or rhizomes at the same time. Generally, rhizomes and tillers nearest the tip of the shoot grow to the greatest length (57). Large, plump rhizomes with large stores of food are more likely to produce shoots that photosynthesize and return carbohydrates to the rhizomes for storage. In contrast, short rhizome pieces may exhaust their food stores before new shoots reach the soil surface. Tissue deterioration of the cut ends of rhizome pieces also contributes to their rapid demise (14).

Quackgrass has been reported to exhibit two types of dormancy (16). The first type, correlative inhibition, occurs during most of the year in undisturbed rhizomes. The terminal bud exerts a strong apical dominance over other lateral buds on the same rhizome. It is estimated that over 95% of the lateral buds remain inactive during the life of the rhizome, unless the rhizomes are disturbed or cut (14). A second

type of dormancy, "late spring" or "summer" bud dormancy has been reported by Johnson and Buchholtz (16). Lateral buds remain dormant even after being disturbed or cut, particularly during the month of June in the northern United States (16). Late spring dormancy has a practical implication in that repeated tillage during the dormant period will expose rhizomes which cannot become reestablished, increasing the likelihood of desiccation and death (57). However, these summer dormancy findings are not in agreement with the behavior of rhizome buds in Sweden and in England (14) or elsewhere in North America, as reported by Hay (11).

Seed Formation. Flowering in quackgrass is influenced by many factors including soil fertility, soil moisture, light intensity and competition for space. In the northern hemisphere, the first flower initials may be seen on meristems from April through May, with flowers generally appearing later in June (14). Quackgrass is often reported to be self-sterile, but seeds set by enclosed spikes have been observed in both the greenhouse and the field (14, 57, 64). This indicates that there is some self-fertilization and a small amount of seed can be produced without cross-pollination. The amount of seed produced is highly variable (14, 64), depending upon growing conditions and clone type (14). Reports range from 15 to 400 seeds produced per plant stem, with 25 to 40 seeds most commonly observed. Seed formation may be much higher at the margin of a large group of quackgrass clones, where cross-pollination is more likely to occur (64).

Alternating temperatures are a requirement for seed germination. Up to 90% germination is achieved when temperatures fluctuate between 15 and 25 C. If buried in the soil, seeds may remain dormant for 2 to 3

years (64). Viability gradually decreases with increased seed age and after 6 years viability is close to zero (14).

Interference with Crop Growth. Dense populations of quackgrass cause severe reductions in crop leaf area, net assimilation rate and ultimately, yield. Experimentally, living quackgrass has been shown to reduce yields of field corn (Zea mays L.), oats (Avena sativa L.), barley (Hordeum vulgare L.), and soybeans (24, 54, 64). Quackgrass interference over a period of more than 4 weeks significantly reduced soybean height and seed yield (69). When barley was grown in the presence of quackgrass, increases in quackgrass shoot density resulted in comparable decreases in barley yield (54). The presence of quackgrass in corn resulted in delayed tasseling and silking and increased ear moisture at harvest (2).

Ohman and Kommedahl (40) have estimated that as much as 42 and 8 metric tons of fresh and dry quackgrass material respectively, may be present on one hectare of land. They observed chlorosis and stunting of crop plants grown in the presence of a well-established quackgrass sods, and attributed this to the greater ability of quackgrass to withdraw available nutrients from the soil solution. The extensive need for nitrogen and other nutrients by microorganisms during periods of decay of quackgrass residues may also produce temporary symptoms of nutrient deficiency in crops. However, these hypotheses have proven difficult to test in greenhouse and field situations because of the number of variables involved.

Bandeen and Buchholtz (2) examined the competitive effects of quackgrass upon corn growth in the northern United States. Because of the morphology of the corn plant, the favorable spring moisture supply

and the concurrent dormancy of quackgrass buds at the time the corn is planted, it is unlikely that competition for light and water were of much consequence. Corn grown in the presence of living quackgrass exhibited severely reduced height and yield, and time required for crop maturation was increased. Quackgrass uptake of N, P and K was relatively high. However, high levels of fertilization and available water did not overcome the effects of quackgrass interference. Kommedahl (24) also found that plant height and seed yield of wheat (Triticum vulgare L.), oats, corn and soybeans were lower when grown in tilled field plots which had been in quackgrass sod for 2 years as compared to areas that had lain in fallow for 2 years. The addition of 280 kg/ha N only partially corrected yield reductions caused by the previous stand of quackgrass. It was postulated that metabolites from quackgrass tissues reduced the nutrient uptake by corn roots (2). Phlak (47) found no relationship between soil mineral content and the detrimental growth changes in a succeeding sugarbeet crop following a quackgrass sod. Soil nutrient level apparently played no role in the detrimental effect of quackgrass on sugarbeets.

Evidence for Allelopathy in Quackgrass. The first evidence of allelopathic activity of quackgrass in the field was reported by Ahlgren and Aamodt in 1939 (1). They observed that white clover (Trifolium repens L.) and red clover (Trifolium pratense L.) were seldom found in dense, closely-grazed quackgrass sod. They observed species interactions which could not be accounted for on the basis of differential response to light, temperature, moisture, fertilization, or management. It was later determined by a number of investigators that barley, wheat, oats, corn, flax (Linum usitatissum L.) alfalfa and

soybeans, when grown in newly plowed quackgrass sod, exhibited reduced crop stands and plant dry weights (19, 22, 24). When living quackgrass rhizomes were present in field plots, populations of five weed species were also reduced, indicating that the detrimental effects of quackgrass were not limited to agronomic species (12).

Quackgrass toxicity has also been demonstrated in the greenhouse under controlled conditions. The addition of dead quackgrass rhizomes to the soil caused a reduction in alfalfa seedling stand as well as severe stunting and chlorosis (22). In wheat, quackgrass residues reduced germination, caused overall stunting and seedlings were more subject to blights and rots (20). Oats and alfalfa were also chlorotic and stunted when grown with large amounts of quackgrass shoot residues in the greenhouse (40).

The observed inhibition may be due to the direct production of toxic substances by quackgrass rhizomes or shoots (8, 23, 26, 41, 43). Inhibitors such as butyric and acetic acid may also arise from the decay of quackgrass materials in the soil (40, 45, 60, 62, 63), or the interaction of decaying quackgrass with pathogens or soil microbes (46). In addition, there may be aggressive competition of well-established quackgrass stands with crop plants.

The decomposition of rhizomes under anaerobic conditions results in the production of substances highly toxic to other plants (40, 45, 60, 62, 63). Toxicity was found to increase with waterlogging of the plant rhizomes (63) and incubation at warmer temperatures (60, 63). Decomposing residues, in the presence of soil saprophytes or root-rot fungi may account for some of the damage incurred by crop plants grown in plowed quackgrass sods (40,46).

Several researchers have studied the allelopathic effect of quackgrass in the laboratory by documenting the production and release of toxic compounds. Ohman and Kommedahl have shown that hot water extracts of roots, stems, rhizomes and leaves decreased alfalfa seedling growth by 60 to 85% as compared to distilled-water controls (19, 39). Water leached through pots of growing quackgrass decreased wheat seed germination and secondary root growth as compared to distilled-water controls (19). Toai and Linscott (60) reported that both rhizomes and shoots contained water soluble inhibitors of crop seed germination.

Extracts of young quackgrass seedlings proved to be as toxic to alfalfa as leaf extracts of older plants. All toxicity could be removed from the solution if rhizome extracts were passed through activated charcoal (39). Germinating seeds of quackgrass also released substances which were toxic to wheat seedlings (19).

LeTourneau and Heggeness (26) found that aqueous extracts of quackgrass rhizomes inhibited root growth of pea and wheat seedlings. The inhibitor was polar, dialyzable and nonvolatile. Separation could not be obtained with the use of anion or cation exchange resin. In this study as in previous work (39), activated charcoal removed the majority of the inhibitor from solution.

In the most comprehensive chemical analyses, LeFevre and Clagett found that aqueous extracts of quackgrass rhizomes inhibited the growth of eight crop species by 50 to 84 %. Upon concentration by paper chromatography and ion-exchange column chromatography, the growth inhibitor was found to be anionic, soluble in polar solvents, insoluble in nonpolar solvents and unstable in strong acids and bases (25).

Grummer (9) isolated a polyene compound called agropyrene from dried quackgrass that exhibited antibiotic activity against fungi and bacteria. Its herbicidal properties were never investigated. Most recently, Gabor and Veatch isolated and purified a toxin from quackgrass rhizomes that inhibited seedling root growth of corn, oats, cucumber (Cucumis sativus L.) and alfalfa. The compound was water and alcohol soluble, partly soluble in semipolar solvents and insoluble in nonpolar solvents. It was tentatively identified as a glycoside with a molecular weight of 460 (8).

Previous work indicates that toxic compounds are produced and contained within both quackgrass rhizomes and shoots. The isolation of these endogenous toxins from quackgrass may help to explain the allelopathic effects caused by this weed. However, it is not known whether quackgrass toxins are released directly into the soil where they act as inhibitors, or if the allelopathic effects observed are due in part to the decomposition of quackgrass residues or exudates by microbes in the soil community that render them toxic. It is also not known whether the particular toxin isolated by Gabor and Veatch is released under natural conditions in the field.

NITROGEN FIXATION

Introduction. The symbiotic association between Rhizobium and legumes is the most highly developed system for biological nitrogen fixation (4, 6). This symbiosis produces an estimated 40% of all biologically fixed nitrogen and nearly all nitrogen fixed by crop plants (4). Soil factors are important in influencing the legume-Rhizobium symbiosis. Infection and nodulation of legumes are usually

reduced by acid root media, high concentrations of soluble nitrogenous compounds, or by temperatures below 7 or above 36 C. Rhizobium survival in soils is influenced by previous plant inhabitants and also by the soil's physical and chemical properties. Rhizobium number and nitrogen-fixing ability have been shown to decrease in the absence of legumes, or under conditions creating high acidity and temperature extremes, or highly competitive situations with other soil microbes such as fungi, actinomycetes and bacteria, or in the presence of toxic chemical exudates from plants (7).

Impacts of Weeds or Allelochemicals. Several researchers have shown inhibition of nodulation and nitrogen fixation by allelopathic higher plants. Pandya and Pota reported that root exudates of the weed Celosia argentea effectively inhibited the proliferation of Rhizobium and Pennisetum typhoides (bajra), besides producing inhibition of radicle elongation and dry weight production of bajra seedlings (44). Rice reported inhibition of the nitrogen fixing bacteria (Azotobacter and Rhizobium) and the nitrifying bacteria (Nitrobacter and Nitrosomonas) by plant extracts of various seed plants. Extracts of Aristida oligantha, Ambrosia eliator, Bromus japonicus, Euphorbia supina and Digitaria sanguinalis were particularly inhibitory to the legume-Rhizobium symbiosis as shown by a reduction in nodule numbers or indicator legumes. Legume indicators used were Korean lespedeza (Lespedeza stipulacea Maxim.), white clover and red kidney bean (Phaseolus vulgaris L.) which showed varying degrees of nodulation inhibition (50, 51, 52). Water extracts of balsam poplar (Populus balsamifera L.) were reported to decrease nodule number and acetylene reduction of green alder species (15). The presence of Aristida

adscensions, a dominant grass species growing under dry conditions in Northern India, was also shown to inhibit the proliferation of associated rhizobia and nodulation of Indigofera cordifolia, a common associate of the grass during initial stages of succession (37). Extracts from the roots were generally more active than those from the shoots (38). Bigalta limpograss (Hemarthria altissima) has been shown to produce phenolic compounds (58) which inhibit nodulation of associated legumes. The root exudates do not directly inhibit the growth of associated rhizobia but instead appear to alter the legume root system so that nodulation is reduced (59). Decomposing rice straw in wet paddy conditions was also shown to produce phenolic compounds inhibitory to three strains of Rhizobium, resulting eventually in a reduction of legume nodule numbers, legume leghemoglobin content and nitrogen fixation, as measured by acetylene reduction. This indicated that moist anaerobic conditions were necessary in this case to favor the synthesis of organic toxins by microbes (53).

Thus, allelochemicals produced by higher plant species or associated soil microbes may inhibit the legume-Rhizobium symbiosis in several ways. Inhibition of Rhizobium metabolism or growth, the infection process, the nitrogenase enzyme system or the alteration of the legume root system may result in a less effective legume-Rhizobium symbiosis and a resultant decrease in nitrogen fixation.

Our recent work indicates that legume development and nodulation are inhibited when they are grown in either living or herbicidally-treated quackgrass sod. The inhibition of plant development, nodulation and nitrogen fixation in legumes by quackgrass presents an important problem to the growers of legume crops since quackgrass is a widespread

perennial weed. Quackgrass is not only a highly competitive weed, but also appears to inhibit plant development through allelopathic effects. The inhibition of plant development and the biological nitrogen fixation process may result not only in reduced crop growth rates and lower crop yields but may require that additional nitrogen fertilizer be supplied. Under intensive cropping situations, the principal factor limiting plant growth is often nitrogen. Because of the increased cost of nitrogen fertilizer and the recent shortages and depletion of fossil fuels, unrestricted usage of supplemental nitrogen is questionable (56). It is, therefore, the goal of many present researchers to enhance plant growth through increased efficiency of biological nitrogen fixation. At the same time it should also be the goal of researchers to study the naturally-occurring stresses which limit biological nitrogen fixation, in an attempt to eliminate potentially detrimental field conditions leading to reduced legume yields. Allelochemicals may be an important source of environmental stress.

LITERATURE CITED

1. Ahlgren, H. L. and D. S. Aamodt. 1939. Harmful root interactions as a possible explanation for effects noted between various species of grasses and legumes. Agron. J. 31: 982-985.
2. Bandeen, J. D. and K. P. Buchholtz. 1967. Competitive effects of quackgrass upon corn as modified by fertilization. Weeds 15: 220-224.
3. Bode, H. R. 1940. Uber die Blattausscheidungen des Wermuts and ihre Werkung auf andere Pflanzen. Planta 30:567-589.
4. Brill, W. J. 1977. Biological nitrogen fixation. Scientific American. March 1977: 68-81.
5. Buchholtz, K. P. 1962. Some characteristics of quackgrass and their relation to control. Proc. Northeast Weed Control Conf. 16: 16-22.
6. Burris, R. H. and P. W. Wilson. 1957. Methods of measurements of nitrogen fixation. Meth. Enz. 4: 355-387.
7. Dart, P. J. 1974. The infection process. In (A. Quispel, ed.) The Biology of Nitrogen Fixation, North-Holland, Amsterdam. pp. 382-429.
8. Gabor, W. E. and C. Veatch. 1981. Isolation of a phytotoxin from quackgrass (Agropyron repens) rhizomes. Weed Sci. 29, 2: 155-159.
9. Grummer, G. 1961. The role of toxic substances in the interrelationships between higher plants. Symp. Soc. Exp. Biol. 15: 219-228.

19. Kommedahl, T. 1957. Quackgrass can be toxic to crop seedlings. Down to Earth. Fall 1957: 4-5.

20. Kommedahl, T. and J. B. Kotheimer. 1957. The role of quackgrass in root rots of cereals. Phytopathology Annual Meeting Abstracts. January. p. 21.

21. Kommedahl, T., J. B. Kotheimer and J. V. Bernardini. 1957. The effects of quackgrass on germination and seedling development of certain crop plants. Weeds 7: 1-12.

22. Kommedahl, T., A. J. Linck, and J. V. Bernardini. 1957. The toxic effects of quackgrass on growth of alfalfa. Phytopathol. 47: 526.

23. Kommedahl, T. and J. H. Ohman. 1960. The role of Agropyron repens in the seedling blight epidemiology of alfalfa and cereals. Proc. Minn. Acad. Science 28: 10-15.

24. Kommedahl, T., K. M. Old, J. H. Ohman and E. W. Ryan. 1970. Quackgrass and nitrogen effects on succeeding crops in the field. Weed Sci. 18: 29-32.

25. LeFevre, C. W. and C. O. Clagett. 1960. Concentration of a growth inhibitor from Agropyron repens (quackgrass). Proc. Northeast Weed Control Conf. 14: 353-356.

26. LeTourneau, D. and H. G. Heggeness. 1957. Germination and growth inhibitors in leafy spurge foliage and quackgrass rhizomes. Weeds 5: 12-19.

27. Linderman, R. G. 1970. Plant residue decomposition products and their effects on host roots and fungi pathogenic to roots. Phytopathol. 60: 19-22.

28. Lovett, J. V. and Levitt, J. 1981. Allelochemicals in the future agriculture. In (B. Stonehouse, ed.) Biological Husbandry. B. Butterworths, London. pp. 169-180.

29. Lovett, J. V. 1982. Allelopathy and self-defense in plants. Australian Weeds 2,1: 33-36.

30. McCalla, T. M. and F. A. Haskins. 1964. Phytotoxic substances from soil microorganisms and crop residues. Bact. Rev., 28: 181-207.

31. McCalla, T. M. and F. A. Norstadt. 1974. Toxicity problems in mulch tillage. Ag. and Environ., 1: 153-174.

32. McClure, J. W. 1977. An ecological role for plant phenolics. Report of the Research Planning Conference on the Role of Secondary Compounds in Plant Interactions (Allelopathy). Mississippi State University. pp. 42-62.

33. Molisch, H. 1937. Der Einfluss einer pflanze auf die audere-allelopathic. Jena:Fischer.

34. Muller, C. H. 1965. Inhibitory terpenes volatilized from Salvia shrubs. Bull. Torrey Bot. Club. 92: 38-45.

35. Muller, W. H., P. Lorber and P. Haley. 1968. Volatile growth inhibitors produced by Salvia leucophylla: Effect on seedling growth and respiration. Bull. Torrey Bot. Club. 95: 225-231.

36. Muller, W. H., and C. H. Muller. 1964. Volatile growth inhibitors produced by Salvia species. Bull. Torrey Bot. Club. 91: 327-330.

37. Murthy, M. S. and T. Nogodra. 1977. Allelopathic effects of Aristidia adscensionis on Rhizobium in Indigofera cordifolia J. Appl. Ecol. 14,1: 279-282.

38. Murthy, M. S. and R. Ravindra. 1975. Allelopathic effects of Aristidia adscensionis. Oecologia 18: 243-249.

39. Ohman, J. H. and T. Kommedahl. 1960. Relative toxicity to extracts from vegetative organs of quackgrass to alfalfa. Weeds 8: 666-670.

40. Ohman, J. H. and T. Kommedahl. 1964. Plant extracts, residues, and soil minerals in relation to competition of quackgrass with oats and alfalfa. Weeds 8: 666-670.

41. Osvald, H. 1948. Toxic exudates from the roots of Agropyron repens. J. Ecol. 38: 192-193.

42. Overland, L. 1966. The role of allelopathic substances in the "smother crop" barley. Amer. J. Bot. 53:5 423-432.

43. Palmiter, D. H. 1959. Evidence of quackgrass toxicity to apple seedlings. Phytopathology 49: 228-229.

44. Pandya, S. M. and K. B. Pota. 1978. On the allelopathic potentials of root exudates from different ages of Celosia argentea Linn, a weed. Nat. Acad. Sci. Letters, Vol. 1, 2: 56-58.

45. Patrick, Z. A., T. A. Toussoun, and W. C. Snyder. 1963. Phytotoxic substances in arable soils associated with decomposition of plant residues. Phytopathol. 53: 152-161.

46. Penn, D. J. and J. M. Lynch. 1982. The effect of bacterial fermentation of couchgrass rhizomes and Fusarium culmorum on the growth of barley seedlings. Plant Pathol. 31: 39-43.

47. Phlak, F. 1967. The effect of quackgrass on succeeding plants. Plant Soil. 27: 273-284.

48. Raleigh, S. M., T. R. Flanagan and C. Veatch. 1962. Life history studies as related to weed control in the Northeast. Quackgrass. Bulletin 365. Agricultural Experiment Station, University of Rhode Island, Kingston, RI.

49. Rice, E. L. 1984. Allelopathy. Academic Press, New York. 422 pp.

50. Rice, E. L. 1974. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. I. Ecol. 45: 824-837.

51. Rice, E. L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. II. Characterization and identification of inhibitors. Physiol. Plant. 18: 255-268.

52. Rice, E. L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. III. Comparison of three species of Euphorbia. Proc. Okla. Acad. Sci. 45: 43-44.

53. Rice, E. L., C. Y. Lin, and C. Y. Huang. 1981. Effects of decomposing rice straw on growth and nitrogen fixation by Rhizobium. J. Chem. Ecol. 7,2: 333-345.

54. Rioux, R. 1982. La mesure de l'interference du chiendent dans l'orge. Can J. Plant Sci. 62: 183-188.

55. Rovira, A. D. 1969. Plant root exudates. Bot. Rev. 35: 35-39.

56. Schubert, K. 1982. The energetics of biological nitrogen fixation. Workshop Summaries - I. Plant Physiol. 28 pp.

57. Stobbe, E. H. 1976. Biology of quackgrass. Proceedings of the North Central Weed Control Conference, Omaha, Nebraska. 31: 151-160.

58. Tang, C.-H., and C.-C. Young. 1982. Collection and identification of allelopathic compounds from the undisturbed root system of Bigalta limpograss (Hemarthria altissima). Plant Physiol. 69: 155-160.

59. Tang, C.-H., 1985. Personal communication.

60. Toai, T. V. and D. L. Linscott. 1979. Phytotoxic effects of decaying quackgrass residues. Weed Sci. 27: 595-598.

61. Tukey, H. B. Jr. 1966. Leaching of metabolites from above ground plant parts and its implications. Bull. Torrey Bot. Club. 93: 385-401.

62. Welbank, P. J. 1960. Toxin production from Agropyron repens, In (J. L. Harper, ed.) The Biology of Weeds. Blackwell Scientific Publications, Oxford, England. pp. 158-164.

63. Welbank, P. J. 1963. Toxin production during decay of Agropyron repens (couchgrass) and other species. Weed Res. 3: 205-214.

64. Werner, P. A. and R. Rioux. 1977. The biology of Canadian weeds. Agropyron repens (L.) Beauv. Can. J. Plant Sci. 57: 905-919.

65. Westra, P. H. and D. L. Wyse. 1981. Growth and development of quackgrass (Agropyron repens) biotypes. Weed Science. 29:1 44-52.

66. Whittaker, R. H. 1970. The biochemical ecology of higher plants. In (E. Sondheimer and J. B. Simeone, eds.) Chemical Ecology. Academic Press, New York. pp. 43-70.

67. Whittaker, R.H. and P. Feeny. 1971. Allelochemics: chemical interaction between species. Science. 171: 757-770.

68. Wyse, D. L. 1980. Vernolate for augmentation of quackgrass (Agropyron repens) control with glyphosate. Weed Sci. 28: 654-657.

69. Young, F. L., D. L. Wyse, and R. J. Jones. 1982. Influence of quackgrass (Agropyron repens) density and duration of interference on soybeans (Glycine max). Weed Sci. 30: 614-619.

CHAPTER 2

INHIBITION OF GROWTH, NODULATION AND NITROGEN FIXATION OF LEGUMES BY QUACKGRASS (Agropyron repens (L.) Beauv.)

ABSTRACT

Quackgrass (Agropyron repens (L.) Beauv.) has been reported to possess allelopathic potential. Both the shoots and rhizomes of quackgrass contain inhibitors of seed germination and crop growth. We recently observed chlorosis and decreases in nodulation in snapbeans that were planted into quackgrass infested sites. The objective of this study was to determine if nodulation, nitrogen fixation and legume growth were inhibited in the presence of living or herbicidally-treated quackgrass. Soybeans (Glycine max (L.) Merr.), navybeans, and snapbeans (Phaseolus vulgaris (L.)) were grown both in greenhouse and field experiments in living or killed quackgrass. Inoculated legumes were seeded in the following regimes: 1) living quackgrass sod which was regularly mowed; 2) glyphosate (N-phosphonomethyl glycine) - treated quackgrass sod 3) soil from quackgrass sod from which plant material was removed by sieving or raking; 4) a control soil of similar type and physical structure free from quackgrass infestation. Nodulation and plant growth were measured by recording nodule number and fresh weight and root and shoot weights before flowering. Nitrogen fixation was estimated by the acetylene reduction assay. Legumes grown in mowed quackgrass sod in the greenhouse and in the field exhibited

decreased nodule number, nodule fresh weight and nitrogen fixation when compared to legumes grown under similar conditions in screened quackgrass soil or the control soil. Shoot and root weights were also significantly decreased in field and greenhouse experiments when legumes were grown in living quackgrass sod. In many cases, legume nodulation and growth were decreased in glyphosate-treated quackgrass sod as compared to screened quackgrass soil or the control soil. Decreases in legume growth and nodulation in the presence of quackgrass may be attributed to allelochemical effects.

INTRODUCTION

Quackgrass is an aggressive perennial that is a widespread weed problem in the temperate zone of the Northern Hemisphere (4). It is a particular problem in soybeans and drybeans as few herbicides are available for selective control in these crops (4, 25).

The detrimental interference of quackgrass on the growth of higher plants has been demonstrated both in the field and under controlled conditions. These effects may be due to the direct production of toxic substances by quackgrass shoots and rhizomes (2, 6, 9, 11, 16), inhibitors such as acetic and butyric acid (14), which arise from the decay of quackgrass materials in the soil (7, 10, 13, 22, 23, 24), the interaction of decaying quackgrass with soil plant pathogens such as Fusarium culmorum (15), or the aggressive competition of well-established quackgrass stands with the crop plants for available light, water and nutrient supplies (5, 10).

The allelopathic effects of quackgrass in the laboratory have been confirmed by documenting the production and release of toxic compounds. Toai and Linscott (22) reported that dried quackgrass rhizomes and leaves contained water-soluble toxins which inhibit alfalfa (Medicago sativa (L.)) seed germination. Decomposing quackgrass residues developed additional toxins after incubation at temperatures of 30°C (22). Two phytotoxins present in quackgrass have been characterized (2, 6); one is tentatively identified as a glycoside (2).

Allelopathic stresses upon crop plants can reduce crop emergence, growth and yield, resistance to plant pathogens, and possibly nodulation and nitrogen fixation (20). In 1982, we repeatedly observed that field-grown snapbeans in herbicidally-treated quackgrass residues were markedly chlorotic, stunted in size, and possessed few, if any, nodules.

Inhibition of nodulation of legumes in the presence of quackgrass or its residues was not previously documented. However, previous work by other researchers has shown evidence for the inhibition of nodulation and nitrogen fixation by other weed species (12, 17, 18, 19). The inhibition of plant growth, nodulation and nitrogen fixation pose a threat to growers of legume crops through reductions in stand, growth rate and yield as well as the need for additional fertilizer nitrogen. The following experiments were initiated to determine if legume growth, nodulation and nitrogen fixation are inhibited by the presence of living quackgrass or herbicidally-treated quackgrass residues.

MATERIALS AND METHODS

Field Experiments. The experiments were conducted at the Horticulture Research farm, East Lansing, MI., on a Marlette sandy loam (Glossoboric Hapludalf, fine- loamy, mixed, mesic). Plots were established on a level site with dense quackgrass sod that had been undisturbed for 15 years. No lime or fertilizer was applied since composite soil analysis showed a pH of 6.4; 12 kg NO_3, 57 kg P_2O_5, and 98 kg K_2O/ha; and ample remaining Ca, Mg, Zn, Fe, and Mn.

Treatments were: 1) living quackgrass sod maintained at a height of 2.5 to 7.5 cm (trimmed weekly with hand shears and a lawnmower) to minimize competitive effects of quackgrass shoots; 2) quackgrass sod treated 13 May with 0.7 kg/ha glyphosate (N-phosphonomethyl glycine) to form a dead rhizome and shoot mulch; Any remaining living quackgrass in treatment 2 was trimmed weekly with hand shears; 3) quackgrass sod plowed to a depth of 25 cm on 2 June. Rhizome and shoot material remaining in the top 15 cm was removed by hand raking. Quackgrass in treatments 1 and 2 was mowed to a 5 cm height on 6 June, 2 days before planting and all loose residue was raked from the plots.

Snapbean 'Bush Blue Lake', navybean 'Seafarer', and soybean 'Corsoy 79' were used as indicator species for growth and nodulation. Two 9.1 m rows, 0.25 m apart, were planted of each cultivar. Twenty seeds/m, were planted 2.5 cm deep on 8 June in each plot with a no-till planter (Moore Uni-Drill). All seed was previously moistened and coated with the appropriate peat-based Rhizobium japonicum, R.

phaseoli, or R. leguminosarum inoculant (Nitragin Co.). The field design was a randomized complete block with 4 blocks. Each cultivar was considered as a separate experiment. Plots were irrigated overhead (<4 cm/wk) as necessary to maintain moist top-soil conditions and hand weeded during the experiment.

To determine the effects on growth and nodulation, legumes were harvested (navybean, 7 July; snapbean, 9 July; soybean, 11 July) before flowering. Root systems were carefully extracted from the soil and gently washed. Nodule number, nodule fresh weight, root fresh weight and shoot dry weight were determined for 10 randomly chosen plants per plot and 40 plants per treatment (Tables 1 and 2).

Nitrogenase activity of nodules as an estimate of nitrogen fixation per plant was measured by acetylene reduction (3) (Table 3). Measurements were made on detached roots of 4-week-old plants, before flowering commenced. All plant collections were made between 0800 and 1000 EDT. During each collection, four plants were harvested from each plot. Soil was carefully removed from the root system, plants were cut at the soil level, and roots were placed into airtight 60 ml serum vials and exposed to 10% acetylene in air for 1 h at ambient temperature. Ethylene concentrations were determined with a gas chromatograph (Varian Model 1900) equipped with flame ionization detector and a stainless steel column packed with Poropak N.

Soil analysis was performed on each treatment at the end of the experiment. Results were similar and the composite analysis showed pH 6.4, 18 kg NO_3, 75 kg P_2O_5, and 150 kg K_2O/ha and ample remaining major nutrients.

Data were analyzed for the randomized complete block experiments using separate analyses of variance for each species and cultivar. Where significant treatment effects were found, means were compared using LSD values (0.05).

Greenhouse Experiments. Snapbeans 'Bush Blue Lake', navybeans 'Seafarer', soybeans 'Corsoy 79', and peas 'Sparkle' were selected as indicators for growth and nodulation in greenhouse experiments. Quackgrass sods (rhizomes and soil) were collected from the same site where field experiments were performed. No additional fertilizer was applied during the experiment.

Quackgrass sod or soil was placed into 30 cm square wooden flats, 15 cm in depth and the following treatments were applied: 1) living quackgrass sod maintained at a height of 2.5 to 6 cm (trimmed weekly with hand shears) to minimize competitive effects of quackgrass shoots; 2) quackgrass sod treated with 0.6 kg/ha glyphosate 2 weeks before planting to form a dead rhizome and shoot mulch. Any quackgrass escapes were maintained at a height of 2.5 to 6 cm by trimming weekly with hand shears; 3) rhizosphere soil obtained from the quackgrass field site that had been sifted with a fine mesh sieve to remove all plant material and in addition; 4) a Marlette sandy loam soil, free of quackgrass infestation and with similar soil analysis, obtained from an adjacent field and sifted with a fine mesh sieve before use. Quackgrass in treatments 1 and 2 was trimmed to a height of 3 cm before planting and all loose residue was removed from the flats.

Indicator seeds were moistened and coated with the appropriate peat-based Rhizobium inoculant (Nitragin Co.) and planted at a 2.5 cm depth. Ten seeds each of snapbean, navybean, and pea were planted in

one flat in three separate rows, 12 cm apart, with a 5 cm spacing within rows. Flats were thinned to 5 plants per row 10 days after planting. Soybean seed was planted in flats in a similar manner; two rows of soybeans 15 cm apart and a 5 cm spacing within rows, and the plants thinned as above. The design for all experiments was a randomized complete block with four blocks. Experiments were repeated twice with plantings on 18 June and 12 November.

Flats were placed under 1000W metal halide lamps with photosynthetic flux density (PPFD) of 425 μmol m^{-2} s^{-1} at the top of the canopy. A 14-h-light/10-h-dark photoperiod at 28/22 C was maintained in the greenhouse. Plants were watered overhead twice daily in sunny weather or once daily in cloudy conditions to maintain moist soil conditions. Weed control in the flats was maintained by hand weeding.

On 15 July and 23 December, before the onset of flowering, legume root systems were carefully extracted from the soil and gently washed. Nodule numbers, nodule fresh weights, root fresh weights and shoot dry weights were collected for five plants of each cultivar per flat (Tables 4 and 5). The acetylene reduction assay (3) was also performed as previously described on two plants per flat of each cultivar to estimate nitrogen fixation per plant (Table 6).

Data from individual species and cultivars were analyzed separately. Data from the two experiments were combined for analysis of variance. Treatment means were compared using the LSD (0.05) test where significant treatment effects were found.

RESULTS AND DISCUSSION

The application of 0.7 kg/ha glyphosate resulted in nearly complete control (> 90%) of quackgrass in the field with little regrowth of shoots. The raked and hand- weeded plots showed little quackgrass regrowth. Snapbeans and navybeans grown in trimmed living quackgrass sod appeared stunted and chlorotic and the root systems were browned in comparison to other treatments.

The response of three legume species were similar in all parameters measured (Tables 1-3). Shoot dry weight and root fresh weight (Table 1) were greatest when 4-week-old plants were grown in either glyphosate-treated quackgrass or when plant material was removed from the plot by raking. Up to 50% reductions in shoot dry weights and root fresh weights were observed when legumes were grown in trimmed living quackgrass sod. There was a 1.3 to 20-fold reduction in nodule number when legumes were grown in living quackgrass sod as compared to treatments with quackgrass material removed (Table 2). Decreased nodule fresh weight per plant in navybeans and soybeans was primarily attributed to fewer nodules per root system (Table 2). Decreased nodule numbers on plants grown in glyphosate-treated quackgrass were also observed as compared to the situation in which quackgrass material was removed from plots. When snapbeans and navybeans were grown in living quackgrass sod, 50 to 80% decreases in nitrogen fixation, as measured by total nitrogenase activity, were observed when compared to plants grown in the absence of quackgrass material (Table 3).

Table 1. Influence of various quackgrass regimes on shoot dry weight and root fresh weight of three legume indicators grown in the field.

Regime	Snapbean	Navybean	Soybean
	----------(g)----------		
		Shoot wt[a]	
Living	1.7 b[b]	1.1 b	1.6 b
Glyphosate	4.6 a	2.9 a	3.7 a
Removed	3.1 ab	2.1 a	3.7 a
		Root Weight[a]	
Living	1.6 b[b]	1.9 b	2.4 c
Glyphosate	3.1 a	3.0 a	4.4 ab
Removed	3.5 a	2.3 ab	5.3 a

[a]Figures are the means of 40 plants harvested per treatment.

[a]Means within columns followed by different letters are significantly different at the 5% level.

Table 2. Influence of various quackgrass regimes on nodule number and nodule fresh weight of three legume indicators grown in the field.

Regime	Snapbean	Navybean	Soybean
		Nodule No.[a]	
Living	2 c[b]	5 b	61 ab
Glyphosate	3 b	5 b	54 b
Removed	44 a	15 a	79 a
		Nodule Weight[a]	
	----------(g)----------		
Living	0.03[b]	0.03 b	0.60 b
Glyphosate	0.10	0.16 a	1.10 a
Removed	0.14	0.07 b	1.20 a

[a]Figures are the means of 40 plants harvested per treatment.

[b]Means within columns followed by different letters are significantly different at the 5% level.

Table 3. Influence of various quackgrass regimes on nitrogen fixing ability of three legume indicators grown in the field.

Regime	Fixed Nitrogen (N_2)[a]		
	Snapbean	Navybean	Soybean
	----------(nmoles plant^{-1}h^{-1})----------		
Living	28 c[b]	212 c	1654
Glyphosate	158 ab	191 b	1702
Removed	198 a	399 a	2871

[a]Figures are the means of 16 plants harvested per treatment. Figures represent total nitrogenase activity.

[b]Means within columns followed by different letters are significantly different.

Decreased nitrogen fixation, as measured by total nodule activity, was most likely caused by decreased nodule numbers, perhaps as a result of reduced overall root growth. Nodule specific activity was calculated but was not significantly different among treatments, indicating that nitrogenase activity per g of nodule tissue did not vary among treatments. Legumes grown in the absence of quackgrass material exhibited greatest nitrogen fixing ability per plant.

Results obtained in the greenhouse experiments generally substantiated observations made in the field experiments. The application of 0.6 kg/ha glyphosate resulted in nearly complete control (> 95%) of quackgrass with little shoot regrowth. Legumes grown in living or glyphosate-treated quackgrass sod often appeared chlorotic, and browning of the root system was observed. Shoot dry weights of snapbeans, navybeans, and soybeans, before the onset of flowering, were greatest in glyphosate-treated quackgrass (Table 4). Root fresh weights of all indicators were decreased up to 70% when grown in living sod as compared to legumes grown in the absence of quackgrass sod (Table 4). Nodule numbers of these three indicators were significantly decreased in the presence of either living or glyphosate-treated quackgrass as compared to controls (Table 5). Nodule fresh weights of snapbeans and navybeans were greatest when grown in the control soil and lowest in the presence of either living or glyphosate-treated quackgrass (Table 5). Nitrogen fixing ability of snapbeans and navybeans (on a per plant basis) was also decreased by more than 70% when legumes were grown in living or glyphosate-treated quackgrass sod as compared to other treatments where quackgrass material had been removed (Table 6). Nodule specific activity (nitrogenase activity/g

Table 4. Influence of various quackgrass regimes on shoot dry weight and root fresh weight of four legume indicators grown in the greenhouse.

Regime	Snapbean	Navybean	Soybean	Pea
	----------(g)----------			
		Shoot wt[a]		
Living	2.3 b[b]	1.5 b	4.2 b	0.5
Glyphosate	4.1 a	2.4 a	6.1 a	0.8
Sifted	2.4 b	1.3 b	2.5 c	0.6
Control	2.4 b	1.7 b	3.5 b	0.6
		Root Weight[a]		
Living	2.0 b[b]	1.7 d	4.2 c	0.7 b
Glyphosate	3.0 b	2.8 c	6.7 bc	1.0 b
Sifted	5.0 a	5.5 a	8.2 ab	2.4 a
Control	4.3 ab	4.0 b	10.6 a	2.2 a

[a]Figures are the means of 40 plants harvested per treatment on 15 July and 23 December.

[b]Means within columns followed by different letters are significantly different at the 5% level.

Table 5. Influence of various quackgrass regimes on nodule number and nodule fresh weight of four legume indicators grown in the greenhouse.

Regime	Snapbean	Navybean	Soybean	Pea
		Nodule No.[a]		
Living	24 c[b]	27 bc	51 b	18
Glyphosate	39 c	14 c	62 b	34
Sifted	134 b	57 b	66 b	36
Control	195 a	107 a	83 a	41
		Nodule Weight[a]		
	----------(g)----------			
Living	0.09 c[b]	0.08 c	1.30	0.02
Glyphosate	0.16 c	0.04 c	1.60	0.05
Sifted	0.64 b	0.26 b	1.10	0.07
Control	0.98 a	0.51 a	1.50	0.08

[a]Figures are the means of 40 plants harvested per treatment on 15 July and 23 December.

[b]Means within columns followed by different letters are significantly different at the 5% level.

Table 6. Influence of various quackgrass regimes on nitrogen-fixing ability of four legume indicators grown in the greenhouse.

Regime	Fixed Nitrogen (N_2)[a]			
	Snapbean	Navybean	Soybean	Pea
	(nmoles plant^{-1}h^{-1})			
Living	66 c[b]	55 b	1919	21
Glyphosate	183 bc	42 b	1749	21
Sifted	325 b	119 b	2257	30
Control	612 a	413 a	2237	43

[a]Figures are the means of 16 plants harvested per treatment on 15 July and 23 December. Figures represent total nitrogenase activity.

[b]Means within columns followed by different letters are significantly different at the 5% level.

nodule tissue) was calculated and was not significantly different among treatments. Overall reductions in plant growth and nodulation are likely responsible for reductions in legume nitrogen fixing activity.

In all experiments, snapbeans and navybeans were the indicator species most sensitive to the presence of quackgrass, as measured by decreased plant size, nodulation and nitrogen fixation. Soybeans appeared to be least inhibited by the presence of living or glyphosate-treated quackgrass while peas produced few nodules for observation in both experiments. If one calculates nodule number or nodule weight/g root tissue these trends are maintained. Snapbeans and navybeans produced many fewer nodules/g root tissue in the presence of quackgrass as compared to control situations, while soybean nodulation/g root tissue was less affected by the presence of quackgrass. Soybean nodulation may be less inhibited in this case due to the morphology of the soybean root. 'Corsoy' 79 soybeans formed the majority of nodules on the upper two inches of root crown tissue while snapbeans and navybeans nodulated throughout the root system. Greatest exposure to leaching, water soluble toxins would be expected to occur around the quackgrass rhizome system. The greatest density of rhizomes occurs several inches below the soil surface, beneath the majority of soybean nodule tissue.

The highly competitive nature of living quackgrass may account for some of the observed inhibition of plant growth, nodulation and nitrogen fixation. Competition for available light, water and nutrients may result in stress imposed upon legume crops grown in a quackgrass sod (5, 10). However, soil nutrient levels in all treatments were sufficient for legume growth, and nitrate

concentrations (< 56 kg/ha) were in the range generally considered not inhibitory to symbiotic nitrogen fixation. In addition, plants were irrigated in a timely manner to prevent moisture stress in either greenhouse or field experiments. Competition for light was decreased by repeated trimming of quackgrass shoots.

Effects of dead quackgrass are most likely attributed to allelopathy. The production and release of toxic water soluble compounds by quackgrass has been previously documented (2, 6, 9, 11, 16). These compounds were implicated in inhibition of seed germination, and root and shoot growth in various higher plants. Toxins released by living and/or decomposing quackgrass material may be partially responsible for the inhibition of root growth, nodulation and nitrogen fixation in legumes. In the laboratory, we have obtained strong inhibition of seed germination and radicle elongation in legume seedlings by aqueous extracts of both quackgrass shoots and rhizomes [Weston and Putnam, (unpublished)]. However, the extracts do not inhibit the growth of several species of Rhizobium. The browned and stunted appearance of legume roots grown in the presence of living and herbicidally-treated quackgrass suggests that a toxin may be released from quackgrass. These inhibitors may have a direct effect on the growth of the legume root system to reduce or prevent root hair formation with the consequent decrease in infection and nodulation by rhizobia.

A marked inhibition of nodulation and nitrogen fixation of legumes grown in glyphosate-treated quackgrass was also observed. The application of glyphosate may somehow enhance toxin production in quackgrass or perhaps increase leakiness of cell membranes resulting in

a release of compounds into the rhizosphere which may act directly or upon decomposition (7, 10, 13, 22, 24). Numerous experiments have shown that legume crops can be successfully grown in soils recently treated with glyphosate (1, 8, 21). Data from this laboratory showed no direct inhibitory effect of glyphosate (10^{-3} - 10^{-5}M) on the growth of three strains of rhizobia (R. japonicum, R. phaseoli, R. leguminosarum) on yeast extract mannitol agar. It is therefore doubtful that the inhibitory effect on nodulation can be attributed to glyphosate per se.

The results of these greenhouse and field experiments show that certain legumes grown in living or glyphosate-treated quackgrass exhibit large reductions in root growth, nodulation and nitrogen fixation. More detailed investigations on the effects of quackgrass on the growth and nodulation process in legumes are warranted. From field and greenhouse studies, one cannot ascertain whether inhibition in legume growth is caused by a direct release of quackgrass allelochemicals or their indirect release through the interactions of soil microbes and quackgrass residues. However, legumes grown in our laboratory under sterile conditions in the presence of aqueous quackgrass extracts exhibited severe root necrosis and stunted growth. This laboratory has recently isolated two compounds from quackgrass which are inhibitory to seed germination and root growth (Weston and Putnam, unpublished). The characterization and identification of these inhibitory compounds will contribute to an understanding of the allelopathic mechanism.

LITERATURE CITED

1. Baird, D. D., R. P. Upchurch, W. B. Homesley and J. E. Franz. 1971. Introduction of a new broadspectrum postemergence herbicide class with utility for herbaceous perennial weed control. Proc. North Cent. Weed Contr. Conf. 26:64-68.
2. Gabor, W. E. and C. Veatch. 1981. Isolation of a phytotoxin from quackgrass (Agropyron repens) rhizomes. Weed Sci. 29. 2:155-159.
3. Hardy, R. F. W., D. R. Holsten, E. K. Jackson, and R. C. Burns. 1969. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.
4. Holm, L. G., D. L. Plucknett, J. V. Pancho, and J. P. Herberger. 1977. The World's Worst Weeds: Distribution and Biology. University Press, Hawaii.
5. Kommedahl, T., K. M. Old, J. H. Ohman, and E. W. Ryan. 1970. Quackgrass and nitrogen effects on succeeding crops in the field. Weed Sci. 18:29-32.
6. LeFevre, C. W. and C. O. Clagett. 1960. Concentration of a growth inhibitor from Agropyron repens (quackgrass). Proc. North Cent. Weed Contr. Conf. 14:353-356.
7. Lynch, J. M. and D. J. Penn. 1980. Damage to cereals caused by decaying weed residues. J. Sci. Food Agric. 31:321-324.
8. Moshier, L. and D. Penner. 1978. Use of glyphosate in sod seedling alfalfa (Medicago sativa) Establishment. Weed Sci. 26:163-166.

9. Ohman, J. H. and T. Kommedahl. 1960. Relative toxicity of extracts from vegetative organs of quackgrass to alfalfa. Weeds. 8:666-670.

10. Ohman, J. H. and T. Kommedahl. 1964. Plant extracts, residues, and soil minerals in relation to competition of quackgrass with oats and alfalfa. Weeds. 12:222-231.

11. Osvald, H. 1948. Toxic exudates from the roots of Agropyron repens. J. Ecol. 38:192-193.

12. Pandya, S. M. and K. B. Pota. 1978. On the allelopathic potentials of root exudates from different ages of Celosia argentea Linn., a weed. Nat. Acad. Sci. Letters, Vol. 1, No. 2:56-58.

13. Patrick, Z. A., T. A. Toussoun, and W. C. Snyder. 1963. Phytotoxic substances in arable soils, associated with decomposition of plant residues. Phytopath. 53:152-161.

14. Penn, D. J. and J. M. Lynch. 1981. Effect of decaying couchgrass rhizomes on the growth of barley. J. of Appl. Ecol. 18, 669-674

15. Penn, D. J. and J. M. Lynch. 1982. The effect of bacterial fermentation of couchgrass rhizomes and Fusarium culmorum on the growth of barley seedlings. Plant Pathology. 31: 39-43.

16. Plhak, F. 1967. The effect of quackgrass on succeeding plants. Plant Soil. 27:273-284.

17. Rice, E. L. 1964. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. I., Ecol. 45:824-837.

18. Rice, E. L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. II., Characterization and identification of inhibitors, Physiol. Plant. 18:255-268.

19. Rice, E. L. 1965. Inhibition of nitrogen-fixing and nitrifying bacteria by seed plants. III., Comparison of three species of Euphorbia, Proc. Okla. Acad. Sci. 45:43-44.

20. Rice, E. L. 1974. Allelopathy, Academic Press, New York. 353 pp.

21. Sprankle, P., W. F. Meggitt and D. Penner. 1975. Rapid inactivation of glyphosate in the soil. Weed Sci. 23:224-228.

22. Toai, T. V. and D. L. Linscott. 1979. Phytotoxic effects of decaying quackgrass residues, Weed Sci. 27:595-598.

23. Welbank, P. J. 1960. Toxin production from Agropyron repens, In (J. L. Harper, ed.) The Biology of Weeds, Blackwell Scientific Publications, Oxford, England. pp 158-164.

24. Welbank, P. J. 1963. Toxin production during decay of Agropyron repens (couchgrass) and other species, Weed Res. 3:205-214.

25. Wyse, D. L. 1980. Vernolate for augmentation of quackgrass (Agropyron repens) control with glyphosate, Weed Sci. 28:654-657.

CHAPTER 3

INHIBITION OF LEGUME SEEDLING GROWTH BY RESIDUES AND EXTRACTS OF QUACKGRASS (*Agropyron repens* (L.) Beauv.)

ABSTRACT

Aqueous extracts of quackgrass (*Agropyron repens* (L.) Beauv.) shoots and rhizomes inhibited seed germination and root growth of alfalfa (*Medicago sativa* L. 'Vernal'), soybean (*Glycine max* (L.) Merr. 'Corsoy 79'), and navybean (*Phaseolus vulgaris* L. 'Seafarer'), as well as curly cress (*Lepidium sativum* L.), at concentrations of less than 2.5 mg dried extract/ml. Extracts of quackgrass shoots were generally more inhibitory to radicle elongation than were extracts of rhizomes. Root and shoot dry weights of snapbeans (*Phaseolus vulgaris* L. 'Bush Blue Lake') grown under sterile conditions were reduced by aqueous extracts of shoots. Root systems were stunted and necrotic and lacked root hairs when observed by scanning electron microscopy. The growth of *Rhizobium* species was not influenced by the presence of 40 or 80 mg/ml concentrations of extracts of shoots or rhizomes. Thus, quackgrass may inhibit indirectly the legume/*Rhizobium* symbiosis by inhibiting root hair formation rather than directly inhibiting *Rhizobium* growth. The presence of soil microorganisms was not necessary for the development of quackgrass toxicity in soil or agar. Soil microorganisms reduced toxicity of quackgrass residues in soil.

INTRODUCTION

Quackgrass is an aggressive perennial plant that reproduces mainly by rhizomes. It is a widespread weed problem in the cooler zones of the Northern Hemisphere (3), where it is a strong competitor in grain, forage, vegetable and fruit crops (4, 21).

Allelopathic influences of weeds upon crop plants have been manifested as reductions in crop germination, stands, root and shoot growth, yield, resistance to plant pathogens, nodulation and nitrogen fixation (16). Previous research in both the greenhouse and field has demonstrated the detrimental interference of quackgrass on the growth of higher plants. However, literature concerning quackgrass interference does not clearly differentiate the relative roles of competition and allelopathy in the total interference (16). Interference by quackgrass has been attributed to the aggressive competition of living quackgrass for available light, water and nutrient supplies (6, 9). Other investigators believe that interference may be due to the direct production of toxins by quackgrass shoots or rhizomes (3, 7, 10, 15, 18), inhibitors such as butyric and acetic acid (12), which arise from the decay of materials in the soil (8, 9, 12, 17, 19), or the interaction of decaying quackgrass with pathogenic soil microbes (14).

The production and release of toxic compounds by quackgrass have been documented in the laboratory. Dried quackgrass rhizomes and leaves were reported by Toai and Linscott (17) to contain water-soluble

toxins which inhibit alfalfa seed germination. After incubation at 30 C, decomposing quackgrass residues developed additional toxins. Two phytotoxins from quackgrass rhizomes have been tentatively characterized (3, 7); one is suggested to be a glycoside (3).

Recent work reported reduced root and shoot growth, nodulation, and subsequent nitrogen fixation by legumes grown in the presence of both living and herbicidally-treated quackgrass (20). In addition, legumes grown in the presence of quackgrass displayed chlorotic leaves and necrotic roots. The results of these experiments indicated that quackgrass or its associated microbes released allelochemicals which inhibited the legume-Rhizobium symbiosis, and interfered with subsequent nodulation and nitrogen fixation.

The following objectives were explored to further investigate the possible allelopathic interference of quackgrass upon the legume-Rhizobium symbiosis under well-controlled conditions: 1) determine the relative content of water extractable inhibitors in rhizomes and shoots of quackgrass, 2) assess the effects of quackgrass extracts on seed germination, root development, and the legume-Rhizobium symbiosis, and 3) to determine the influence of soil microorganisms upon the relative toxicity of quackgrass residues.

MATERIALS AND METHODS

Tissue Preparation. Quackgrass sod (rhizomes and soil) was collected at the Horticulture Research Farm, East Lansing, MI, on November 11, 1983 from a level site with a dense quackgrass stand that had been undisturbed for 15 years. Soil type was a Marlette sandy loam

(Glossoboric Hapludalf, fine-loamy, mixed, mesic). The sod was placed in 30 cm square wooden flats, 15 cm in depth. Flats were placed under metal halide lamps in the greenhouse with photosynthetic photon flux density (PPFD) of 425 umol $m^{-2}s^{-1}$ at the top of the canopy. A 14-h-light/10-h-dark photoperiod at 28/22 C was maintained in the greenhouse. Flats were fertilized overhead once weekly with 600 ml soluble 20-20-20 fertilizer at a rate of 3.9 g/L of water.

When quackgrass shoots reached a height of 20 to 25 cm, they were harvested with shears by trimming 3 to 4 cm above the soil surface on December 16, 1983. Rhizomes were carefully removed from the flats and washed thoroughly with tap and distilled water. Rhizome and shoot material were then placed in a drying oven at 35 to 40 C for 4 days. Plant material was ground in a mill (mesh screen size 1 mm).

Powdered shoot and rhizome tissues (100 g) were extracted separately for 24 h with 3 L distilled water on a shaker in a 4 C coldroom. The mixture was filtered through four layers of cheesecloth and centrifuged for 20 min at 16,300 g to remove particulate material. Extracts were filtered through # 4, #1, and #42 grade filter paper (Whatman) using a Buchner funnel. The clear extracts were lyophilized at -75 C and the residue was stored under vacuum at room temperature. Two hundred and fifty mg residue were extracted from 1.0 g of shoot tissue. Five hundred and twenty mg residue were extracted from 1.0 g of rhizome tissue.

Our studies were designed to assess the allelopathic potential of quackgrass under well-controlled conditions. For example, lower temperatures of 40 C for drying were assumed to prevent alteration of potentially-allelopathic compounds. Water was used as the solvent for

extraction of plant material rather than organic solvents, and extraction proceeded for only 24 h. In addition, extraction was carried out in a cold room at 4 C and plant material was rapidly lyophilized to deter microbial growth and breakdown of plant extracts. The pH of both aqueous and shoot extracts applied in these experiments was approximately 6.5.

Influence of quackgrass extracts on radicle growth. Four experiments were designed to compare the toxicity of aqueous extracts of quackgrass rhizomes and shoots on the root growth of four indicator species. Lyophilized extracts were redissolved in distilled water to form a dilution series of rhizome or shoot extracts ranging in concentration from 53.3 to 0.4 mg/ml. Plastic petri dishes (60 by 15 mm) were lined with filter paper (Whatman #1) and 1.5 ml of each treatment solution was applied on ten seeds per dish. When Vernal alfalfa or Burpee curly garden cress were used as bioassays, extracts of rhizomes and shoots were applied at 80 to 0.63 mg/petri dish along with distilled water controls. In 'Corsoy 79' soybean and 'Seafarer' navybean assays, extracts of rhizomes and shoots were applied at 80 to 2.5 mg/petri dish along with distilled water controls. Four replicates of each treatment were arranged in a completely randomized design and placed in a 26 C germination chamber with relative humidity near saturation. Radicle lengths of all species were measured at 72 h. Data were subjected to analysis of variance and standard errors were calculated. Percentage inhibition (Figure 1) was expressed as a percentage decrease in radicle elongation in comparison to the control.

Influence of quackgrass extracts on Rhizobium species. Pure cultures of Rhizobium japonicum ((Kirchner) Buchanan 1926, #61A115), Rhizobium meliloti (Dangeard 1926, #102F51), Rhizobium phaseoli

(Dangeard 1926, #127K44), and Rhizobium leguminosarum ((Frank) Frank 1889, #128C53) were obtained commercially.[1] Cultures were maintained under sterile conditions at 35 C on a yeast extract plus mannitol (YEM) agar (1.5%) until use. Each Rhizobium species was transferred under sterile conditions into separate YEM broths. The broths were maintained for 4 days on a slow shaker (130 oscillations/min) in a growth chamber at 26 C. Broths were harvested when cells were in the early log phase of growth ($2x10^6$ cells/ml). The Rhizobium inoculum (0.1 ml) of each species was mixed with 2.5 ml YEM agar (0.75%), and this mixture spread uniformly across each of 18 sterile 1.5% YEM agar plates per Rhizobium species, for a total of 72 plates. After the plate surfaces had solidified for 1 h, sterile 21 mm filter paper discs (Whatman #540) were infiltrated with sterile treatment solutions and one was placed on the center of the plate. Solution treatments consisted of quackgrass extracts of shoots or rhizomes at 40 and 80 mg/ml; streptomycin sulfate at 80 mg/ml (a known inhibitor of Rhizobium growth), used only as a check on the ability of the procedure to detect antibiotic activity; and distilled water. All solutions were sterilized by filtration through a 0.2 um filter unit. Three replicates of each treatment were arranged in a completely randomized manner and placed in an incubator at 30 C. The zone of inhibition around the filter paper disc was measured for the slow-growing R. japonicum treatments at 120 h, whereas the faster growing species were measured at 48 h. When inhibition occurs, a clear zone void of bacterial growth is present around the

[1]Nitragin Corporation, 3101 West Custer Avenue, Milwaukee, WI 53209

disc and when it is absent bacteria will grow directly up to the filter paper disc. The experiment was repeated under the same conditions and data from the six replicates were combined for analysis.

Influence of quackgrass extracts upon root growth of snapbeans under sterile conditions. One hundred and twenty five ml of a 1% agar Fahraeus medium (an agar highly suitable for legume growth under sterile conditions) (2) were poured into 250 ml Ehrlenmeyer flasks. The use of Fahraeus medium allows culture of legumes in sterile agar culture, excluding microorganisms which may alter the toxicity of plant compounds. Snapbeans were selected for this bioassay because they can be easily cultured in agar media as opposed to other large or small seeded legume crops. Flasks were closed with styrofoam stoppers and autoclaved at 121 C for 25 min. Bush Blue Lake snapbean seeds were placed in a 0.5% solution of calcium hypochlorite for 4.5 min for surface sterilization and then rinsed for 1 min with sterile distilled water. Seeds were placed on moist sterile filter paper in glass petri dishes in a 30 C growth chamber for 4 days in the dark. When seedling radicles were approximately 3 cm in length, the Fahraeus agar in the flasks was remelted by autoclaving at 121 C for 10 min (agar was made beforehand for convenience). Treatment solutions were then added and mixed before the agar solidified; these consisted of lyophilized extracts of rhizomes or shoots dissolved in water (80 mg/ml). To each flask, 2.5 ml (200 mg) of filter-sterilized extract solution was added to give a final concentration of 1.6 mg/ml agar medium. In the controls, 2.5 ml distilled water was added to the agar. Pregerminated seeds were planted into the treated agar at a 1.5 cm depth, one seed per flask. Seeds were inoculated with 0.1 ml *R. phaseoli* in the

exponential phase of growth in YEM broth. Flasks were placed in a lighted growth chamber at 27 C day and 23 C night temperatures. A 16 h photoperiod under fluorescent and incandescent bulbs was maintained with an average PPFD of 175 umol $m^{-2}s^{-1}$ at the top of the flasks. The treatments in each experiment were replicated ten times, a replicate consisting of one flask and arranged in a completely randomized design. Only the plants remaining sterile were harvested after 30 days, when beans reached the second to third trifoliate leaf stage. The experiment was later repeated as above, applying shoot extract at concentrations ranging from 0 to 4 mg/ml to establish a dose response curve. Sterile plants were harvested after 21 days, when beans reached the second to third trifoliate leaf stage. Root and shoot dry weights were recorded. Data from the two experiments were analyzed separately using analysis of variance and mean separation by LSD (.05) test.

Observations were made on the visual appearance of the snapbeans, and scanning electron microscopy was used to examine the root tissues. Samples were fixed according to standard electron microscopy procedures (alcohol dehydration, critical point drying and sputter-coating with gold) (5) and viewed with a JEOL JSM-35 C scanning microscope.

Influence of soil microorganisms on the phytotoxicity of quackgrass shoots. Quackgrass was collected, placed in 30 cm square wooden flats, and grown in the greenhouse as previously reported. After 4 weeks, quackgrass shoots were trimmed with shears at soil level and placed in a drying oven at 35 to 40 C for 4 days. Dried shoot material was cut into 1.5 cm pieces. Shoot material was placed into an atmosphere of propylene oxide (0.1 %) and air for 48 h for surface sterilization. A Parker bioassay (11) was used for observation of toxicity of quackgrass residues on seed germination and radicle elongation. Spinks sandy loam

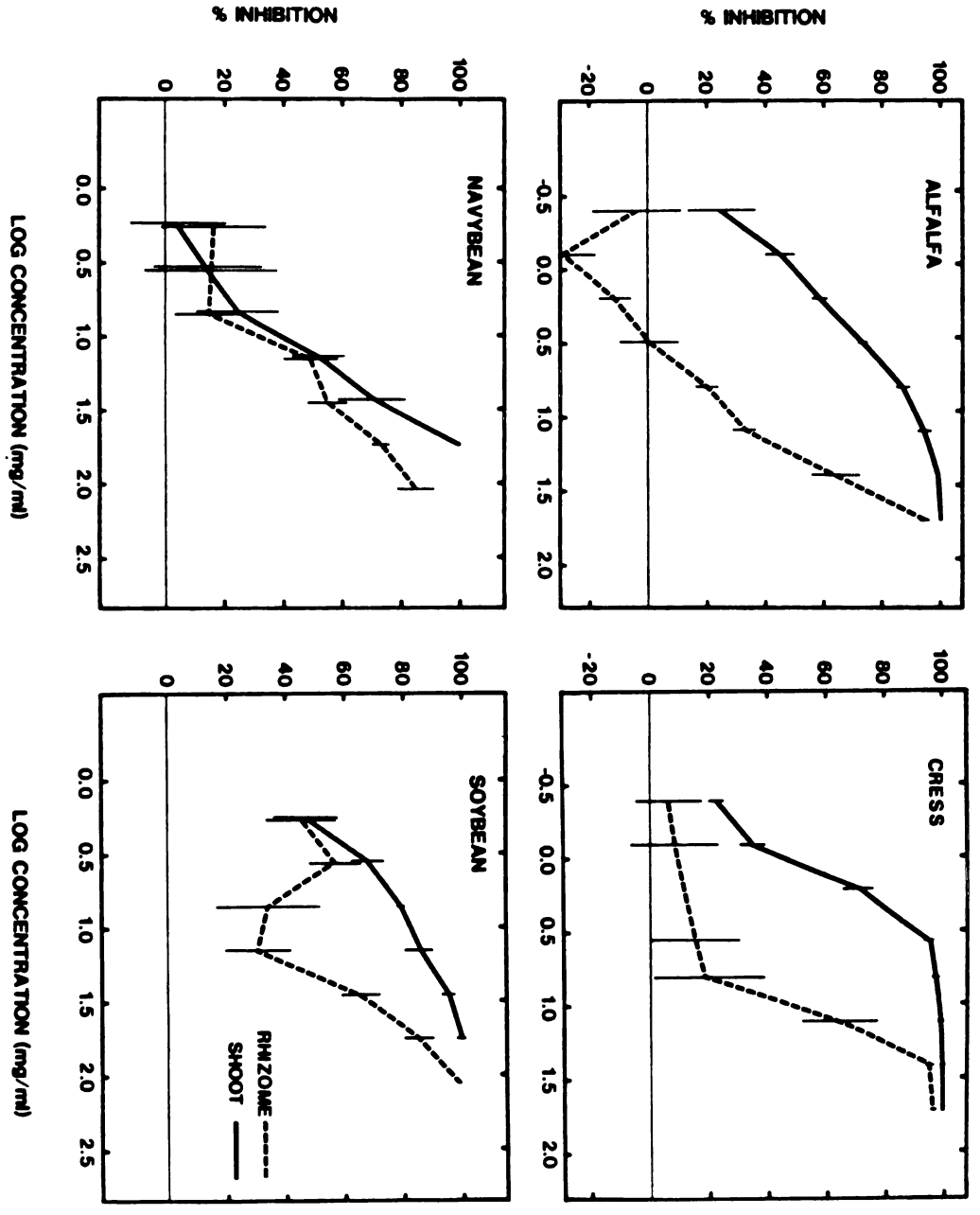
(Psammentic Hapludalf, sandy, mixed, mesic) was sifted and placed in a 40 C oven for 7 days until dry. One portion of this soil was autoclaved at 121 C for 1 h on 2 consecutive days to sterilize the soil. Treated soil (100 g) was placed into each 100 by 15 mm square plastic petri dish. Next, 0.5 g of the quackgrass material was spread uniformly across each soil surface. Finally, a thin layer of the appropriate Spinks sandy loam (50 g) was placed on the surface of each residue to cover the residue. The control dishes for this experiment contained 0.5 g of propylene oxide-treated paper toweling cut into 1.5 cm pieces. Treatments included quackgrass shoots placed on top of autoclaved soil, paper toweling placed on top of autoclaved soil, quackgrass shoots placed on top of non-sterile soil, and paper toweling placed on top of non-sterile soil. Dishes were then moistened with 35 ml of distilled water. A 9 by 8 cm piece of filter paper (Whatman #1) was placed on the soil surface of each dish. Ten seeds of each of two indicator species were uniformly placed on either end of the dish for bioassay. Lettuce (Lactuca sativa L. 'Ithaca'), Burpee curly garden cress, proso millet (Panicum miliaceum L.) and barnyardgrass (Echinochloa crus galli L. Beauv.) were utilized as bioassays. Dishes were covered, taped shut and placed in a vertical position to encourage downward root growth. Dishes were placed in a 26 C growth chamber and radicle length was measured for each seed 3 days later. Experiments were analyzed separately by species, as completely randomized designs with eight replicates per treatment. Data were subjected to analysis of variance and means separated by the LSD (.05) test.

RESULTS AND DISCUSSION

Influence of quackgrass extracts on radicle growth. Extracts of quackgrass rhizomes and shoots caused significant decreases in radicle length of four crop species after 72 h growth. (Figure 1). Radicle length was a more sensitive indicator of crop inhibition than seed germination or shoot length (data not presented). Shoot extracts were generally more inhibitory to radicle growth of the indicator species (mean I_{50} value, 3.8 mg/ml) than were the rhizome extracts (mean I_{50} value, 13.8 mg/ml), with the exception of navybean. The aqueous shoot extracts were more inhibitory to smaller seeded alfalfa and cress (I_{50} 1.3 to 2.0 mg/ml) than to larger seeded legumes (I_{50} 2.0 to 10.0 mg/ml). These findings support those of Toai and Linscott (17), who reported quackgrass rhizome and shoot materials both to contain water soluble germination indicators. Our experiments also indicated that aqueous shoot extracts of quackgrass were inhibitory to radicle growth of a variety of crop species when present at relatively low rates. At higher rates seed germination was severely inhibited (data not presented).

Influence of quackgrass extracts upon root growth of snapbeans under sterile conditions. Snapbeans grown with extracts of quackgrass rhizomes exhibited a slight brownish discoloration of the root system and a normal appearance of the shoots when compared to the controls. No significant decreases in root or shoot dry weights of snapbeans occurred in the presence of the rhizome extract as compared to the

Figure 1: Inhibition of seedling radicle elongation of four indicator species by extracts of quackgrass shoots and rhizomes after 72 h of growth. Bars represent $\pm$ one standard error unit.


% INHIBITION
ALFALFA
CRESS
NAVYBEAN
SOYBEAN
RHIZOME
SHOOT
LOG CONCENTRATION (mg/ml)
LOG CONCENTRATION (mg/ml)

control (Table 1). However, snapbean shoot and root dry weights were decreased by over two-fold in the presence of the sterile shoot extract. Snapbeans grown in the presence of shoot extract exhibited severe generalized darkening of root tissue, swelling of roots, and lack of root hair formation. In many cases, necrotic lesions appeared at the base of the stem. In comparison, the controls produced a white fibrous root system with a healthy appearance of roots and shoots.

The dose response curve indicated that the greatest inhibition of both snapbean root and shoot weight by extracts of quackgrass shoots occurred at the highest application rate of 4 mg/ml (Figure 2). When observed by scanning electron microscopy, the control roots appeared slender and straight, and exhibited numerous root hairs (Figure 3a). The treated roots showed an extreme reduction in root hair number and the appearance of large root appendages (Figures 3b and 3c). Quackgrass toxins may reduce nodulation and subsequent nitrogen fixation in legumes (20), as a result of the inhibition of legume root growth (Figure 2) and root hair formation (Figure 3), thereby eliminating suitable sites for Rhizobium infection (1). Alternatively, the toxins might inhibit Rhizobium growth. Thus, possible direct inhibition of Rhizobium growth was tested.

Influence of quackgrass extracts on Rhizobium species. No inhibition of either slow-growing (R. japonicum) or the fast-growing (R. leguminosarum, R. meliloti, R. phaseoli) species was noted by relatively high concentrations of extracts of rhizomes or shoots (data not presented). A smooth white "lawn" of Rhizobium grew directly around the filter paper disc as it did in the controls. However, streptomycin sulfate (a known inhibitor of Rhizobium growth) at 80 mg/ml did

Table 1: Influence of sterile aqueous extracts (1.6 mg/ml) of quackgrass shoots and rhizomes on the dry weight of aseptically-grown snapbeans.

Treatment	Shoot weight[b]	Root weight[b]
	(mg)[a]	
Control	190	60
Rhizome	200	70
Shoot	80	20
F value	**	**
LSD (.05)	80	30

[a]Mean weights of 8 plants per treatment.

[b]*5% level of probability, **1% level of probability, NS = no significant difference.

Figure 2: Dose response curve of the extract of quackgrass shoots upon root and shoot dry weights of aseptically-grown snapbeans. The LSD (.05) value was 65 mg for shoot dry weight and 20 mg for root dry weight.

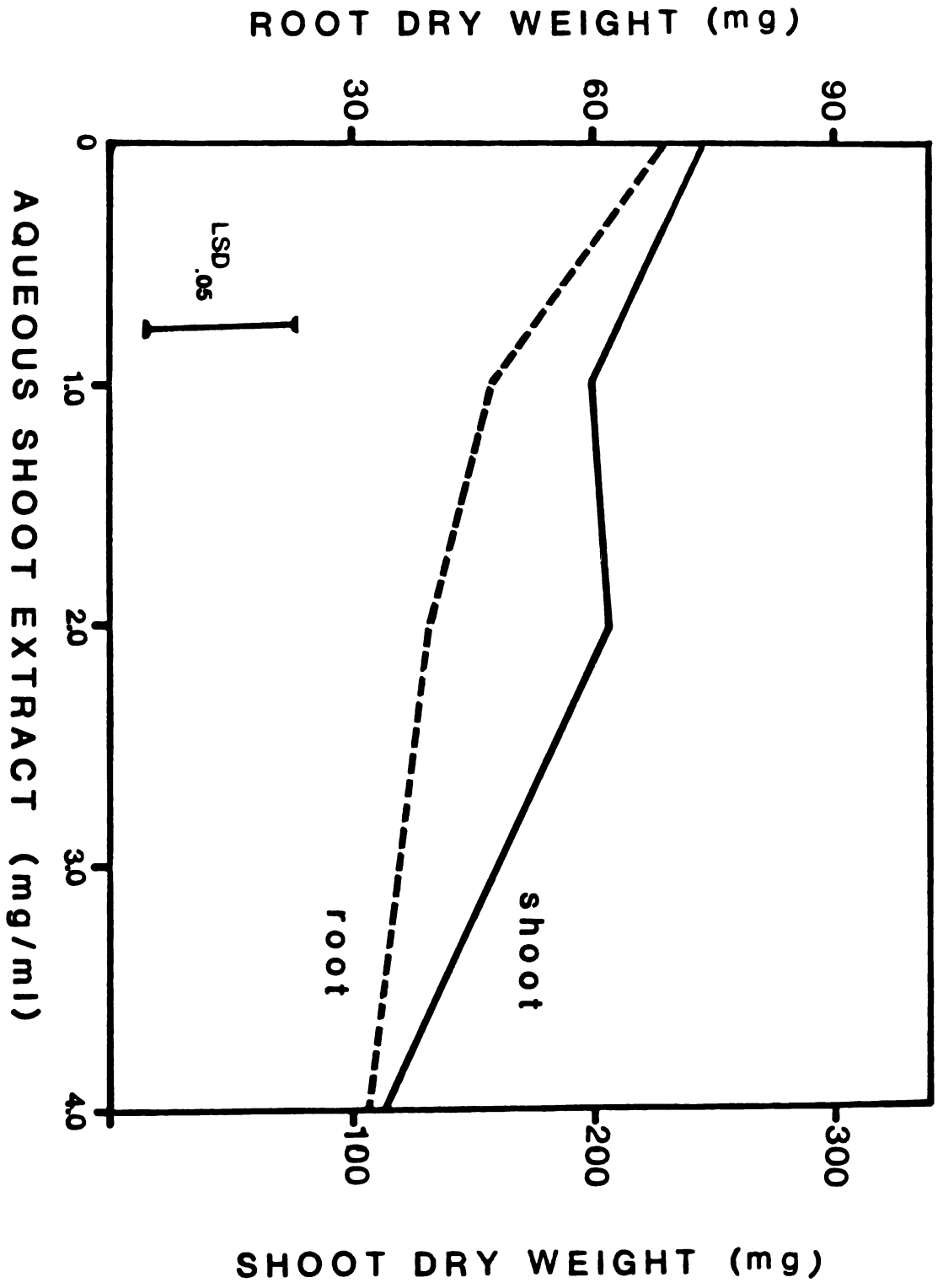
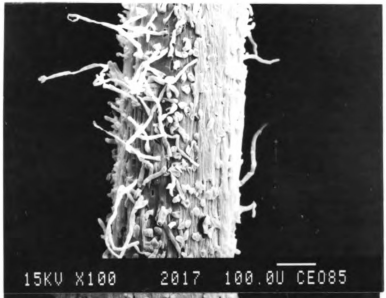
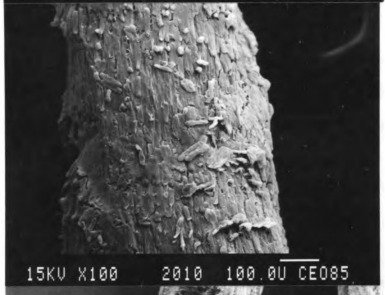

ROOT DRY WEIGHT (mg)
30
60
90
0
1.0
2.0
3.0
4.0
AQUEOUS SHOOT EXTRACT (mg/ml)
LSD .05
root
shoot
100
200
300
SHOOT DRY WEIGHT (mg)

Figure 3a: Scanning electron micrograph (x 100 magnification) of a typical untreated aseptically-grown control snapbean root. Root segment was taken 1.5 cm from root tip. Note the presence of numerous root hairs.

Figure 3b: Scanning electron micrograph (x 100 magnification) of a typical aseptically-grown snapbean root grown in extract of quackgrass shoots. Root segment was taken 1.5 cm from root tip. Note the absence of root hairs and flaking of epidermal tissue.

Figure 3c: Scanning electron micrograph (x 32 magnification) of an aseptically-grown snapbean root grown in extract of quackgrass shoots. Segment was taken 5.0 cm from root tip. Note the presence of swollen root appendages.


15KV X100 2017 100.0U CE085


15KV X100 2010 100.0U CE085


15KV X32 2013 1000.0U CE085

significantly inhibit the growth of four different species around the paper disc. This indicated that quackgrass compounds do not play a direct role in inhibiting the growth of the four common species of Rhizobium that participate in the legume-Rhizobium symbiosis.

Influence of soil microorganisms on the phytotoxicity of quackgrass shoots. In all cases, the quackgrass residue inhibited radicle elongation more under sterile than under non-sterile soil conditions (Table 2). As observed in sterile agar culture with quackgrass extracts (Figure 2), the presence of soil microbes is not necessary for quackgrass extracts to produce toxicity. In fact, residues were significantly less toxic under non-sterile soil conditions (Table 2), indicating that soil microbes may detoxify quackgrass toxins. In general, the small-seeded broadleaf species, cress and lettuce, were inhibited more by the quackgrass residues than were the grass species, barnyardgrass and millet.

Experiments performed under controlled laboratory conditions indicated that extracts of quackgrass shoots indirectly inhibit nodulation and nitrogen fixation in legumes by releasing toxins which inhibit root growth and root hair formation. Quackgrass shoot extracts and residues were also inhibitory to seed germination and radicle elongation. Previous studies concerned with the allelopathic interference of quackgrass have generally involved only quackgrass rhizome material (3, 7, 10). However, the marked toxicity of quackgrass shoot residues and extracts observed in our laboratory may explain the detrimental interference of quackgrass residues on crop growth in the field (6, 8, 15, 19, 20). Our experiments indicate that the toxicity of quackgrass extracts and shoot residues is not dependent on the presence of soil microbes. It is not neccessary for soil microbes to metabolize

Table 2: Influence of quackgrass shoot residues upon radicle length of four indicator species grown in sterile and non-sterile soil conditions.

Treatments	Radicle Length			
	Lettuce	Cress	Barnyardgrass	Proso Millet
	(mm)[ab]			
Nonsterile control	17.4	56.8	35.5	43.5
Nonsterile quackgrass	10.9	36.1	30.3	31.6
Sterile control	11.5	43.4	27.8	44.3
Sterile quackgrass	3.1	4.2	10.8	26.5
F value	**	**	**	**
LSD (.05)	0.5	6.0	5.1	6.2

[a]Measurements of radicle length were collected 72 h after initiation.

[b] *5% level of probability, **1% level of probability, NS = no significant difference.

quackgrass compounds into simpler, toxic forms for inhibition to occur as suggested by others (8, 13, 14). Instead, soil microorganisms reduced the toxicity of quackgrass residues in soil.

LITERATURE CITED

1. Dart, P. J. 1974. The infection process. Pages 382-429 in A. Quispel, ed. The Biology of Nitrogen Fixation. North Holland Press, Amsterdam.
2. Dazzo, F. B. 1982. Leguminous root nodules. Pages 431-446 in R. G. Burns and J. L. Slater, ed. Experimental Microbial Ecology. Blackwell Scientific. Oxford, U.K.
3. Gabor, W. E. and C. Veatch. 1981. Isolation of a phytotoxin from quackgrass (Agropyron repens) rhizomes. Weed Sci. 29:155-159.
4. Holm, L. G., D. L. Plucknett, J. V. Pancho, and J. P. Herberger. 1977. Pages 153-168 in The World's Worst Weeds, Distribution and Biology. University Press, Hawaii.
5. Hooper, G. R., K. K. Baker and S. L. Flegler. 1979. pages 1-144 in Exercises in Electron Microscopy: A Laboratory Manual for Biological and Medical Sciences. MSU University Press, Michigan. 144 pp.
6. Kommedahl, T., K. M. Old, J. H. Ohman, and E. W. Ryan. 1970. Quackgrass and nitrogen effects on succeeding crops in the field. Weed Sci. 18:29-32.
7. LeFevre, C. W. and C. O. Clagett. 1960. Concentration of a growth inhibitor from Agropyron repens (quackgrass). Proc. North Cent. Weed Contr. Conf. 14:353-356.
8. Lynch, J. M. and D. J. Penn. 1980. Damage to cereals caused by decaying weed residues. J. Sci. Food Agric. 31:321-324.

9. Ohman, J. H. and T. Kommedahl. 1964. Plant extracts, residues, and soil minerals in relation to competition of quackgrass with oats and alfalfa. Weeds. 12:222-231.

10. Osvald, H. 1948. Toxic exudates from the roots of Agropyron repens. J. Ecol. 38:192-193.

11. Parker, C. 1966. The importance of shoot entry in the action of herbicides applied to soil. Weeds 14(2):117-121.

12. Patrick, Z. A., T. A. Toussoun, and W. C. Snyder. 1963. Phytotoxic substances in arable soils, associated with decomposition of plant residues. Phytopathology. 53:152-161.

13. Penn, D. J. and J. M. Lynch. 1981. Effect of decaying couchgrass rhizomes on the growth of barley. J. of Appl. Ecol. 18:669-674.

14. Penn, D. J. and J. M. Lynch. 1982. The effect of bacterial fermentation of couchgrass rhizomes and Fusarium culmorum on the growth of barley seedlings. Plant Pathol. 31:39-43.

15. Phlak, F. 1967. The effect of quackgrass on succeeding plants. Plant Soil. 27:273-284.

16. Rice, E. L. 1984. Pages 1-422 in Allelopathy. Academic Press, New York. 353 pp.

17. Toai, T. V. and D. L. Linscott. 1979. Phytotoxic effects of decaying quackgrass residues. Weed Sci. 27:595-598.

18. Welbank, P. J. 1960. Toxin production from Agropyron repens. Pages 158-164 in J. L. Harper, ed. The Biology of Weeds. Blackwell Scientific Publications, Oxford, England.

19. Welbank, P. J. 1963. Toxin production during decay of Agropyron repens (couchgrass) and other species. Weed Res. 3:205-214.

20. Weston, L. A. and A. R. Putnam. 1985. Inhibition of growth, nodulation and nitrogen fixation of legumes by quackgrass (Agropyron repens). Crop. Sci. 25:561-565.

21. Wyse, D. L. 1980. Vernolate for augmentation of quackgrass (Agropyron repens) control with glyphosate. Weed Sci. 28:654-657.

CHAPTER 4

ISOLATION, CHARACTERIZATION AND ACTIVITY OF PHYTOTOXIC COMPOUNDS FROM QUACKGRASS (*Agropyron repens* (L.) Beauv.)

ABSTRACT

Previous experiments showed that legumes grown in the presence of living or herbicidally-treated quackgrass residues or extracts exhibited reduced seedling root and shoot growth and decreased nodulation and nitrogen fixation. Aqueous extracts of quackgrass shoots were most inhibitory to plant growth. Upon sequential partitioning of an aqueous extract of quackgrass shoots, the ether extract possessed the most activity and caused 50% reductions in radicle elongation of eight crop and weed species at concentrations of less than 240 µg/ml (small seeded species) and 1000 µg/ml (large seeded species). Snapbeans (*Phaseolus vulgaris* L. 'Bush Blue Lake') grown aseptically in agar containing an ether extract at 100 and 200 µg/ml exhibited severe root browning, lack of root hair formation and a two to three fold reduction in root and shoot dry weights. The ether extract of quackgrass shoots had no inhibitory effect on the growth of four *Rhizobium* species in petri dishes or two species in broth culture. Toxins present in the ether extract may inhibit the legume *Rhizobium* symbiosis indirectly by reducing legume root growth and root hair formation. The ether extract of quackgrass shoots was separated using high pressure liquid, thin layer and liquid column chromatography in an attempt to isolate and

identify the toxins responsible for the inhibition of seedling growth. Two closely related flavonoid toxins were isolated from the ether extract. One was identified as 5,7,4'-trihydroxy 3',5'-trimethoxy-flavone (tricin). Both flavonoids caused 50% inhibition of radicle elongation in cress (Lepidium sativum L. 'Burpee curly') seeds at concentrations of less than 125 µg/ml. Both flavonoids were found in ether extracts of quackgrass shoots and rhizomes, but the largest amounts of both compounds occurred in quackgrass shoots collected from the field.

INTRODUCTION

Quackgrass is a highly aggressive perennial plant that reproduces mainly by rhizomes (5). It is a widespread weed problem in grain, forage, vegetable and fruit crops grown in the cooler zones of the Northern Hemisphere (5, 23).

Previous research in both the field and the greenhouse has demonstrated the detrimental interference of quackgrass on the growth of higher plants. However, the literature concerning quackgrass interference does not clearly differentiate the relative roles of allelopathy and competition in the total interference (15). In the field, quackgrass has been reported as an aggressive competitor for light, water and nutrients (6, 10). Others have reported considerable allelopathic activity by quackgrass due to the direct production of toxins by quackgrass shoots or rhizomes (7, 11, 14, 19), inhibitors such as acetic and butyric acid (12) (which arise from the decay of materials in the soil (8, 10, 12, 17, 20)), or the interaction of decaying quackgrass with pathogenic soil microbes (13).

In the field and greenhouse, Weston and Putnam (21) reported reduced root and shoot growth, nodulation and nitrogen fixation by legumes grown in the presence of living and herbicidally-treated quackgrass. Legumes grown in the presence of quackgrass displayed chlorotic leaves and necrotic roots. In the laboratory, extracts of quackgrass shoots were particularly inhibitory to seed germination, radicle elongation and root growth of legumes. Root systems were stunted, necrotic and lacked root hairs when grown in the presence of shoot or rhizome extracts, indicating that quackgrass indirectly inhibits the legume/Rhizobium symbiosis by inhibiting root hair formation rather than directly inhibiting Rhizobium growth (22).

The production and release of toxic compounds by quackgrass have been documented in the laboratory. Dried quackgrass rhizomes and leaves were reported by both Toai and Linscott (17) and Ohman and Kommedahl (10) to contain water soluble toxins which inhibit seedling growth. Two toxins from quackgrass rhizomes have been tentatively characterized by Gabor and Veatch (2) and LeFevre and Clagett (7); one is suggested to be a glycoside. However, the structures of these toxins have never been fully elucidated. Characterization of toxins present in quackgrass shoots, the portion of the plant most toxic to seedling growth (22), has not been previously reported.

To further explore the possible allelopathic potential of quackgrass shoots under well-controlled conditions we attempted to: 1) assess the effect of partitioned quackgrass shoot extracts on seed germination, root development, and the legume-Rhizobium symbiosis 2) isolate and identify the toxin(s) present in quackgrass shoots responsible for the inhibition of seedling root growth and 3) quantify

the amount of the phytotoxin(s) in aqueous extracts of both field and greenhouse-grown rhizome and shoot tissue.

MATERIALS AND METHODS

Procedure for collection of quackgrass material. Quackgrass was collected from the Horticultural Research Farm, East Lansing, MI. The site had a soil classified as a Marlette sandy loam (Glossoboric Hapludalf, fine-loamy, mixed, mesic) and supported a community dominated by quackgrass that had grown undisturbed for approximately fifteen years. On June 10, 1984, field-grown quackgrass shoots (25 to 30 cm in height) were collected and placed in a drying oven at 35 to 40 C for 4 days. Rhizomes were also carefully removed from field soil at this time, washed thoroughly with tap and distilled water and placed in a drying oven under similar conditions.

Sods (0.3 by 0.3 m) were collected from this field site and placed in square wooden flats, 15 cm in depth. Flats were placed under metal halide lighting in the greenhouse with photosynthetic photon flux density (PPFD) of 425 μmol m^{-2} s^{-1} measured at the top of the canopy. The greenhouse was maintained with a 14-h-light/10-h-dark photoperiod at 28/22 C. Flats were fertilized once weekly with 600 ml soluble 20-20-20 fertilizer at a rate of 3.9 g/L of water. When quackgrass shoots reached a height of 20 to 25 cm, they were repeatedly harvested with shears by trimming 3 to 4 cm above the soil surface. After 60 days, rhizomes were carefully removed from the flats and washed thoroughly with tap and distilled water. Rhizomes and shoot material were then placed in a drying oven at 35 to 40 C for 4 days. The entire process of

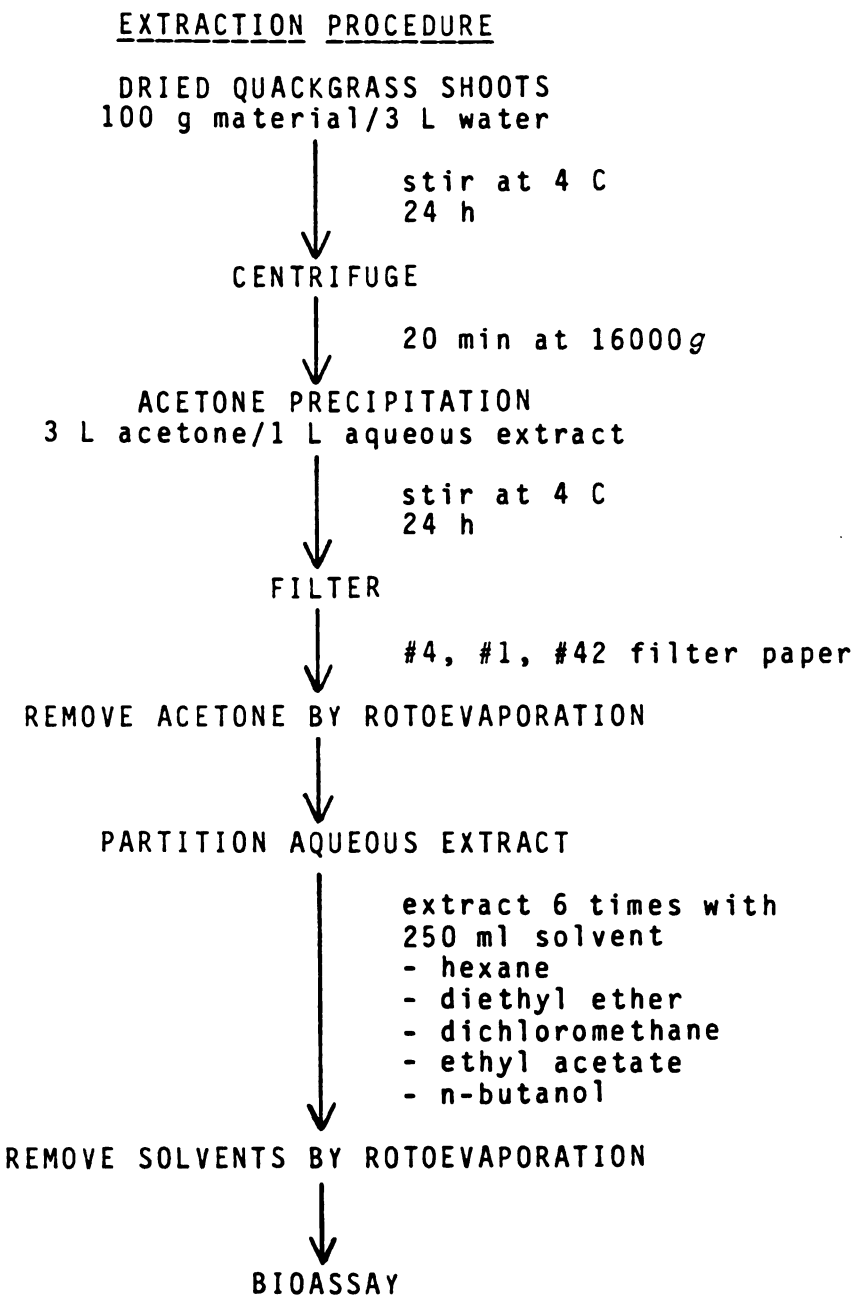
collecting material from the greenhouse was repeated six times between 1983 and 1985 to gather sufficient material for experimental use.

All field-grown and greenhouse-grown plant material was ground separately in a Wiley mill (mesh screen size 1 mm). Powdered material was stored in tightly closed glass containers until used for extraction and quantification of quackgrass phytotoxins.

Procedure for extraction of quackgrass shoot material. Shoot tissue (100 g) was extracted for 24 h with 3 L of distilled water on a shaker in a 4 C coldroom (Figure 1). The mixture was filtered through four layers of cheesecloth and centrifuged for 20 min at 16,000 g to remove particulate material. Nine liters of chilled acetone were slowly added to the aqueous extract. The mixture was stirred at slow speed on a stir plate for 24 h in a 4 C coldroom. Proteinaceous material precipitated out of solution in the presence of acetone and was removed by vacuum filtration through Whatman #4, #1, and #42 filter paper and discarded. (This procedure prevents dense emulsions from forming during partitioning). Acetone was removed from the extract by rotary evaporation at 35 C. The clear, aqueous extract was sequentially partitioned with the following solvent series ranging from least polar to most polar: hexane, diethyl ether, dichloromethane, ethyl acetate and n-butanol. Each liter of the aqueous shoot extract was sequentially partitioned six times with 250 ml of each of the five solvents. Solvent was removed from each fraction by rotary evaporation at 35 C. The entire process of extraction and partitioning was repeated five times to gather enough material for experimentation.

Each fraction from the liquid/liquid extractions was redissolved in methanol to form solutions at a concentration of 0.5 mg/ml. One ml

Figure 1: Flow diagram of the procedure for extraction and partitioning of dried quackgrass shoots.


EXTRACTION PROCEDURE
DRIED QUACKGRASS SHOOTS
100 g material/3 L water
stir at 4 C
24 h
CENTRIFUGE
20 min at 16000g
ACETONE PRECIPITATION
3 L acetone/1 L aqueous extract
stir at 4 C
24 h
FILTER
#4, #1, #42 filter paper
REMOVE ACETONE BY ROTOEVAPORATION
PARTITION AQUEOUS EXTRACT
extract 6 times with
250 ml solvent
- hexane
- diethyl ether
- dichloromethane
- ethyl acetate
- n-butanol
REMOVE SOLVENTS BY ROTOEVAPORATION
BIOASSAY

of each fraction was placed in separate glass petri dishes (60 by 15 mm), which were lined with filter paper (Whatman #1). Methanol was allowed to evaporate from each dish and 1.5 ml distilled water was added to form a concentration of 0.33 mg material/ml. In addition to the fractions obtained from the liquid/liquid extractions, the following materials were also tested for toxicity: a distilled water control (applied to the filter paper after evaporating 1.0 ml of methanol), crude aqueous extract of quackgrass shoots, and the aqueous extract of quackgrass shoots remaining after partitioning. Twenty seeds of 'Burpee curly' cress were then added to each dish. Radicle elongation of curly cress was consistently used as a bioassay throughout our experiments because of its availability, uniformity and sensitivity. Three replicates of each treatment were arranged in a completely randomized design and placed in a 26 C growth chamber with relative humidity near saturation. Radicle lengths of seeds were measured at 72 h. Data were subjected to analysis of variance and means separated by LSD (.05).

Influence of ether extracts on radicle growth. Ether extracts were redissolved in methanol to form a dilution series ranging in concentration from 1600 µg/ml to 25 µg/ml. One ml of each treatment solution was applied separately to glass petri dishes. After the addition of 1.5 ml distilled water per dish, concentrations ranged from 1066.7 µg/ml to 16.7 µg/ml. A distilled water control was also included and twenty seeds were arranged per dish. Seed indicators used were: 'Burpee curly' cress, lettuce (Lactuca sativa L. 'Ithaca'), alfalfa (Medicago sativa L. 'Vernal'), redroot pigweed (Amaranthus retroflexus L.), soybean (Glycine max (L.) Merr. 'Corsoy 79'), navybean (Phaseolus

vulgaris L. 'Seafarer'), barnyardgrass (Echinochloa crus galli (L.) Beauv.) and smooth crabgrass (Digitaria ischaemum (Schreb. ex Schweig.) Schreb. ex Muhl.). Two replicates of each treatment were arranged in a completely randomized design and placed in a 26 C growth chamber with relative humidity near saturation. Radicle lengths of all species were measured at 72 h and data were analyzed as described previously. Standard errors were calculated for each mean.

Influence of ether extracts on aseptically-cultured snapbeans. Fahraeus medium (125 ml of 1% agar) was poured into 250 ml Ehrlenmeyer flasks. This medium is well suited for culture of legumes in sterile agar culture (1), excluding microorganisms which may alter the toxicity of plant compounds. Snapbeans were selected for this bioassay because they can be easily cultured in agar media as opposed to other legume crops. Flasks were closed with styrofoam stoppers and autoclaved at 121 C for 25 min. 'Bush Blue Lake' snapbean seeds were placed in a 0.5% solution of calcium hypochlorite for 4.5 min for surface sterilization and then rinsed for 1 min with sterile distilled water. Seeds were placed on moist sterile filter paper in glass petri dishes in a 27 C growth chamber for 4 days in the dark. When seedling radicles were approximately 3 cm in length, the Fahraeus agar in the flasks was remelted by autoclaving at 121 C for 10 min (agar was made beforehand for convenience). Treatment solutions were then added and mixed before the agar solidified; these consisted of ether extracts of quackgrass shoots dissolved in water at 2 concentrations (12.5 mg/ml and 6.25 mg/ml). To one-third of the flasks, 2.0 ml (25 mg) of filter-sterilized extract solution was added to give a final concentration of 200 µg ether extract/ ml agar medium. To another third of the flasks, 2.0 ml

(12.5 mg) of filter-sterilized extract solution was added to give a final concentration of 100 µg/ml agar medium. In the controls, 2.5 ml distilled water were added to the agar. The pH of the agar mixtures ranged from 5.2 to 5.4. Pregerminated seeds were planted in the treated agar at a 1.5 cm depth, one seed per flask. Seeds were inoculated with 0.1 ml of R. phaseoli in the exponential phase of growth in YEM broth. Flasks were placed in a lighted growth chamber at 27 C day and 23 C night temperatures. A 16 h photoperiod under fluorescent and incandescent bulbs was maintained with an average PPFD of 175 µmol m^{-2} s^{-1} at the top of the flasks. The treatments in the experiment were replicated five times (one flask each) and arranged in a completely randomized design. The experiment was repeated, also using five replicates. Only the plants remaining sterile were harvested after 21 days, when beans reached the second to third trifoliate leaf stage. Root and shoot dry weights were recorded. Data from the two experiments were combined and analyzed using analysis of variance and mean separation by LSD (.05) test.

Influence of ether extracts on the growth of Rhizobium species. Pure cultures of Rhizobium japonicum (Kirchner) Buchanan 1926, #61A115), Rhizobium meliloti (Dangeard 1926, #102F51), Rhizobium phaseoli (Dangeard 1926, #127K44), and Rhizobium leguminosarum ((Frank) Frank 1889, #128C53) were obtained commercially[1]. Cultures were maintained under sterile conditions at 35 C on a yeast extract mannitol (YEM) agar (1.5%) until use. Each Rhizobium species was transferred

[1]Nitragin Corporation. 3101 West Custer Avenue, Milwaukee, WI 53209.

under sterile conditions into a separate YEM broth. The broths were maintained for 2 days on a slow shaker (130 oscillations/min) in a growth chamber at 26 C. Broths were harvested when cells were in the exponential phase of growth. The Rhizobium inoculum (0.1 ml) of each species was mixed with 2.5 ml YEM agar (0.75 %) and this mixture spread uniformly across each of 16 sterile 1.5 % YEM agar plates per Rhizobium species, for a total of 64 plates. After the plate surfaces had solidified for 1 h, sterile 21 mm filter paper discs (Whatman #540) were infiltrated with sterile treatment solutions and one disc was placed on the center of the plate. Solution treatments consisted of ether extract of quackgrass shoots at 4.0 and 0.4 mg/ml; streptomycin sulfate at 4.0 mg/ml (a known inhibitor of Rhizobium growth, used only as a check on the ability of the procedure to detect antibiotic activity); and distilled water. All solutions were sterilized by filtration through a 0.2 µm filter-unit. Four replicates of each treatment were arranged in a completely randomized manner and placed in an incubator at 30 C. The zone of inhibition around the filter paper disc was measured for the slow growing R. japonicum treatments at 120 h, whereas the faster growing species were measured at 48 h. When inhibition occurs, a clear zone void of bacterial growth is present around the disc, and when it is absent bacteria will grow directly up to the filter paper disc.

Rhizobium phaseoli (127K44) and Rhizobium japonicum (61A115) were also cultured in two broth media (yeast extract mannitol (YEM) and modified Bergersen's III (BIII)) in the presence of ether extract of quackgrass shoots to determine the influence of quackgrass toxins on Rhizobium growth in broth culture. YEM is a nutrient rich medium,

while BIII is a carefully defined medium, less rich in nutrients. When stock cultures of both Rhizobium species grown in YEM broth were in the exponential phase of growth, 0.1 ml inoculum was seeded into 125 ml of YEM or BIII broths in 250 ml bevelled Ehrlenmeyer flasks. At this time, treatments were also incorporated into the broths and included: 2.0 ml distilled water, 2.0 ml of ether extract at a concentration of 12.5 mg/ml (25.0 mg) to form a final concentration of 200 µg extract/ml agar, and 2.0 ml of streptomycin sulfate at a concentration of 12.5 mg/ml to form a final concentration of 200 µg/ml agar. Flasks were maintained under sterile conditions on a slow shaker (130 oscillations/min) in a 27 C growth chamber. At periodic intervals over a 72 h period (approximately every 8-10 h) one 2.0 ml sample was withdrawn from each flask under sterile conditions. Absorbance of the samples at 660 nm was measured on a spectrophotometer (Sequoia Turner Model 390, single beam) immediately after collection. A growth curve was constructed for each of the treatments by plotting absorbance versus time. Data points represent the means of 3 absorbance readings per sample. Using only the exponential growth phase data for each treatment (graphed on semi-log graph paper), growth parameters including specific growth rate, doubling time and cell yield were calculated (Table 4). Replication of the experiment was not attempted as the data during exponential phase of growth fit a straight line when graphed on semi-log paper (3) and a prohibitive amount of ether extract was required for the experiment.

Isolation and characterization of toxins from the ether extracts of quackgrass shoots. The ether extract was further separated in an attempt to isolate and identify the compounds in quackgrass shoots

responsible for inhibition of seedling root growth (Figure 4). When the dried ether fraction (240.0 mg) was redissolved in dichloromethane (40 mg/1 ml) a gold crystalline precipitate was formed. The precipitate was collected in a fritted glass filter and washed with 100 ml hexane followed by 100 ml diethyl ether. The precipitate was dried, weighed (11.3 mg) and bioassayed. The standard bioassay used throughout these separation procedures was the inhibition of radicle elongation of 'Burpee curly' cress measured at 72 h. Since the precipitate possessed considerable inhibitory activity (I_{50}= 149.0 µg/ml) further separation was conducted using silica gel thin layer chromatography (TLC) plates (Merck, .25 mm, F-254). Plates were run in 8:1 chloroform:methanol. Visualization under shortwave ultraviolet (UV) light at 254 nm and exposure to 5% vanillin in sulfuric acid showed three distinct bands (rf= 0.20, 0.35 and 0.40). The bands appeared bright yellow, and band 3 (rf= 0.40) appeared to make up the majority of the mixture. Band 3 (rf= 0.40) was scraped off the plate and the scrapings were eluted with 4:1 chloroform:methanol through a fritted glass filter. Band 3 was bioassayed and also subjected to spectral analyses. All spectral analyses were performed by Basil A. Burke at the ARCO Plant Cell Research Institute. UV spectra were obtained with a Perkin Elmer Lambda 5 UV/VIS spectrophometer, while chemical ionization mass spectroscopy (MS) was performed with a VG 7070E direct probe inlet using isobutane or ammonia as the ionizing gas. Nuclear magnetic resonance spectroscopy (NMR) was performed in deuterated methanol or chloroform on a Varian XL 300 MHz instrument. A dose response curve was constructed to determine the concentration of this material (compound 1) required for 50% inhibition of cress radicle elongation (I_{50}).

The ether fraction was also separated using liquid column chromatography (LC). This crude material (500 mg) was loaded onto a silica gel (Baker, 200-250 mesh) flash column (Figure 4). Pressure for the column was provided with a laboratory airline at a rate of 40 ml solvent/sec and eluted with 500 ml 65:35 dichloromethane:ethyl acetate, followed by 500 ml of ethyl acetate and 500 ml methanol. Fractions (25 ml) were collected, examined by TLC (silica gel -eluted with 65:35 dichloromethane:ethyl acetate) and combined to provide 11 distinct fractions. The 11 fractions were bioassayed separately for inhibitory activity using 250 µg material/dish, or 166.7 µg/ml. Activity was concentrated in fractions six through eight and the material from these fractions was combined. This material (55.5 mg) was loaded on a silica gel TLC plate and run in 10:1 chloroform:methanol. Seven distinct bands were scraped off the plate after visualization under UV (254 nm) light. Plate scrapings were eluted with 4:1 chloroform:methanol through a fritted glass filter and the eluent from each zone was collected, dried, weighed and bioassayed at 250 µg material/dish or 166.7 µg/ml. The majority of the activity occurred in zones 2 (rf= 0.10 to 0.21) and 3 (rf= 0.21 to 0.30). This material was combined (15.0 mg) and further separated by high pressure liquid chromatography (HPLC) using a Waters, uBondapak C_{18}, 8 mm by 10 cm radial compression column. The UV detector (Waters Lambda-max Model 481) measured absorbance at 380 nm. The solvent system used was 75:25 methanol:water at a flow rate of 1.5 ml/min. The mixture was resolved into two peaks and two fractions were collected after repeated injections. The two fractions were bioassayed on an equal weight basis at 200 µg material/petri dish or 133.3 µg/ml after the removal of the solvents by rotary evaporation. Strong

inhibitory activity was associated only with fraction 1, which appeared as one large, distinct, needle-shaped peak in a variety of HPLC solvent systems. The material was dried, weighed (6.5 mg) and a small amount was subjected to analysis by TLC (silica gel in 10:1 chloroform:methanol). Visualization by UV (254 nm) light showed one distinct band at rf= 0.21. Exposure to 5% vanillin in sulfuric acid showed the same band (rf= 0.21) which turned a pale pink. The material was subjected to spectral analysis by UV, MS, and NMR (as described previously). A dose response curve was constructed to determine the concentration of this material (compound 2) required for 50% inhibition of cress seed radicle elongation (I_{50}).

HPLC quantification of toxins from various quackgrass tissues. An attempt was made to extract and quantify the amounts of compounds 1 and 2 (characterized in the previous section) in rhizomes and shoots grown in two different environments. Treatments consisted of 100 g each of field- or greenhouse-grown rhizome or shoot tissue, which were extracted separately for 24 h as described previously. The four extracts were sequentially partitioned with the same solvent series and the ether extract of each tissue treatment was retained and weighed. Each extract (40 mg) was loaded separately onto a silica gel TLC plate and run in 10:1 chloroform:methanol. Bands tentatively identified as compounds 1 and 2 were observed under UV (254 nm) light in all tissue treatments. Plate scraping was used to separate the mixture before further evaluation by HPLC. Three zones were scraped off each plate (rf= 0.14 to 0.34, rf= 0.34 to 0.45 and rf= 0.45 to 0.55) which contained variable amounts of compounds 1 and 2. All scrapings were eluted in 4:1 chloroform:methanol and the eluent was filtered and dried. The HPLC system described previously (75:25 methanol:water) was used to separate

and quantitate the amount of compound 1 and 2 in each fraction. Standard curves of each purified compound were generated and used to estimate the total amount of compound 1 and 2 in each tissue extract.

RESULTS AND DISCUSSION

Extraction of quackgrass shoot material. Aqueous extraction of quackgrass shoot material was performed at 4 C to prevent the breakdown of secondary products by microorganisms. Greenhouse-grown tissue was used because of its uniformity in size, its availability and because it was exposed to more uniform growing conditions throughout the year. The greatest amount of material (mg) was removed from the aqueous shoot extract through partitioning with n-butanol (Table 1). However, the most inhibitory activity occurred in the hexane, ether and ethyl acetate fractions, as measured by the inhibition of cress radicle elongation at 72 h. Repeated partitioning with a solvent series was successful in removing considerable toxicity from the crude aqueous extract of shoots. Since the greatest toxicity and a comparatively large weight of material were concentrated in the ether fraction, the crude ether extract was used in further separations and bioassays in an attempt to isolate quackgrass toxins responsible for inhibition of seedling growth.

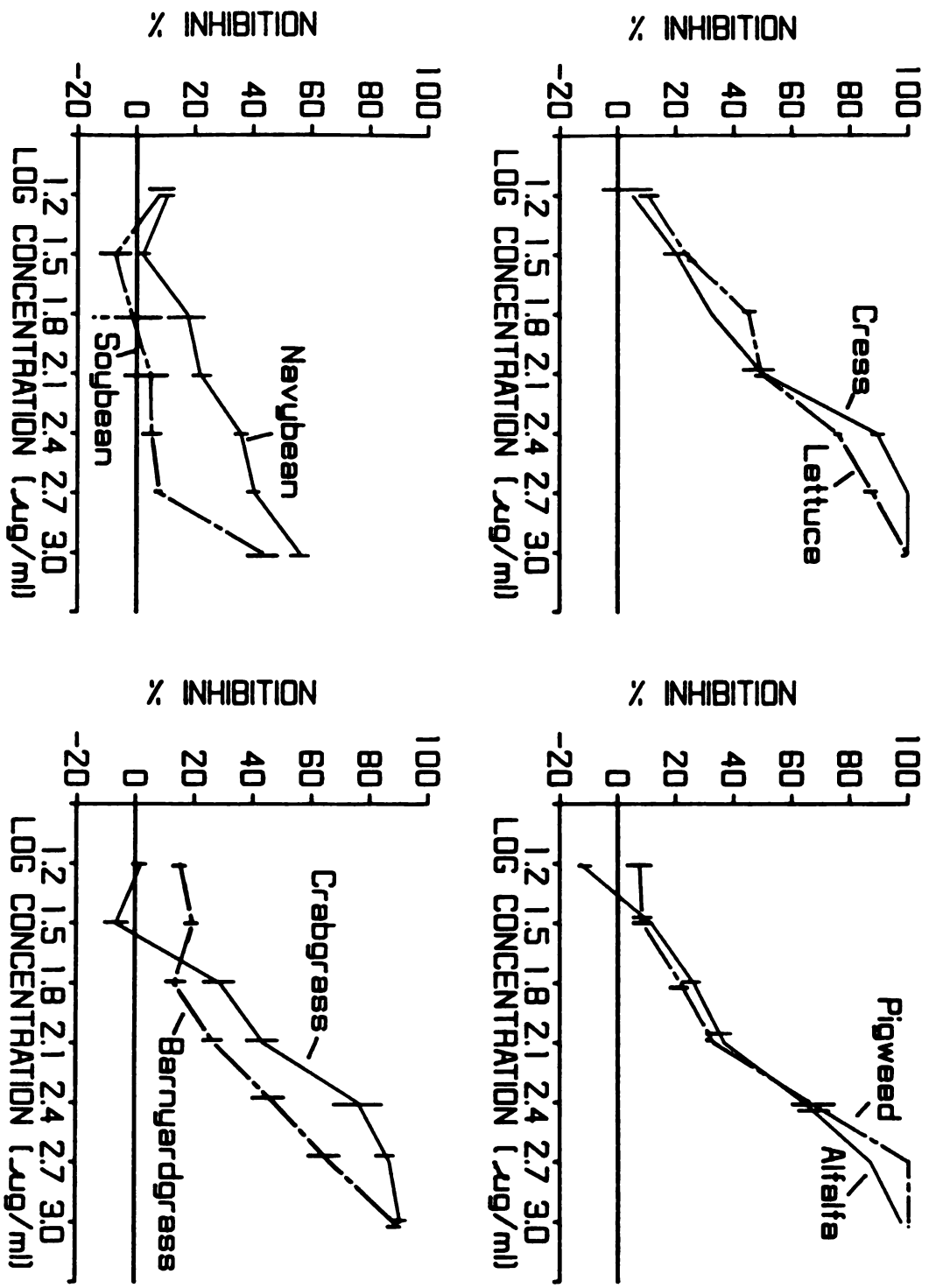
Influence of ether extracts on radicle growth. The ether extract of quackgrass shoots also caused significant decreases in radicle length of eight crop and weed species after 72 h of growth (Figure 2). Radicle

Table 1: Influence of quackgrass shoot extracts (0.33 mg/ml) on radicle length of curly cress at 72 h and quantity extracted from 100 g dried shoot material.

Treatment	Quantity Extracted (mg)	Radicle Length (mm)[a]
Distilled water control	---	21.2
Crude Aqueous	---	13.9
Hexane	14.9	1.0
Diethyl ether	65.4	0.0
Dichloromethane	34.5	6.1
Ethyl acetate	31.4	1.6
Butanol	2759.7	11.9
Remaining Aqueous	13416.7	18.9
F Value	---	**
LSD (.05)	---	5.1

[a]*5% level of probability, **1% level of probability, NS = no significant difference.

Figure 2: Inhibition of seedling radicle elongation of eight indicator species by ether extracts of quackgrass shoots after 72 h of growth. Bars represent ± one standard error unit.


% INHIBITION
100
80
60
40
20
0
-20
Cress
Lettuce
1.2 1.5 1.8 2.1 2.4 2.7 3.0
LOG CONCENTRATION (µg/ml)
% INHIBITION
Navybean
Soybean
1.2 1.5 1.8 2.1 2.4 2.7 3.0
LOG CONCENTRATION (µg/ml)
% INHIBITION
Pigweed
Alfalfa
1.2 1.5 1.8 2.1 2.4 2.7 3.0
LOG CONCENTRATION (µg/ml)
% INHIBITION
Crabgrass
Barnyardgrass
1.2 1.5 1.8 2.1 2.4 2.7 3.0
LOG CONCENTRATION (µg/ml)

length was a more sensitive indicator of inhibition than seed germination or shoot length (data not presented). The crude ether extract was more inhibitory to smaller seeded alfalfa, cress, redroot pigweed and lettuce (mean I_{50} value, 170 µg/ml) than to larger seeded soybeans and navybeans (mean I_{50} value, 1000 µg/ml). The smaller-seeded monocots (barnyardgrass and smooth crabgrass) were also inhibited by ether extracts (mean I_{50} value, 242 µg/ml). These experiments indicate that ether extracts of quackgrass shoots are inhibitory to the radicle growth of a variety of crop and weed species when present at relatively low rates.

Influence of ether extracts on aseptically-cultured snapbeans. Snapbeans grown with 100 or 200 µg/ml ether extract of quackgrass shoots exhibited severe brown discoloration of the root system, swelling of the roots, and a lack of root hair formation. Leaves appeared green but were smaller and cupped. Similar concentrations had also reduced radicle elongation of most seed indicator species (previous section). In comparison, the controls produced a white fibrous root system with a healthy appearance of roots and shoots. A two to three fold decrease in both root and shoot dry weights occurred in the presence of ether extract as compared to the control (Table 2). These experiments indicate that quackgrass toxins are effectively removed from extracts of shoots by ether. Ether-soluble quackgrass toxins may reduce nodulation and nitrogen fixation in legumes (21) as a result of inhibition of legume root growth and root hair formation, thereby eliminating suitable sites for Rhizobium infection.

Influence of ether extracts on the growth of Rhizobium species. In previous experiments, aqueous extracts of quackgrass shoots or rhizomes had no effect on the growth of Rhizobium bacteria (22). In this case,

Table 2: Influence of sterile ether extract of quackgrass shoots on the dry weight of aseptically-grown snapbeans.

Treatment	Shoot weight[b]	Root Weight[b]
	(mg)[a]	
Distilled water control	222	78
Ether extract (100 µg/ml)	91	28
Ether extract (200 µg/ml)	71	26
F value	**	**
LSD (.05)	40	20

[a]Measurements were taken 21 days after planting and are the means of 8 plants per treatment.

[b]*5% level of probability, **1% level of probability, NS= no significant difference.

the ether fractions from quackgrass shoot extracts at 4.0 or 0.4 mg/ml also had no effect on the growth of Rhizobium (Table 3). A smooth white "lawn" of both slow growing (R. japonicum) and fast growing (R. phaseoli, R. meliloti, and R. leguminosarum) grew directly around the filter paper disc as it did in the controls. Streptomycin sulfate (a known inhibitor of Rhizobium growth) at 4.0 mg/ml inhibited the growth of the four species around the filter paper disc.

When the ether extract was incorporated into YEM or BIII broths at a concentration of 200 µg/ml, no inhibition of either R. phaseoli or R. japonicum growth occurred as compared to a control of distilled water (Table 4 and Figure 3). Instead, the specific growth rate and cell yields during the exponential growth phase were slightly increased and doubling time of the population was decreased for both Rhizobium species in the presence of ether extract. This was observed in both the nutrient rich YEM broth and the defined BIII broth, indicating that this lack of inhibition occurred over a wider range of growing conditions and for several species of Rhizobium. When streptomycin sulfate was incorporated into the media at a similar 200 µg/ml concentration, severe decreases in cell yields and specific growth rates were observed, while doubling time increased by at least 2.5 times. Apparently the quackgrass toxins which inhibit seedling growth do not play a direct role in inhibiting the growth of several common species of Rhizobium that participate in the legume-Rhizobium symbiosis.

Isolation and characterization of phytotoxins from the ether extracts of quackgrass shoots. Two toxic compounds were isolated from the ether extract of greenhouse-grown quackgrass material (Figure 4).

Table 3: Influence of the ether extract of quackgrass shoots on the growth of four *Rhizobium* species.

Treatment	Zone of inhibition			
Extract Conc.	*R. leguminosarum*	*R. meliloti*	*R. phaseoli*	*R. japonicum*
	(cm)			
Ether extract (4.0 mg/ml)	0	0	0	0
Ether extract (0.4 mg/ml)	0	0	0	0
Distilled water	0	0	0	0
Streptomycin sulfate (4.0 mg/ml)	4.0 (0.14)[b]	3.1 (0.19)[b]	4.6 (0.10)[b]	4.8 (0.51)[b]

[a]Figures are the means of 4 plates.

[b]Standard deviation.

Table 4: Influence of the ether extract of quackgrass shoots upon the growth parameters of Rhizobium raised in two types of growth media.

Media	Treatment	Specific Growth Rate[b] (T)	Doubling Time (h)	Cell Yield[c] (A)
		Rhizobium phaseoli[a]		
YEM	Distilled water	.126	5.5	1.276
YEM	Ether extract	.173	4.0	1.224
YEM	Streptomycin sulfate	.036	19.0	0.007
BIII	Distilled water	.114	6.1	0.684
BIII	Ether extract	.124	5.6	1.228
BIII	Streptomycin sulfate	.046	15.0	0.004
		Rhizobium japonicum[a]		
YEM	Distilled water	.095	7.3	0.043
YEM	Ether extract	.098	7.1	0.055
YEM	Streptomycin sulfate	.030	23.4	0.007
BIII	Distilled water	.062	11.1	0.017
BIII	Ether extract	.087	8.0	0.032
BIII	Streptomycin sulfate	.026	26.4	0.006

[a]Growth parameters were measured during the exponential phase of growth which occurred between 10 and 40 h for all species and treatments.
[b]Specific growth rate is presented as absorbance at 660 nm per hour.
[c]Total cell yield is presented as maximum absorbance at 660 nm.

Figure 3: Growth response of Rhizobium phaseoli and Rhizobium japonicum in YEM or BIII broth to ether extracts of quackgrass shoots as measured by absorbance at 660 nm.

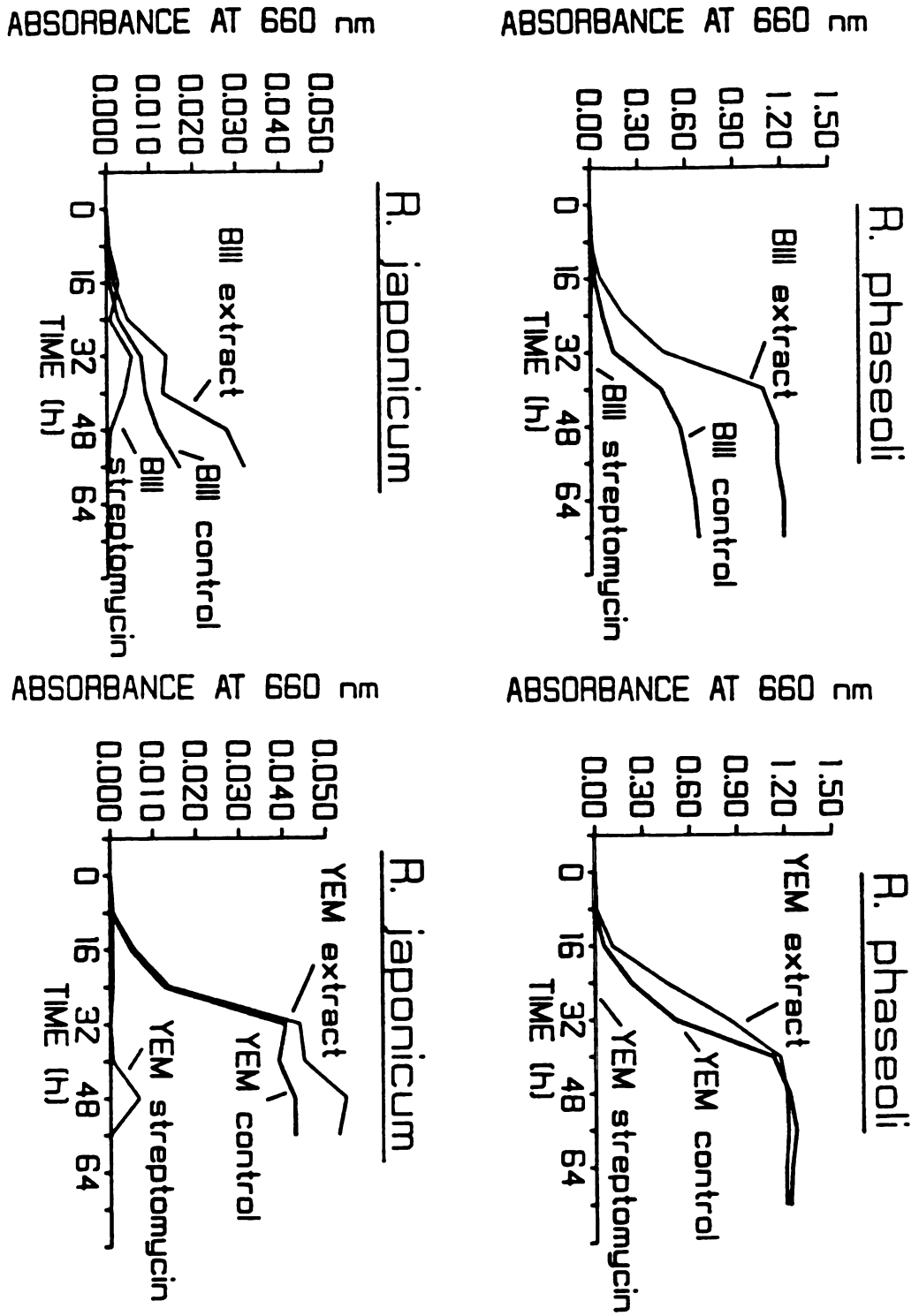
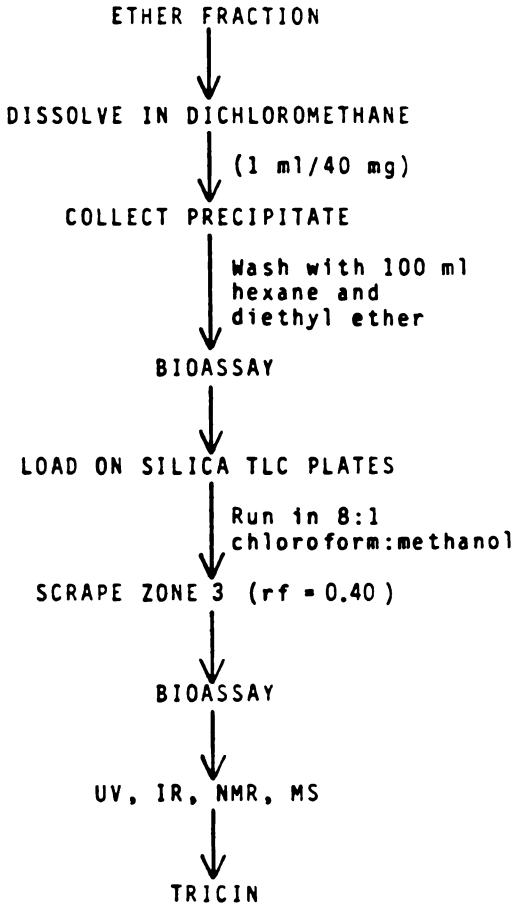

R. phaseoli
ABSORBANCE AT 660 nm
1.50
1.20
0.90
0.60
0.30
0.00
0
16
32
48
64
TIME (h)
BIII extract
BIII control
BIII streptomycin
R. japonicum
ABSORBANCE AT 660 nm
0.050
0.040
0.030
0.020
0.010
0.000
BIII extract
BIII control
BIII streptomycin
YEM extract
YEM control
YEM streptomycin

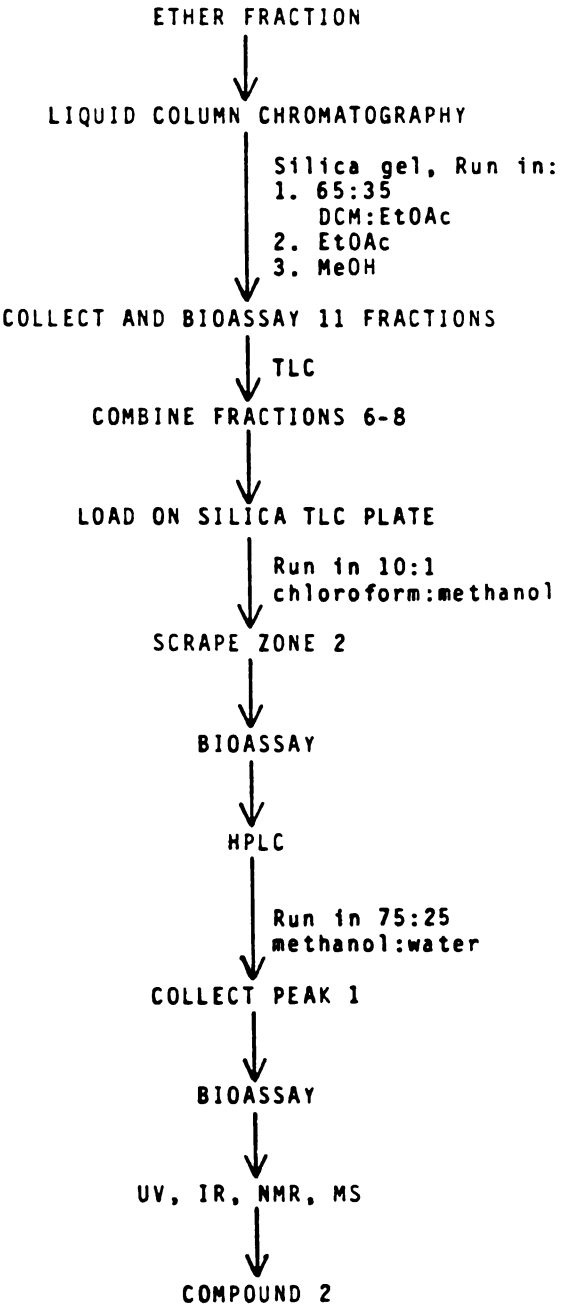
Figure 4: Flow diagram of the separation procedures used for purification of the ether extract of quackgrass shoots.

ISOLATION OF ACTIVE COMPOUNDS FROM ETHER FRACTION

COMPOUND 1



COMPOUND 2



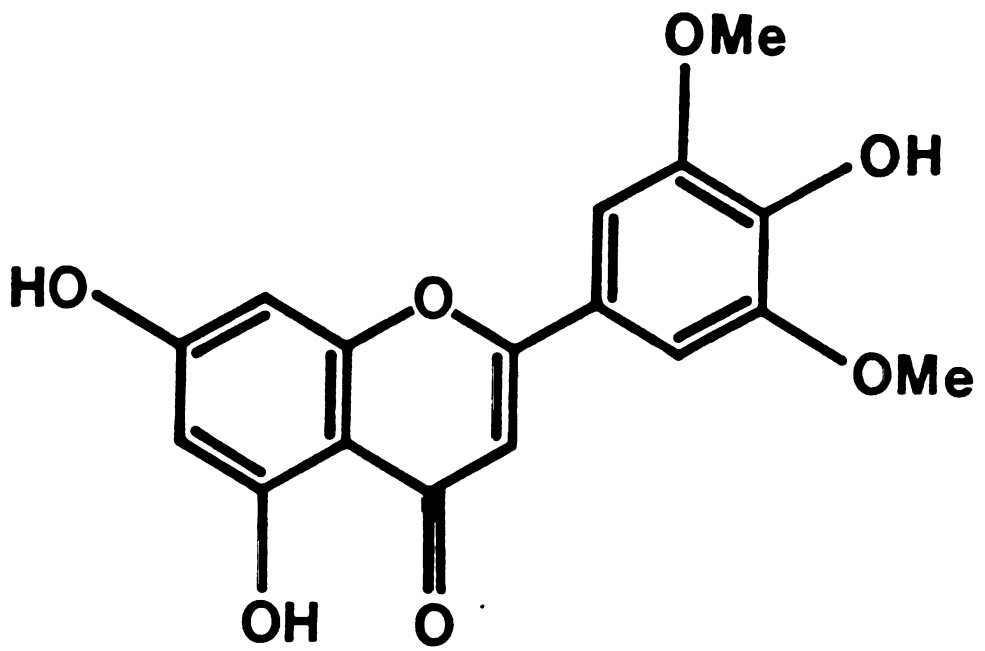
It was neccessary to extract relatively large amounts of dried quackgrass shoot material (500 g) to obtain enough purified product for the spectroscopic analyses. Since the ether extract was a complex mixture, the isolation of both compounds required the use of a number of chromatographic separation techniques.

Compound 1 was isolated from a gold crystalline material which precipitated when the ether fraction was dissolved in dichloromethane. Compound 1 was eventually identified as 5,7,4'-trihydroxy 3',5'-dimethoxyflavone (tricin) (Figure 5). It is yellow gold in color, soluble in alcohols and has a molecular weight of 330.

Examination of the mass spectrum of compound 1 showed a fragmentation pattern (Figure 6) indicative of a simple hydrocarbon structure. A closer examination showed the first significant fragment to be at m/z 69. Fragments of m/z 15 and 31 indicate methyl and methoxy substitution and loss of CH_3 and OCH_3. The apparent molecular ion occurred at m/z 330, indicating a probable molecular weight of 330.

Analysis of the (^{1}H) NMR spectrum (Figure 7) of compound 1 in deuterated methanol disclosed information suggesting a flavonoid structure. Closer examination of the spectrum showed several impurites; a singlet at δ 1.3, most likely due to plasticizer, a singlet at δ3.3 corresponding to the presence of methanol, a singlet a δ7.9 due to residual chloroform from the separation procedure, and a singlet at δ 4.9 attributed to partially deuterated water. The large singlet at δ 3.95 is attributed to two methoxy groups (six protons off the 3' and 5' positions) shifted downfield because the protons are adjacent to oxygen. Smaller singlets at δ6.25, 6.5 and 6.7 are attributed to interactions of H within the A rings (one proton at #6, one proton at #8, one proton at #3 position, respectively), while a large singlet

Figure 5: The molecular structure of 5,7,4'-trihydroxy 3',5'-dimethoxyflavone (tricin).



Tricin

Figure 6: Chemical ionization mass spectrum of 5,7,4'-trihydroxy 3',5'-dimethoxyflavone (tricin).

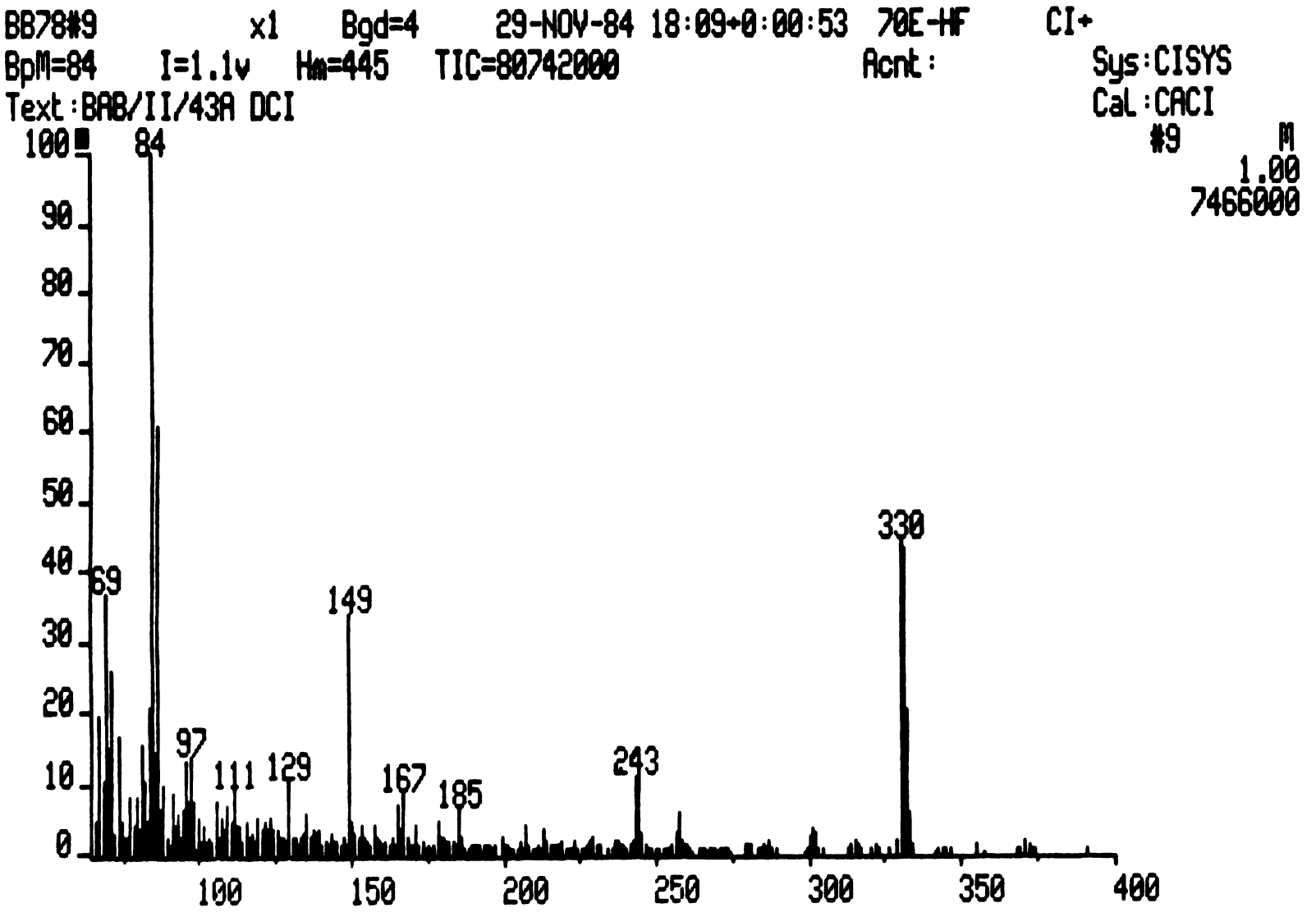
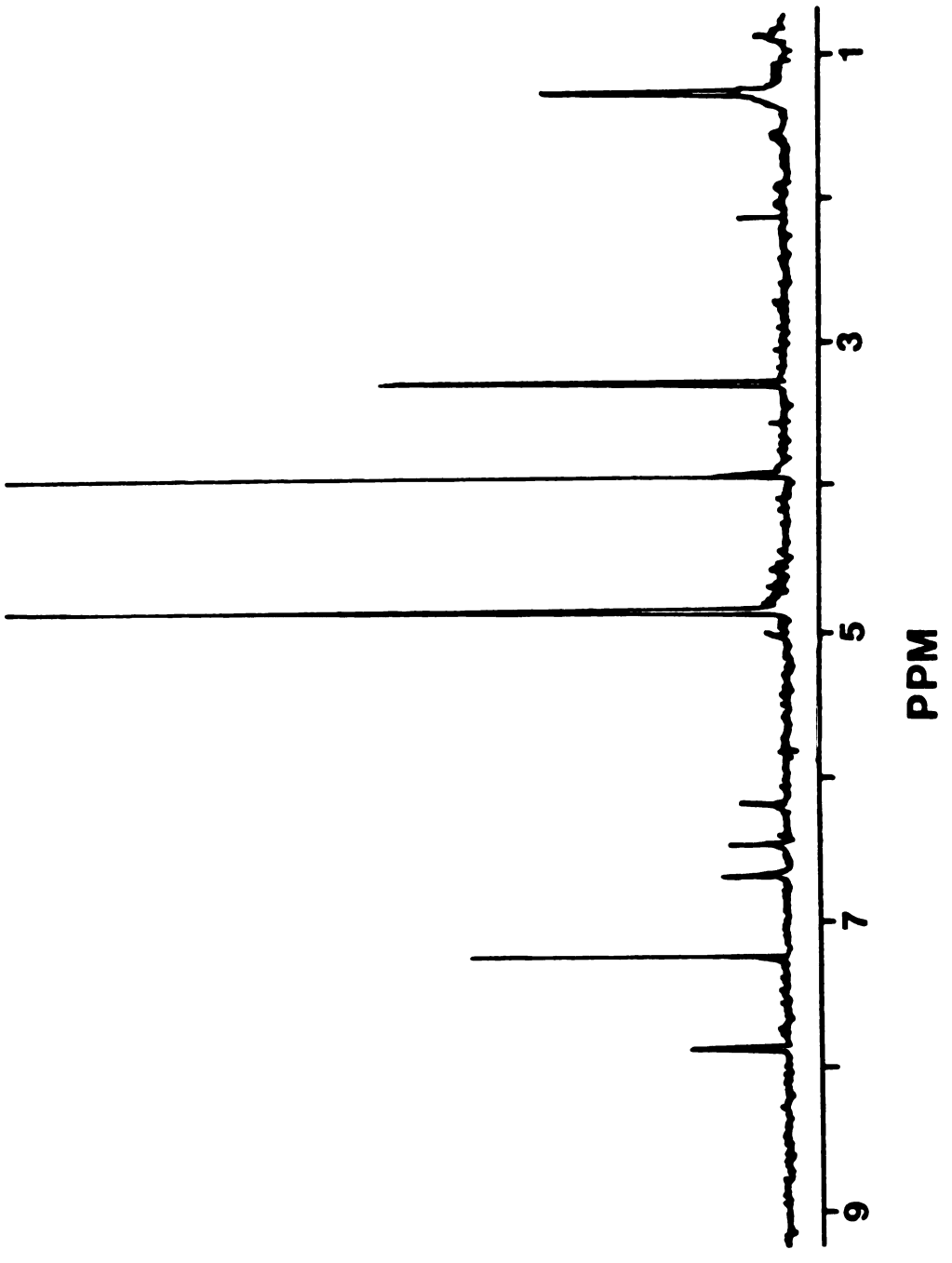

BB78#9 x1 Bgd=4 29-NOV-84 18:09+0:00:53 70E-HF CI+
BpM=84 I=1.1v Hm=445 TIC=80742000 Acnt: Sys:CISYS
Text:BAB/II/43A DCI CaL:CACI
#9 M
1.00
7466000
100
84
69
97
111
129
149
167
185
243
330
90
80
70
60
50
40
30
20
10
0
100
150
200
250
300
350
400

Figure 7: (^{1}H) nuclear magnetic resonance spectrum of 5,7,4'-trihydroxy 3',5'-dimethoxyflavone (tricin) dissolved in deuterated methanol.


9
7
5
3
1
PPM

at δ 7.25 is most likely due to the interaction of protons within the B ring (two protons in the 2'and 6' positions).

The (^{1}H) NMR spectrum indicated a substituted methoxyflavone, but it was difficult to identify the exact placement of the methoxy and hydroxy groups without standards available for comparison. Therefore, the compound 5,7-dihydroxy 3',4',5'-trimethoxyflavone was synthesized by Basil A. Burke at the ARCO Plant Cell Research Institute. It was dissolved in deuterated methanol and analyzed by (^{1}H) NMR (Figure 8). The spectrum of the trimethoxyflavone was different than that of compound 1 as a singlet at δ 3.8 (three protons off the 4' position) appeared, due to an additional methoxy group substitution on the B ring. The singlet at δ 7.3 also corresponds to H interaction on the B ring between protons in the 6' and 2' position.

In 1983, Voiron (18) published a paper on the identification of methoxyflavones with different patterns of OH and OCH_3 substitution using UV spectral differentiation. The UV spectrum is observed in methanol and also in the presence of methanol plus aluminum chloride, a spectral shift reagent. The use of (^{1}H) NMR requires mg quantities for analysis while UV spectral differentiation uses only a minute amount. The majority of flavonoids may be easily identified by their purple fluorescence in UV light and by their spectra in methanol since they exhibit an absorption maxima in the long UV range (band 1) above 326 nm, and generally the ratio of the absorption of band I to band II is greater than 0.7. Compound 1 exhibits a UV maxima at 268 and 350 nm in methanol and when in the presence of aluminum chloride exhibits a bathochromic shift to 277 and 393 nm (Figure 9) (18). This, along with other spectral information, is characteristic only of tricin (5,7,4'-

Figure 8: (^{1}H) nuclear magnetic resonance spectrum of synthetic 5,7-dihydroxy 3',4',5'-trimethoxyflavone dissolved in deuterated methanol.

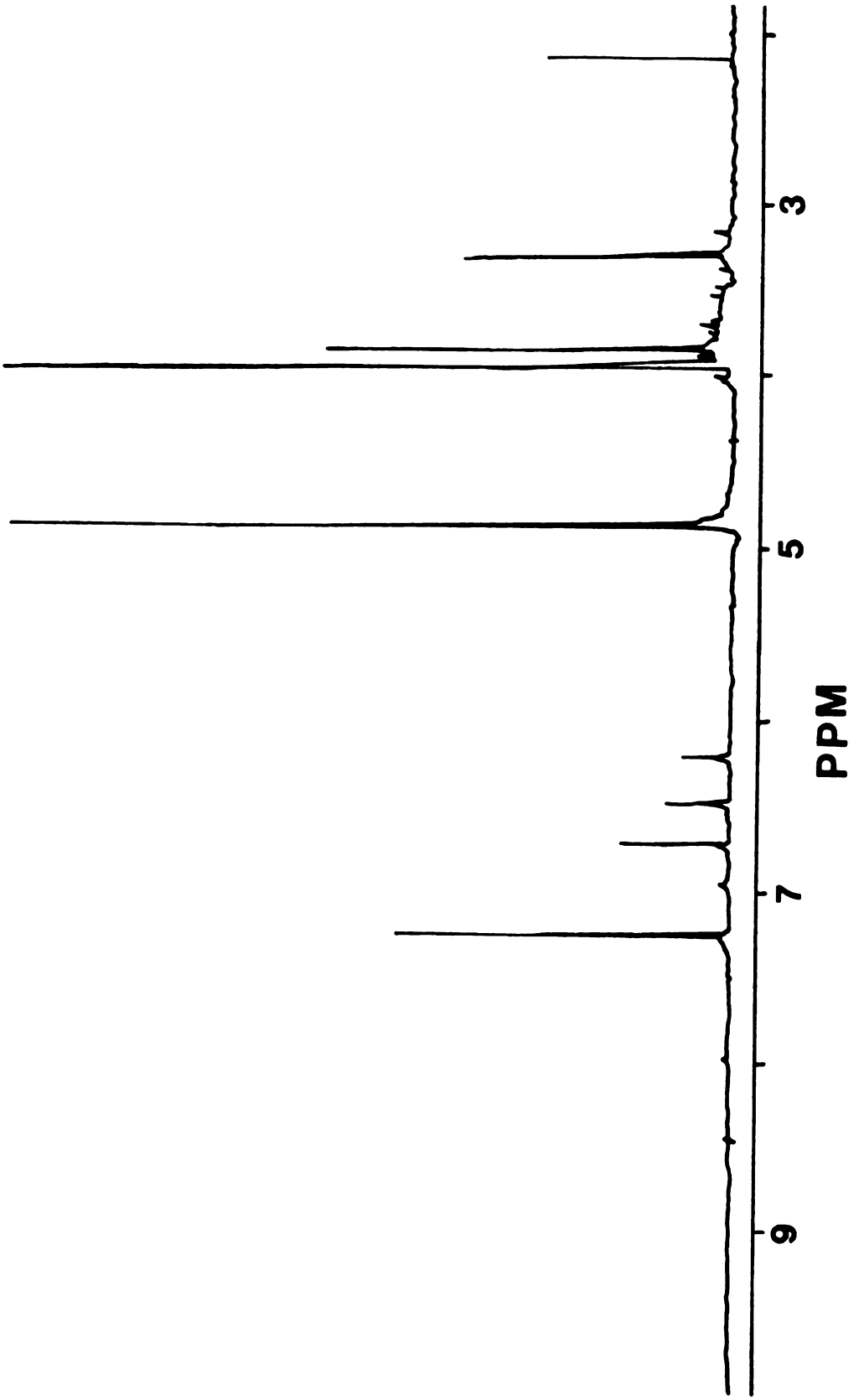
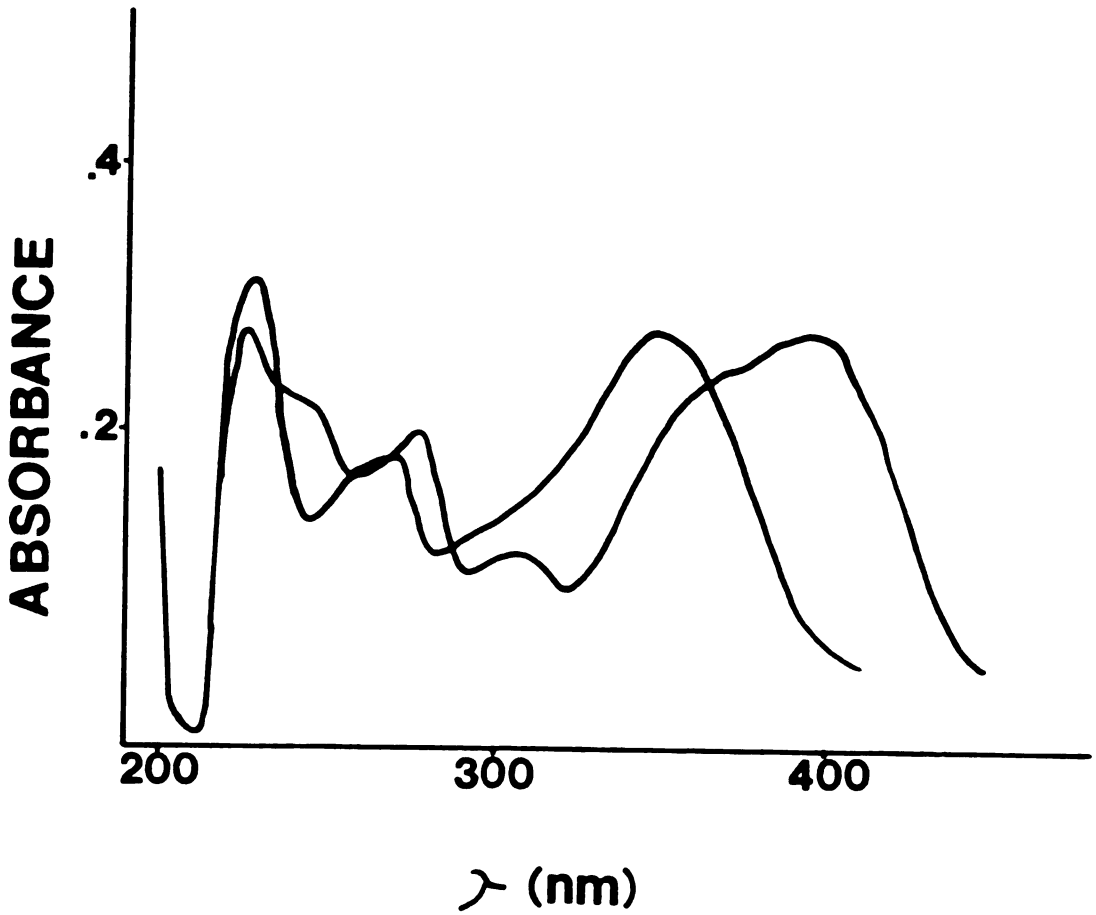

3
5
7
9
PPM

Figure 9: Ultraviolet absorbance spectrum of 5,7,4'-trihydroxy 3',5'-dimethoxyflavone (tricin) in methanol and its corresponding bathochromic shift when placed in methanol plus aluminum chloride.


ABSORBANCE
.4
.2
200
300
400
λ (nm)

trihydroxy 3',5'-trimethoxyflavone).

Compound 2 is also golden yellow in color, soluble in alcohols and fluoresces purple under UV (254 nm) light. We believe that compound 2 is also a flavonoid, closely related in structure to tricin. From the HPLC (Figure 10) and TLC data obtained, we believed that we had purified the mixture to only one component. The mass spectrum (Figure 11) also shows the fragmentation pattern of a large hydrocarbon structure. One of the first significant fragments also occurs at m/z 69. The loss of fragments of m/z 17 and m/z 31 also indicate methoxy and hydroxy substitution. The apparent molecular ion in the mass spectrum could be at m/z 300, indicating a possible molecular weight of 300.

However, the lack of clarity in the(^{1}H) NMR spectrum indicates that an impurity is still present (Figure 12). However, analytical HPLC using a variety of solvent systems did not resolve compound 2 into anything other than one needle shaped peak. This, along with the NMR spectrum, points out that the impurity is most likely one or more very closely related flavonoids. Looking closely at the NMR spectrum, singlets at δ 3.9 indicate at least one methoxyl group substitution on the B ring, along with a possibility of trimethoxyl substitution. A singlet at δ 7.2 possibly corresponds to protons in the 2' and 6' position on the B ring. Singlets at δ 6.2, 6.5 and 6.7 are attributed to interactions of protons within the A ring (one proton at 6 position, one proton at the 8 position, and one proton at the 3 position. Multiplets at δ 6.7 and a singlet at δ 6.9 are not clearly resolved, but may indicate impurities and/or the interaction of the protons within the A ring. Again, a large peak at δ 4.9 denotes partially deuterated water, and a peak at δ 3.3 is attributed to methanol.

Figure 10: Drawing 1 represents the HPLC tracing of the crude mixture containing compound 2; drawing 2 represents the HPLC tracing of the purified compound 2 after HPLC separation.

Figure 11: Chemical ionization mass spectrum of compound 2.

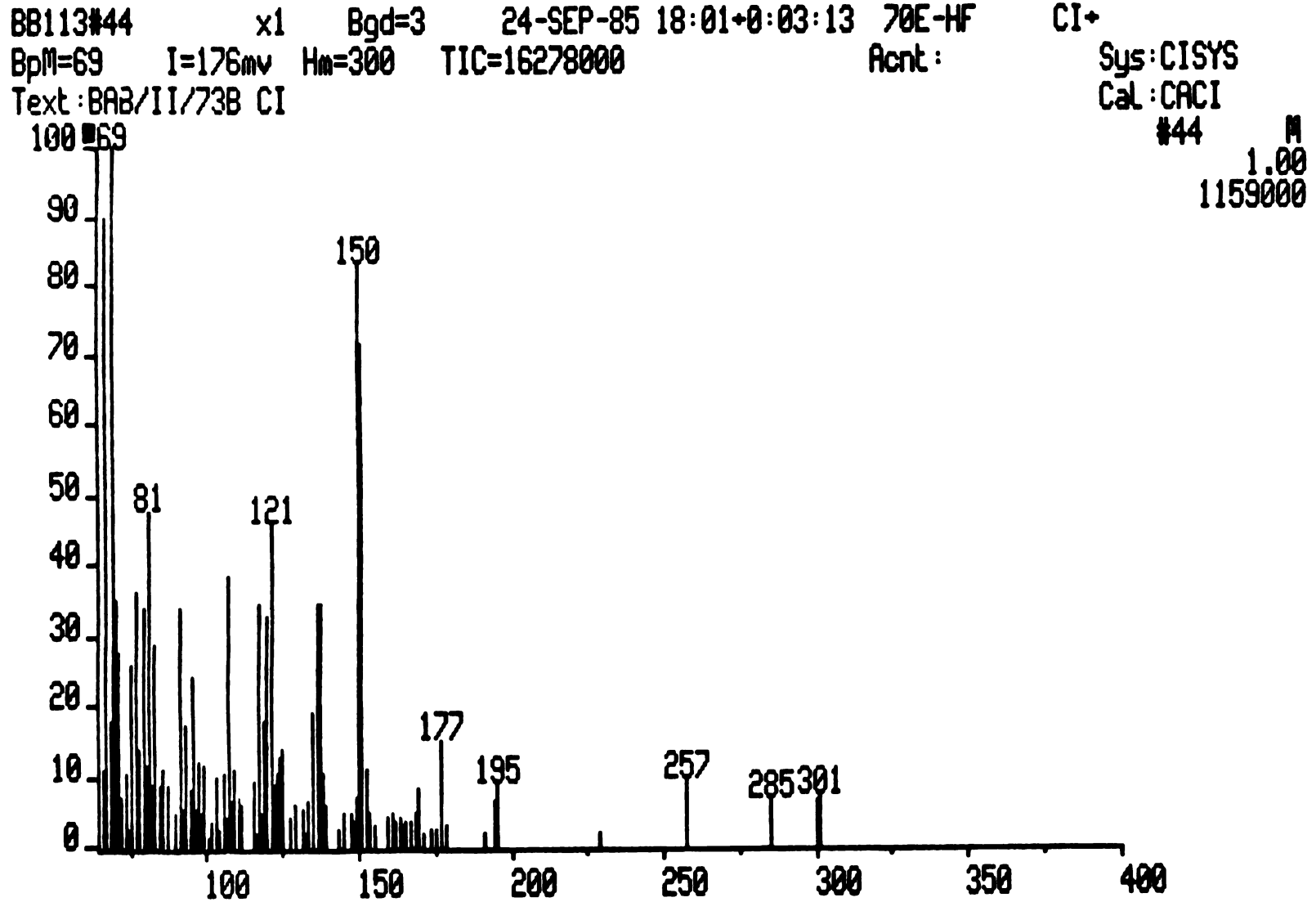
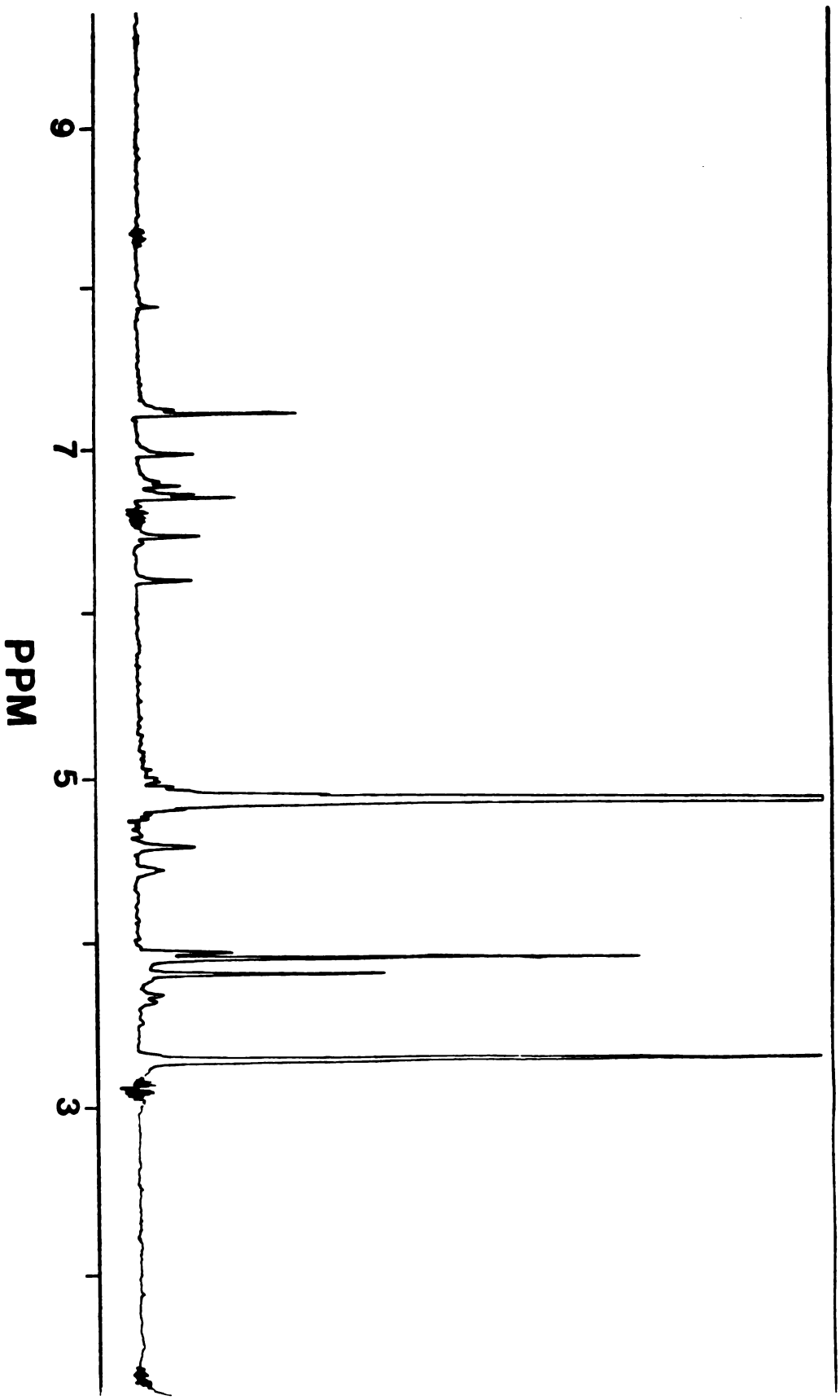

BB113#44 x1 Bgd=3 24-SEP-85 18:01+0:03:13 70E-HF CI+
BpM=69 I=176mv Hm=300 TIC=16278000 Acnt: Sys:CISYS
Text:BAB/II/73B CI CaL:CACI
#44 M
1.00
1159000
100
90
80
70
60
50
40
30
20
10
0
69
81
121
150
177
195
257
285
301
100
150
200
250
300
350
400

Figure 12: (^{1}H) nuclear magnetic resonance spectrum of compound 2 dissolved in deuterated methanol.


PPM
6
7
5
3

The UV spectrum for compound 2 (Figure 13) shows absorption maxima at 210 and 285 nm, and a bathochromic shift occurs from 210 to 320 nm in the presence of aluminum chloride. However, the UV spectrum is broad and is probably not useful for identification purposes until the mixture containing compound 2 is entirely purified. The MS data and NMR data obtained are somewhat conflicting. Given that the molecular weight is indeed 300, compound 2 is most likely 5,7,3'-trihydroxy 4'-methoxyflavone or 5,7,4'-trihydroxy 3'-methoxyflavone and possible structures are presented (Figure 14). However, the NMR data strongly suggests trimethoxy substitution on the B ring and a larger molecular weight. A possible structure is presented (Figure 15).

Flavonoids are secondary phenolic compounds which are closely related to both the coumarins and the substituted cinnamic acids. The structural attribute which appears to confer biological activity upon these compounds is substitution upon the benzene moiety (9). Many possible roles for flavonoids in the physiology of higher plants have been postulated. Flavonoids may play a role as screening pigments for the protection of plant tissues from UV light. They may also act as growth regulators (cofactors in IAA oxidation, or abscisic acid); antioxidants, antibiotics or phytoalexins; serve as pollination attractants; or act as inhibitors of respiration and photophosphorylation (4,9).

The resinous oils of many plants are particularly rich in flavonoid aglycones. Flavonoids are excreted by the root systems of various plants, and some have shown considerable allelopathic activity. For example, in wheat roots, striking inhibition of ion uptake occurs in the presence of various flavones, flavonols and isoflavones. Various

Figure 13: Ultraviolet absorbance spectrum of compound 2 in methanol and its corresponding bathochromic shift when placed in methanol plus aluminum chloride.

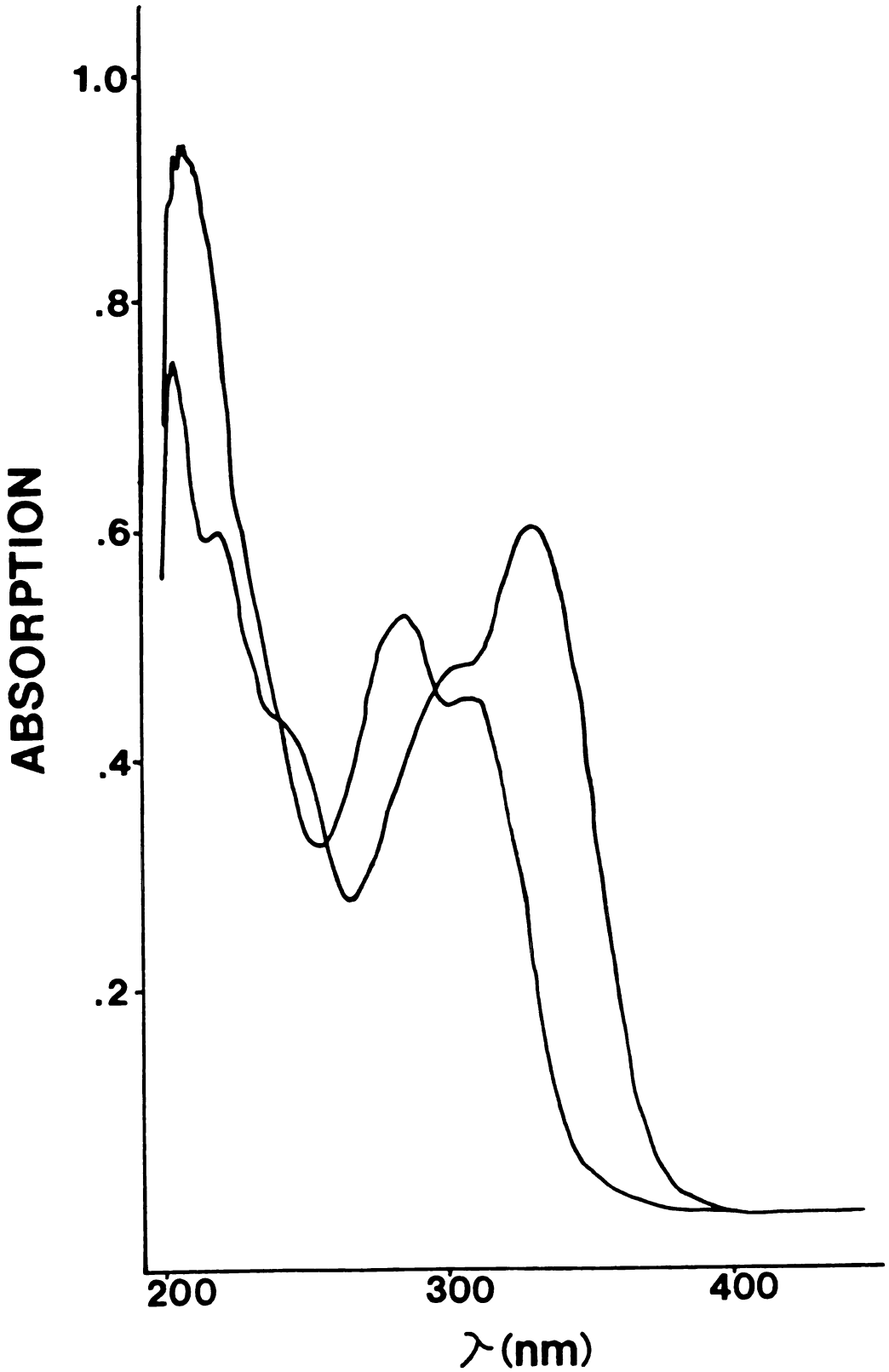
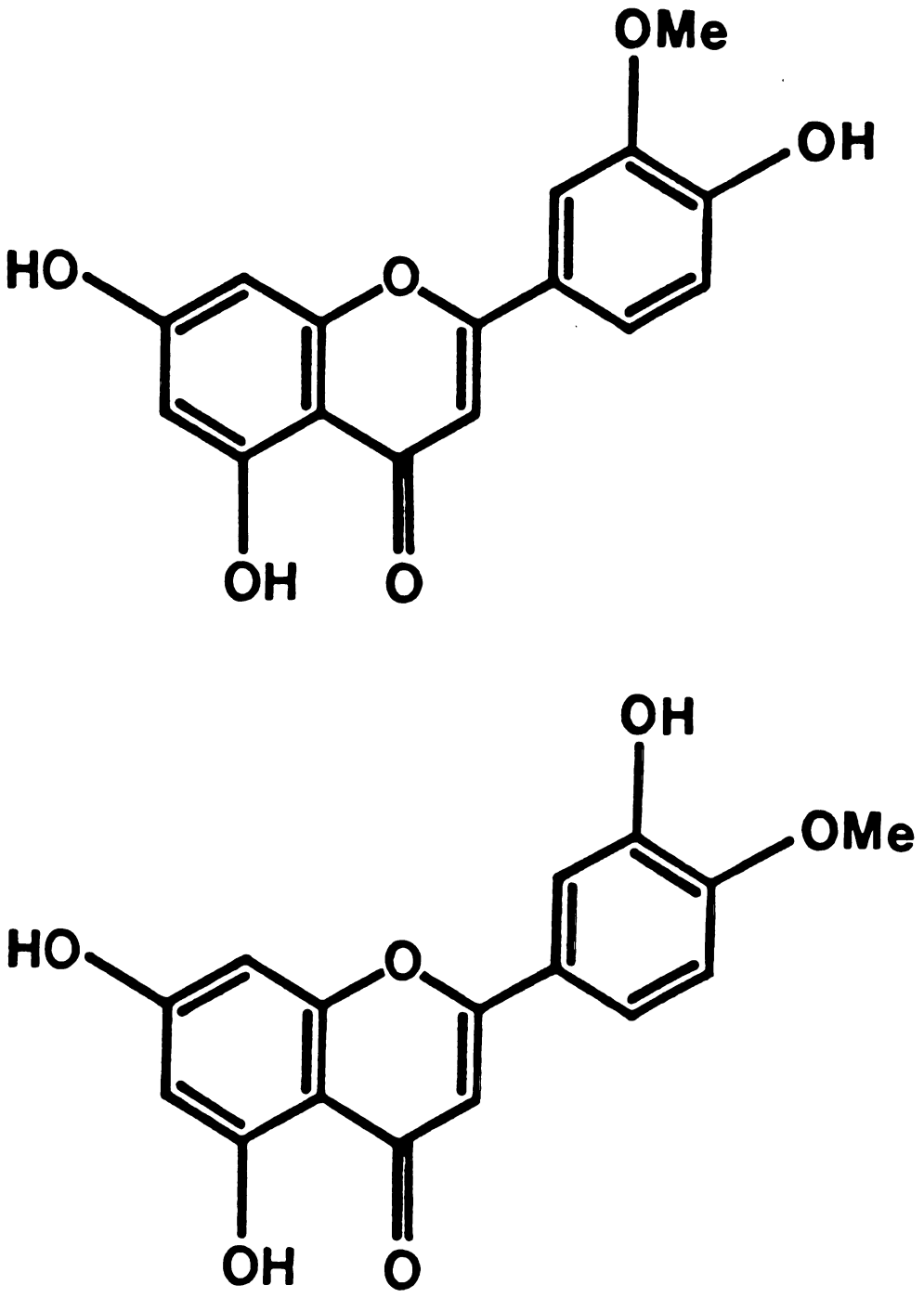
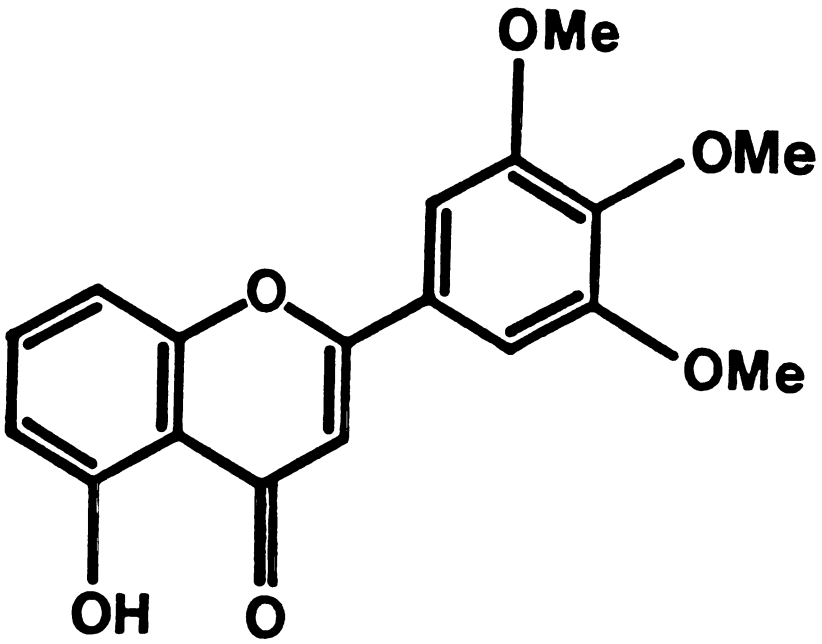

1.0
.8
.6
.4
.2
ABSORPTION
200
300
400
λ (nm)

Figure 14: The proposed molecular structure(s) of compound 2, based primarily on mass spectral data.



Compound 2

Figure 15: A proposed molecular structure of compound 2, based primarily on nuclear magnetic resonance data.



Compound 2

flavonoids also inhibit photophosphorylation in plant mitochondria at extremely low concentrations (9).

Quackgrass extracts contain at least two related flavonoid compounds (tricin and compound 2) which inhibit cress seedling germination and radicle elongation (Table 5). The I_{50} value for tricin upon cress seed radicle elongation is 123.3 µg/ml, while compound 2 has an I_{50} of 59.3 µg/ml. These compounds are both inhibitory to radicle elongation in cress seeds at relatively low concentrations in comparison to several other alleged allelochemicals (15). Neither compound was previously screened for phytotoxic activity. The flavone tricin was also isolated from the leaves of several Triticum species. It appears to be restricted in distribution to a few monocotyledonous plants (4). The ether extract of quackgrass shoots not only contains tricin and compound 2, but also appears to contain a number of other closely related flavonoids. These may possess varying degrees of biological activity depending upon the substitution pattern upon the flavonoid rings (9). Previously, Gabor and Veatch (2) isolated a golden phytotoxin, soluble in alcohol, which was extracted from quackgrass rhizomes and characterized as a glycoside. It is possible that the aglycone portion of that molecule was also a flavonoid.

HPLC quantification of phytotoxins from various quackgrass tissues. Quackgrass shoots and rhizomes collected from the field produced three to five times more dried ether extracts per 100 g of dried tissue than did shoots or rhizomes grown in the greenhouse (Table 6, Figure 16 a). The amounts of the two phytotoxic compounds (tricin and compound 2) in field- and greenhouse-grown shoots or rhizomes were also compared by TLC and HPLC quantification (Table 6, Figure 16 b). Quackgrass shoots

Table 5: Influence of tricin and compound 2 on the inhibition of radicle elongation of curly cress at 72 h.

	I_{50} value (µg/ml)[a]
Tricin	123.3
Compound 2	59.3

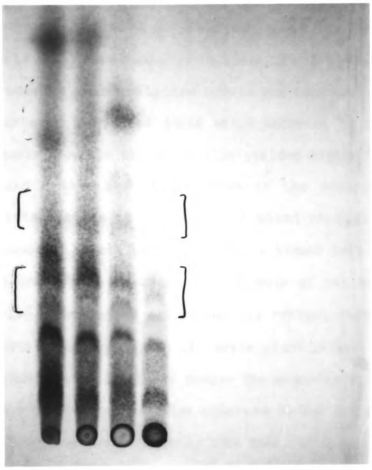
[a] I_{50} values represent the point at which 50% inhibition of radicle elongation of curly cress occurred. A dose response curve was calculated on the basis of mean elongation of 40 seeds at 5 concentrations of the chemicals.

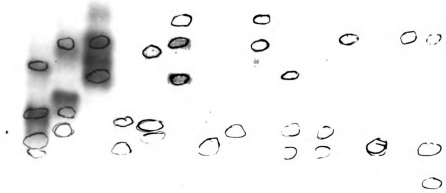
Table 6: Quantity of ether extract, tricin and compound 2 obtained from partitioning and HPLC quantification of different sources of quackgrass material.

Quackgrass Source	Ether extract	Tricin	Compound 2
	(mg/100 g)		
Greenhouse shoot	66	10	6
Field shoot	208	30	36
Greenhouse rhizome	62	1	8
Field rhizome	375	5	9

Figure 16a: Silica thin layer chromatography plate run in 10:1 chloroform:methanol. Each lane contains 200 µg of the ether extracts of the following quackgrass material. From left to right, lane 1 contains greenhouse-grown shoot material; lane 2 contains field-grown shoot material; lane 3 contains greenhouse-grown rhizome material; lane 4 contains field-grown rhizome material.

Figure 16b: Silica thin layer chromatography plate run in 10:1 chloroform:methanol. Lanes 1-12 contain 150 µg of the ether extracts of the following quackgrass materials, separated further by plate scraping. Lanes 1-3 contain greenhouse-grown shoot material; lanes 4-6 contain field-grown shoot material; lanes 7-9 contain greenhouse-grown rhizome material; lanes 10-12 contain field-grown rhizome material. Lane labelled #2 contains compound 2, lane labelled #1 contains compound 1. Lane labelled c contains crystallized material from ether extracts of greenhouse-grown shoots. Circled portions represent areas visible under UV (254 nm) light.





1 2 3 4 5 6 7 8 9 10 11 12 •2 •1 C

grown under field conditions yielded the greatest amounts of both tricin and compound 2 (29.8 and 35.5 mg/100 g dried tissue, respectively) when compared to other tissues. Rhizomes grown in both environments also contained tricin and compound 2, but in generally smaller quantities than those which occurred in the shoots. Rhizomes and shoots grown in the field also yielded higher levels of tricin and compound 2 than did those grown in the greenhouse. This is not surprising, since it is known that plant tissues exposed to greater environmental stress generally produce higher levels of flavonoids (9). The figures shown represent an estimate of the quantities present in the ether extracts and may or may not reflect relative amounts of the two phytotoxins present in the whole plant before extraction. However, partitioning with ether did remove the majority of flavonoid compounds from the aqueous extracts (as observed by TLC and the cress bioassay).

These experiments clearly show that quackgrass shoots and rhizomes possess at least two phytotoxins which are flavonoid compounds. These flavonoid compounds are inhibitory to radicle elongation and root growth. They are present in larger proportions in the ether extracts of quackgrass shoots as compared to extracts of rhizomes. Extracts of quackgrass shoots are also more toxic to seedling root growth in a number of plant species than are extracts of rhizomes (22). Tricin and compound number 2, along with other related compounds, may be released from plant tissues by exudation or degradation and may play a role in the alleged allelopathic activity of living and dead quackgrass. This observed allelopathic activity includes reductions in root and shoot growth of crop and weed species and inhibition of legume nodulation and nitrogen fixation in the presence of quackgrass or its extracts in the field, greenhouse and laboratory (21, 22). The specific mode of action

of the flavonoid compounds, their rate of release from quackgrass tissue and their role in the soil rhizosphere should be further investigated.

LITERATURE CITED

1. Dazzo, F. B. 1982. Leguminous root nodules. In: R. G. Burns and J. L. Slater, eds. Experimental Microbial Ecology. Blackwell Scientific. Oxford, U.K. pp. 431-446.
2. Gabor, W. E. and C. Veatch. 1981. Isolation of a phytotoxin from quackgrass (Agropyron repens) rhizomes. Weed Sci. 29: 155-159.
3. Gerhardt, P. et al. 1981. In: Manual of Methods for General Bacteriology. American Society of Microbiology, Washington. pp. 202-203.
4. Harborne, J. B. 1965. Flavonoid Pigments. In: J. Bonner and J. E. Varner, eds. Plant Biochemistry. Academic Press, New York. pp. 618-640.
5. Holm, L. G., D. L. Pluckett, J. V. Pancho, and J. P. Herberger. 1977. Agropyron repens (L.) Beauv. In: The World's Worst Weeds, Distribution and Biology. University Press, Hawaii. pp. 153-168.
6. Kommedahl, T., K. M. Old, J. H. Ohman, and E. W. Ryan. 1970. Quackgrass and nitrogen effects on succeeding crops in the field. Weed Sci. 18: 29-32.
7. LeFevre, C. W. and C. O. Clagett. 1960. Concentration of a growth inhibitor from Agropyron repens (quackgrass). Proc. North Cent. Weed Contr. Conf. 14: 353-356.
8. Lynch, J. M. and D. J. Penn. 1980. Damage to cereals caused by decaying weed residues. J. Sci. Food Agric. 31: 321-324.

9. McClure, J. W. 1977. An ecological role for plant phenolics. In: Report of the Research Planning Conference on the Role of Secondary Compounds in Plant Interactions (Allelopathy). Mississippi State University. pp. 42-62.

10. Ohman, J. H. and T. Kommedahl. 1964. Plant extracts, residues, and soil minerals in relation to competition of quackgrass with oats and alfalfa. Weeds 12: 222-231.

11. Oswald, H. 1948. Toxic exudates from the roots of Agropyron repens. J. Ecol. 38: 192-193.

12. Patrick, Z. A., T. A. Toussaun, and W. C. Snyder. 1963. Phytotoxic substances in arable soils, associated with decomposition of plant residues. Phytopathology 53: 152-161.

13. Penn, D. J. and J. M. Lynch. 1982. The effect of bacterial frementation of couchgrass rhizomes and Fusarium culmorum on the growth of barley seedlings. Plant Pathol. 31: 39-43.

14. Phlak, F. 1967. The effect of quackgrass on succeeding plants. Plant Soil 27: 273-284.

15. Rice, E. L. 1984. In: Allelopathy. Academic Press, New York. pp 1-422.

16. Robinson, T. 1980. In: The Organic Consituents of Higher Plants: Flavonoids and Related Compounds. Cordus Press, Massachussetts. pp. 191-225.

17. Toai, T. V. and D. L. Linscott. 1979. Phytotoxic efficts of decaying quackgrass residues. Weed. Sci. 27: 595-598.

18. Voiron, B. 1983. UV spectral differentation of 5-hydroxy- and 5-hydroxy-3-methoxy flavones with mono-(4';)-, di-(3',4')- or tri-(3',4',5')-substituted B rings. Phytochemistry 22(10): 2107-2145.

19. Welbank, P. J. 1960. Toxin production from Agropyron repens. In: J.L. Harper, ed. The Biology of Weeds. Blackwell Scientific Publications, Oxford, England. pp. 158-164.

20. Welbank. P. J. 1963. Toxin production during decay of Agropyron repens (couchgrass) and other species. Weed Res. 3: 205-214.

21. Weston, L. A. and A. R. Putnam. 1985. Inhibition of growth, nodulation, and nitrogen fixation of legumes by quackgrass (Agropyron repens). Crop Sci. 25: 561-565.

22. Weston, L. A. and A. R. Putnam. 1986. Inhibition of legume seedling growth by residues and extracts of quackgrass (Agropyron repens). Weed Sci. (in press).

23. Wyse, D. L. 1980. Vernolate for augmentation of quackgrass (Agropyron repens) control with glyphosate. Weed. Sci. 28: 654-657.