5-1974

Biochemical and Physiological Studies on Phytotoxicity of Selected Pesticides and Allergens During Seed Germination of Some Food Crops

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BIOCHEMICAL AND PHYSIOLOGICAL STUDIES ON PHYTOTOXICITY OF SELECTED PESTICIDES AND ALLERGENS DURING SEED GERMINATION OF SOME FOOD CROPS

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology

UTAH STATE UNIVERSITY
Logan, Utah

1974

ACKNOWLEDGMENTS

I extend my sincere gratitude to my major professor, Dr. D. K. Salunkhe, for his valuable guidance and help, for constructive suggestions and constant encouragement throughout this study, and also for sharing his knowledge in preparation of this manuscript.

I express my deep sense of appreciation to my committee members, Drs., W. A. Brindley, W. F. Campbell, R. K. Olsen, and J. L. Shupe for their advice and review of this work. I would like to thank Drs., B. Singh and R. P. Sharma for serving on my committee for nearly two years. I especially express my appreciation to Dr. Singh for his useful suggestions throughout this work.

My thanks are also due to Dr. W. C. Chang for his help in histochemical and ultrastructural work. I sincerely thank Dr. Campbell for making his laboratory facilities available for the light and electron microscopy work. I wish to thank Dr. H. P. Stanley for the use of electron microscope.

I earnestly extend my deep appreciation to my wife, Rekha, for her patience and encouragement throughout my graduate studies at Utah State University.

I am indeed grateful to Shri V. R. Palande for his incessant love and affection, inspiration, and encouragement in completing this task.

R. R. Dalvi

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ABSTRACT

Biochemical and Physiological Studies on Phytotoxicity of Selected Pesticides and Allergens during Seed Germination of some Food Crops

by

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Utah State University, 1974

Major Professor: Dr. D. K. Salunkhe
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Germination of mung bean, Phaseolus mungo L., and wheat, Triticum aestivum L., seeds was used for bioassay to demonstrate the toxic effects of selected pesticides--menazon, disulfoton, and GS-14254-- and allergens--alantolactone and usnic acid. The ability of gibberellic acid to counteract the toxic effects of these chemicals on germination and seedling growth was studied. Chemical composition of the treated and untreated seeds was made with special attention to starch and protein degradation. Effect of these toxicants on the synthesis of amylase, ATPase, and protease enzymes during germination was studied since these enzymes are synthesized de novo during germination. To ascertain their effect on protein synthesis in storage tissue of the germinating seeds, uptake and incorporation of ^{14}C-L-leucine into protein was studied in potato tuber slices and germinating mung beans.

Correlation of biochemical data and histochemical changes in the treated and untreated seeds of mung bean was obtained with menazon and usnic acid. Furthermore, ultrastructural changes were studied in order to relate functional and structural changes in the seeds in conjunction with phytotoxic actions of these chemicals.

Among the insecticides, menazon (250 ppm) was found to be more toxic to both species than was disulfoton. GS-14254 (100 ppm) also was equally inhibitory to seed germination and seedling growth of mung bean and wheat seeds. When a solution of the herbicide GS-14254 (100 ppm) was added to either of the insecticides at their maximum concentrations the inhibitory effect of the combined pesticides on seed germination and seedling growth was more pronounced, especially with wheat.

Usnic acid (50 to 250 mg/1) and alantolactone (100 mg/1) significantly inhibited germination and root and shoot growth in both mung bean and wheat seeds. These two compounds appeared to be more phytotoxic than the pesticides.

Gibberellic acid partially counteracted the inhibitory effects of the pesticides and allergens, thus these chemicals showed no antiauxin activity.

Before any growth is observed there is a marked increase in respiration during germination that releases energy from food materials already present in usable form in the cells. At their maximum concentrations, menazon, disulfoton, GS-14254, alantolactone, and usnic acid significantly blocked the respiration of the germinating seeds at the end of 72 h after

treatment. In all cases except alantolactone respiration of wheat seeds was considerably more affected than that of the mung beans.

Compared to control seeds, pesticide chemicals as well as allergenic compounds caused significant reduction in the amounts of soluble reducing sugars and free amino acids after 72 h germination period. Similarly, starch degradation was less in the treated seeds. Among the species of seeds, considerably less amounts of reducing sugars and amino acids were formed in the pesticide-treated wheat seeds than in the mung beans as compared to their respective controls. Such differences in the inhibitory effects were not observed in seeds treated with allergenic compounds.

The development of amylase and ATPase activity in the seeds treated with maximum concentrations of pesticides tended to be lower than that in the control seeds. In case of menazon, inhibition of amylase activity was more pronounced than that of disulfoton or GS-14254. Proteolytic activity in control and disulfoton- and menazon-treated seeds was not significantly different during germination period, but in case of GS-14254, it was considerably lower.

Usnic acid at highest concentration tested completely inhibited the development of amylase activity in mung beans whereas it was significantly lower in seeds treated with the maximum concentration of alantolactone. The inhibition of amylase activity in wheat seeds treated with these compounds was more or less similar. ATPase inhibition in seeds treated with usnic acid was more severe than that in alantolactone-treated seeds. However, proteolytic activity in control and treated seeds showed almost the same trend during the germination period.

The activity per se of amylase isolated from mung bean and wheat seeds germinated for 3 days was not significantly inhibited by the presence of the pesticides or allergens in the reaction mixture indicating that these chemicals do not inhibit already synthesized amylase enzyme.

Observations with potato tissue and germinating mung beans indicated that both total uptake and incorporation of ^{14}C-L-leucine into protein were significantly inhibited by menazon, disulfoton, GS-14254, and alantolactone. On the other hand, the uptake in germinating mung bean treated with usnic acid was not affected although both uptake and incorporation were inhibited in potato tissue.

Menazon and usnic acid were then selected as the representative chemicals for pesticides and allergens, respectively, and their toxic effects were studied histochemically in 3-day germinating mung beans. It was observed that total nucleic acid content and RNA content in seeds treated with these chemicals were considerably less than that in the control seeds. Similarly, treated seeds showed more starch grains and protein bodies indicating less metabolic activity in these seeds.

At the ultrastructural level, menazon- or usnic acid-treated mung bean cotyledons at day 3 of germination contained no vacuoles but many undigested protein bodies were observed. In contrast, fully developed mitochondria, endoplasmic reticulum with ribosomes, and vacuoles were seen in control cells indicating protein (enzyme) synthesis and digestion of the food reserves.

(152 pages)

INTRODUCTION

Pesticides applied on or into the soil have obtained a wide acceptance among farmers and scientists. Undoubtedly, these chemicals protect plants from attack by a wide variety of noxious pests affecting agricultural production. The use of these organic pesticides including insecticides, herbicides, and fumigants has been growing annually by 16 per cent and by 1975 pesticide sales at the consumer level are expected to equal 3 billion dollars (Neumeyer, Gibbsons, and Trask, 1969a, 1969b). The economic value and worldwide importance of the chemicals as tools for agricultural production have been well recognized. However, their indiscriminate use threatens environmental contamination owing to their accumulating residues in all segments of the biosphere--air, soil, water and ultimately the plants and animals that live therein. This warrants an urgent need for the knowledge of their behavior in the biosphere, and also for proper surveillance and intelligent control over their use to prevent further deterioration of our environment.

It is true that these man-made chemicals combat many highly destructive pests, but at the same time they are destructive to hosts also. For example, many of the soil insecticides, which are synthetic organic chemicals, are acetylcholinesterase inhibitors and thus kill insects. Unfortunately, mammals and insects have the same enzyme system and as such they are toxic to man also. Similarly, herbicides used on pest plants are also toxic to

economic plants and animals. Thus, it is very difficult to achieve selective toxicity although the word pesticide implies selective toxicity.

Much of our environment is already associated with a variety of pesticides some of which are persistent for years (Edwards, 1965). It is possible that the toxicity of these contaminants may increase or decrease. However, soil contamination is of serious concern since it is the major source through which persistent pesticides penetrate roots and are translocated within the plants (Lichtenstein, Myrdal, and Schulz, 1965). Penetrated residues in the plants may be taken up by animals from their feed to appear later in our food products. Consequent effects may result in public health hazards. Many of these contaminants have not been previously studied or characterized as poisons; but recently their latent effects are being assessed with respect to their potential contribution to toxicological responses,

Substantial contribution to soil residues of pesticides that mainly include insecticides and herbicides is derived from spraying and seed treatments. However, agricultural soils usually contain residues of various types of pesticides owing to their direct application or fallout following crop spraying. Moreover, soils can be contaminated by leaching of the chemicals from one place to another. Plants containing pesticide residues may add to the soil contamination by decomposing in the soil-water system after the completion of their life-cycle. In addition to pesticides, many other toxic compounds of plant and microbial origin might be accumulating in the soil. One of the groups of such

substances is the allergens of plant origin. In this context, it is interesting to note that allergic disease still maintains number 1 position in terms of the number of Americans affected by any disease. One out of every 7 people in the U.S.A., or about 31 million, suffer from some sort of allergic disease. Over $135 million is the annual cost to allergy victims for medicine (Furbush, 1972). Unfortunately, adequate attention has not been given to this important class of toxicants.

Plant life begins with seed germination in soils. Naturally, seeds sowed in the contaminated soil come in contact with the accumulated pesticides and also with toxic compounds naturally occurring in plants that finally persist in soils after the plants complete their life-cycle. Among the economic crops, cereals and legumes are the species of vital importance to man for his living. These species are perennially grown in soils. Therefore, logically the seeds of these crops become the first victims of pesticides and also of some naturally occurring plant toxicants present in contaminated soils. Thus, seeds of our economic crops are exposed to these toxicants by several ways such as soil treatment, seed treatment, persistent residues of man-made and natural chemicals and their leaching in soils, broadcasting of crops, and fumigation of stored grains.

Examination of certain root and oil crops indicates that some pesticides may accumulate in the root and oil seeds. Presence of pesticides in potatoes, peanuts, and soybeans have been associated with residues in soil (Walker, 1970). Furthermore, accumulated residues in the soil can seriously affect

seed germination. For example, atrazine is a very commonly used herbicide for corn, but annual application of atrazine, which is quite persistent, has been reported to interfere with crops that follow corn in a program of crop rotation (Neumeyer et al., 1969a). On the other hand, a large number of natural substances have been found to inhibit germination (Mayer and Poljakoff-Mayber, 1963). Coumarin is widely distributed in plants and due to its strong inhibitory action it is known to be a natural germination inhibitor. There is a need for thorough investigation on natural phytotoxicants in soils that may affect seed germination of our food crops.

The observation that pesticides and other natural toxicants interfere with germination suggested the possibility of using germination as a means for the detection of toxic materials. Apparently, seed germination as an indicator of toxic substances may prove to be a simple, time-saving, and economically feasible test. In addition, this method may serve as a diagnostic tool to determine the biological actions of toxic compounds (Anderson, 1965) as in the case of Chlorella which has been used for the detection and estimation of toxic mold metabolites (Ikawa et al., 1969). Furthermore, in many parts of the world sprouted beans are used in the oriental foods as chief source of vitamin C. Malt and brewery industries are essentially based on germination of grains. As such, if the seeds containing high concentrations of pesticides or other toxicants are used for commercial production of sprouts, industry as well as public health will be in jeopardy, consequently creating food toxicological implications.

In addition to the inhibition of seed germination of our economic crops, the presence of pesticides in our foods is hazardous since some of these chemicals have been found to be carcinogenic and teratogenic in laboratory animals. In a review by Golberg (1970), it has been reported that a variety of organophosphates and organochlorines have carcinogenic and teratogenic effects in mice, whereas the herbicide 2,4,5-T exhibited teratogenic properties. On the other hand, s-triazine derivatives have been found to be carcinogenic (Pliss and Zabezhinskii, 1970). Thus, these and several other reports warn us of the disastrous problems ahead. The situation warrants a need for interdisciplinary approaches to solve these problems since the research in modern toxicology is complex.

The objectives of this dissertation are as follows:

1. To determine the inhibitory effects of selected pesticides and allergenic compounds on germination of wheat and mung bean seeds;
2. To investigate the ability of gibberellic acid, a growth regulator, to counteract the inhibition.
3. To study the effects of the test chemicals:
   a. on the rate of respiration of germinating seeds
   b. on the chemical composition (starch, reducing sugars, and amino acids) of germinating seeds
   c. on the development of some hydrolytic enzymes (amylase, ATPase, and protease) during germination of the seeds
   d. on the protein synthesis in germinating seeds

4. To correlate the biochemical data thus obtained with histochemical and ultrastructural changes in the control and treated germinating seeds.

LITERATURE REVIEW

Germination

Germination has been defined as the resumption of active growth on the part of the embryo resulting in rupture of the seed coats (or pericarp). The exact physiological and biochemical changes which occur prior to, during, and subsequent to germination are complex and not too well understood. There has been considerable variation in descriptions of the process by various investigators (Amen, 1963). A number of events are involved: 1) imbibition and absorption of water; 2) hydration of the tissues; 3) absorption of oxygen; 4) increased enzymatic activity and digestion; 5) initiation of cell division and cell enlargement; 6) increased respiration and assimilation; 7) increased cell division and elongation; 8) cellular differentiation; 9) increased reducing sugar content; 10) embryo emergence. The exact sequence of these changes has not yet been determined. Many factors affect the germination of seeds. These factors include the availability of water, gases (oxygen and carbon dioxide), temperature, light for some species, and the presence of inhibitory or stimulatory substances both internal and external (Crocker and Barton, 1957; Mayer and Poljakoff-Mayber, 1963; Toole et al., 1956; Toole and Toole, 1961; Vegis, 1964). Seed germination evidently reflects a balance between germination inhibitors and promoters with the relative proportion being specific for each seed type (Toole et al., 1956). Blocks to germination may be physical or chemical, internal or environmental.

Each kind of seed must absorb a fairly definite proportion of water before germination will start. The first visible evidence of germination is the breaking of the root tip through the seed covering. Before any growth is observed, however, there is a marked increase in respiration which releases energy from the food materials already present in usable form in the cells (Toole and Toole, 1961). The increase in hydration of the seed coats usually causes pronounced increases in their permeability to O_2 and CO_2 which facilitates the increases in respiration (Ortenzio, 1951).

The metabolic changes which occur during germination are complex and vary with the plant species. They consist of three main types: the breakdown of certain materials in the seed, the transport of materials from one part of the seed to another, and the synthesis of new materials from the breakdown products formed. The only substances taken up by seeds during germination are water and oxygen. The metabolic changes occurring in the early stages of germination are the results of the activities of various enzymes. These enzymes must either be present in the dry seed as zymogens or be synthesized de novo to break down storage materials as germination proceeds. The nucleotide content of the seed and seedling rises in all cases during germination. Protein synthesis is an important part of the process of germination. Inhibition of protein synthesis by substances such as chloramphenicol prevents the rise of phosphatase and amylase activity which normally occurs when seeds germinate (Young and Varner, 1959).

The typical structure and stages in germination of a dicot (mung bean) and a monocot (wheat) are shown in Figures 1 and 2, respectively.

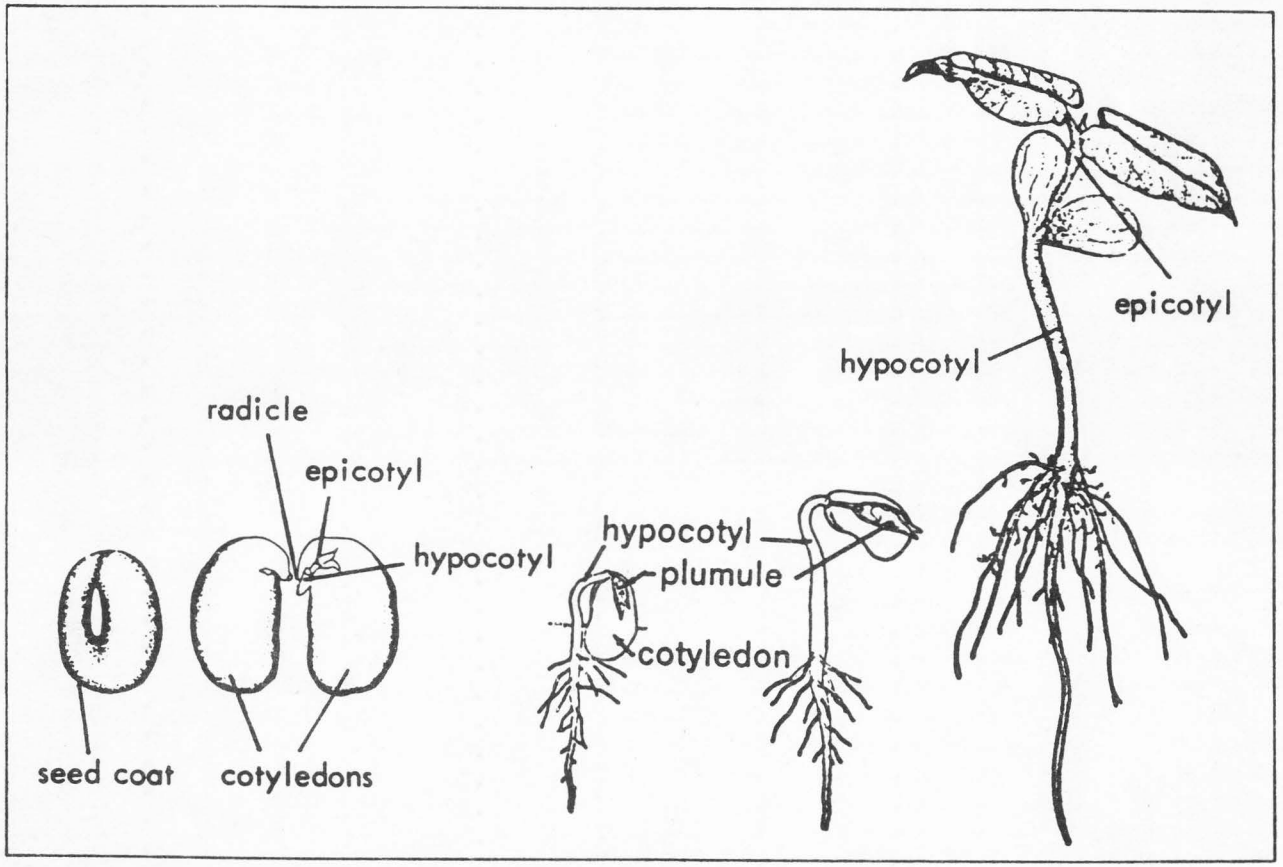


Figure 1. The structure of mung bean and stages in germination.

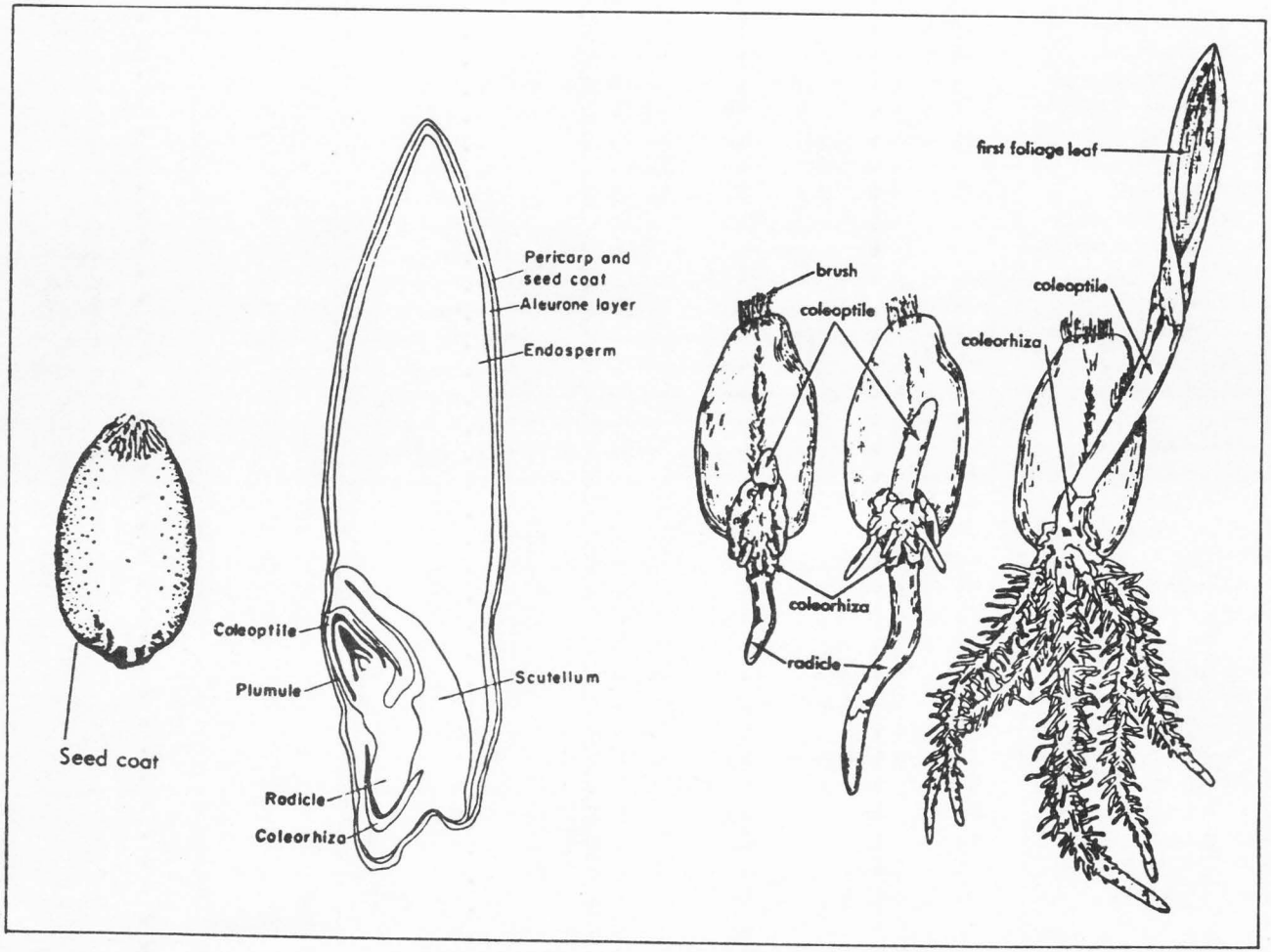


Figure 2. The structure of wheat seed and stages in germination.

Factors affecting germination response

As with most biological phenomena, the response of seeds to toxic chemicals is governed by a series of complex and interacting factors arising within (endogenous) and without (exogenous) the seed. The following review describes some of these and explains their significance.

Endogenous factors. The genetic constitution of a seed has an obvious bearing on its tolerance to a foreign chemical as well as its other attributes such as its resistance to disease and its yield characteristics. Wichramasinghe and Fernando (1962) reported seed-germination differences in susceptibility of bean varieties to endrin used as a seed-soak, as did Lange, Seyman, and Leach (1956) with seed treatments on lima beans. Sclallett and Kurusz (1964) interpreted their results with germination of barley steeped in 2,4-D as differences in varietal susceptibility.

Other variables affecting germination response are age and the physical conditions of the seed. Roane and Starling (1958) found Ceresan M to be severely phytotoxic to chipped wheat seed, slightly toxic to cracked seed, and nontoxic to sound seed. In general, almost any factor having a detrimental influence on seed vigor or quality can increase the likelihood of adverse effects of pesticides or other toxic materials.

Exogenous factors. The chemical nature of the toxic substance and its dose have marked effects on the response of a seed. Within these factors lies the basis for the selective use of many pesticides, particularly herbicides.

Most pesticides impose a dose-range response. Therefore, excessive rates of application of most pesticides can produce harmful secondary effects.

Another factor is the type of pesticide used; e.g. fumigant, seed dressing, and soil treatment. These factors are common to almost all uses. The effect of seed moisture in fumigated storage varies among species, but 10 per cent or less moisture is generally satisfactory to avoid injury (Strong and Lindgren, 1961). High oil content of seeds also has been related to injury, because it can act as a reservoir for methyl bromide and can delay germination for as long as a month (Blackith and Lubatti, 1960).

Lange (1959) has identified in detail a number of factors affecting utilization of insecticides as seed treatments. De Zeeuw et al. (1959) found that prolonged storage increased phytotoxicity to beans and peas from volatile mercurial seed-dressings. Seed damage can be influenced by different formulations of the same compound. Thus, Duran and Fischer (1959) found marked differences among proprietary formulations of benzene hexachloride (BHC) injury to seed.

In liquid pesticide applications to seeds, stickers are usually employed. Methyl cellulose is commonly employed for this purpose, but other materials, such as paraffin oil and linseed oil, are also used. An adverse effect of these oils on germination has been reported by Skoog and Wallace (1964) and by Fletcher (1964).

Often, insecticides and fungicides are applied simultaneously as seed-dressings (Reynolds et al., 1957). Although of obvious benefit from a

protection viewpoint, the mixtures increase the possibility of phytotoxicity through additive or synergistic effects.

Another class of compounds that exogenously impair seed germination are the natural substances derived from microorganisms or plants. Mycotoxins invading cereal grains and phenolic compounds inhibiting seed germination are the well known examples.

It is difficult to describe soil factors that influence effects of foreign chemicals on seed germination because experimental observations have usually been limited to emergence of the seedlings. Relatively little is known about the accumulation of pesticides and other toxic chemicals by imbibing seeds and the internal and external factors that influence it.

Pesticide uses affecting seed germination

Prematurity applications. Various pesticides are often employed during the maturation of seeds on the parent plant. That such treatments can influence the subsequent germinability of the seed crop has been well demonstrated. Seeds from cotton plants affected by 2,4-D showed poor germination and malformed root tips of surviving seedlings (Dunlap, 1948). Similarly, seed germination of mangoes is inhibited by several phenoxy compounds when applied to control fruit (Arora and Singh, 1964). In some cases, germination may not be affected but seedlings are malformed. It is now recognized that 2,4-D and other pesticides can accumulate in immature seeds and be retained for long periods of time (Wheeler et al., 1967).

Cotton seed harvested from plants treated with sprays of the herbicide dalapon displayed retarded seed germination and subsequent growth malformations (Foy and Miller, 1963). Carlson (1959) treated a beet root seed crop with a series of insecticide mixtures and found low seed viability following the application of a mixture of DDT and disulfoton. Indirect residues in plants and seeds may result from absorption, translocation, and subsequent metabolism by plants growing in soils that have been contaminated by the applications to previous crops (Wheeler et al., 1967).

Fumigation during storage. Fumigants in common use for the control of various insect pests of stored products are basically nonselective in their phytotoxic effects. Methyl bromide, for instance, is also commonly employed as a sterilant in field applications where complete kill of weed seeds, nematodes, soilborn insects, and disease is sought. In experiments where one or more fumigants have been compared for effects on seed germination of a number of species, the results inevitably show a relative order of sensitivity to the fumigants (Cobb, 1956, 1958). Thus, Richardson (1951) tested a number of fumigants on seed corn and found rather wide differences in phytotoxicity. Among the most harmful were acrylonitrile, acrylonitrile-carbon tetrachloride mixture (50:50), chloropicrin, and ethylene dibromide.

Another type of fumigation is often suspected of inducing injury to seeds, seeds are occasionally stored where they are subjected to vapors of stored pesticides. Very little attention has been focused on this problem. Furuya and Okaki (1955) found that the per cent of germination of resting seeds of beans exposed to vapors of the herbicide methyl 2,4-dichlorophenoxyacetate

for various periods was not lowered, but seedlings displayed various malformations.

Seed treatments. Pesticides are used to protect seeds and seedlings by applications to seeds during processing in the seed hopper, or by application in the row. Any treatment placing the chemical in intimate contact with the seed or seedling may cause damage. It has been reported by Hanna (1956) that germination and early growth was impaired as a result of such treatment. These secondary effects on seed germination are more likely to occur from seed treatments because the chemical remains in contact with the seeds for a longer period of time. Effective insecticidal treatments originated with the development of chlorinated hydrocarbons. However, problems in the use of insecticidal seed dressings have been realized and are reviewed in detail by several workers (Lange, 1959; Reynolds, 1948).

Early work conducted with technical BHC soon uncovered phytotoxicity and other side effects. Finlayson (1957) reported that lindane applied as a seed treatment to onions was extremely phytotoxic. A mixture of trichlorobenzenes resulting largely from the breakdown of the α-isomer of BHC possesses the ability to cause plant deformation to a large degree. Hocking (1949) found that even low doses inhibit germination. The symptoms are a delay in germination, slight stunting of growth, nonabsorption of cotyledons in case of certain beans, and reduction in seedling weight. Bravo (1956) found that seeds soaked in lindane induced mitotic alterations in rye seedlings causing various morphological injuries.

Generally, aldrin, dieldrin, or heptachlor have not been as injurious as lindane to germination, but injury still occurs. Thus, Finlayson (1957) found aldrin less toxic than lindane to onion seeds. Tiittanen and Varis (1960) reported that seed treatments with aldrin, dieldrin, heptachlor, lindane, and parathion reduced the germination of turnips and rutabaga.

Seed treatment with systemic insecticides has been done by seed-soaks or, more recently, with impregnated coatings. Many of these compounds are phytotoxic. Materials such as Carbowax 6000, activated charcoal, and methyl cellulose have been used as coatings. Skoog and Wallace (1964) reported studies to reduce phytotoxicity of phorate and disulfoton to wheat using various coatings. Bardner (1960) also treated wheat, mustard, and sugarbeet seeds with dressings containing certain systemic insecticides and various stickers and fillers and found reduced toxicity of the insecticides to seedlings. Much work, however, remains to be done before the phytotoxicity problem is solved.

In early work on seed treatments with the organochlorines, it was established that use of an insecticide alone often resulted in reduced germination. The inclusion of a fungicide in the seed treatment resulted in improved germination. However, similar to insecticides fungicides also reduce seed germination.

Some of the earliest work with seed- treatments utilized various inorganic salts of heavy metals. Phytotoxicity was not uncommon. With the appearance of organic fungicides, use of these salts became limited. Walker (1948) reviewed three basic types of seed treatment. Seed disinfestation

with compounds such as inorganic mercury salts destroys only surface organisms. Volatile organic mercury compounds and other fumigants are often used for seed disinfection of surface and internal organisms. Seed protectants, such as captan, provide control of organisms during seed germination but do not eliminate prior infections. The relative order of phytotoxicity is usually disinfestants > disinfectants > protectants.

Soil treatments. Although probably all types of herbicides can influence seed germination under certain conditions, herbicides showing selective pre-emergence activity are more apt to affect seeds than are other types of pesticides. Because, they have the chemical properties to control biologically similar plant growth. In the control of insects and diseases affecting plants, biological differences are often great enough to allow wider margins of tolerance from a chemical and dosage standpoint. Therefore, one of the more challenging aspects of many soil-applied herbicides is the effect of the herbicide on seed germination and early development of seedlings of economic crops, especially since several of these herbicides have little effect on established plants (Ashton, Penner and Hoffman, 1968).

It is now recognized that depending on their concentrations, soil residues of s-triazine herbicides (Holly and Roberts, 1963; Sheets and Shaw, 1963; Ercegovich, 1965) may cause damage to the germinating seeds in the soil. GS-14254, 2-methoxy-4-isopropylamino-6-butylamino-s-triazine, is a member of the s-triazine family and appears to be more phytotoxic and persistent (Harris et al., 1968). Tas (1961) studied the effect of various

herbicides and found that 2,4-D and 2,4-DB reduced or prevented seed germination of several species at high rates. Similar results have been reported by Sund and Nomura (1963) who observed that DNBP, pentachlorophenol, and diquat were phytotoxic to germinating seeds of radish and sudan grass at levels of 10 ppm or less. They also found 2,4,5-T, dichlobenil, and diquat very toxic to germinating cucumber seeds.

Several workers (Harris et al., 1968; Hanna, 1956; Lange, Carlson, and Leach, 1953) have reported reduced germination of seeds when subjected to organochlorine insecticides. Guyer et al. (1958) found that phorate when used as seed treatment adversely affected wheat germination. Gifford, Burkhardt, and Somsen (1959) also noted that wheat germination was reduced and seedling survival was lower due to phorate treatment. Unlike organochlorine insecticides organophosphates are relatively less persistent but can remain in the soil for several months (Edwards, 1965). Nevertheless, as a replacement for persistent chlorinated hydrocarbons, they are finding increased use for protection of crops. However, they appear to be more phytotoxic especially when used as seed dressings (Scopes, 1969). Furthermore, simultaneous presence of herbicides and insecticides may still pose a very serious problem since their interactions most commonly result in increased phytotoxicity due to an inhibition of herbicide degradation by insecticides (Kaufman et al., 1970; Chang, Smith, and Stephenson, 1971).

Many insecticides and fungicides used in seed treatment are applied to soils. Because of seed size or other problems it often is not possible to

provide enough pesticides on the seed to ascertain protection without causing phytotoxicity. In such cases, various soil applications are attempted. Repeated use of the insecticides and herbicides, therefore, could lead to the build-up of residues in the soil. As already reviewed, it is clear that seed germination can be affected by a variety of uses of pesticides when they are directed at target organisms. However, effects on germination arise from a complex of factors, often not well understood. Certain factors such as dosage have nearly universal significance and are often readily controllable. Yet, fundamental studies on response of seeds to defined doses of pesticides are not available in many cases. Most other variables are even less well defined. There is a need for better understanding of the problems involved and for finding ways to cope with them.

Natural compounds inhibiting seed germination

It is known that phenolic compounds of various kinds inhibit germination. Because of the widespread occurrence and distribution of phenolic compounds in plants and fruits it has been suggested that these substances might act as natural germination inhibitors (van Sumere, 1960). The inhibitory action of coumarin and cinnamic acid derivatives has been studied on a wide variety of seeds and it has usually been found that these inhibit germination. Coumarin itself is characterized by an aromatic ring and an unsaturated lactone structure.

Recently, it has been reported (Mitchell et al., 1970) that a sesquiterpene lactone (alantolactone) present in several species of compositae caused

allergic dermatitis in forest workers. Previous studies by Mitchell and Armitage (1965) have shown that lichens could be another group of plants responsible for contact dermatitis in forest workers. In this case, the allergy-causing substance is usnic acid, a phenolic compound, and the skin disease in the forest workers is commonly referred to as "cedar poisoning." The ubiquitous occurrence of lichens and compositae in forest areas and in the cultivated lands as common weeds is known. Like pesticides, these natural substances may prove to be contaminants of our environment. Some times, animals eat these lichens and mosses and may cause poisoning in the animals. In other instances, spores of the lichens may be carried out through air or through water to crop lands or food storage area and may contaminate the food directly resulting in an unknown cause of allergy. In cultivated lands the presence of these compounds as residues derived from common weeds might reduce the germination percentage of crop seeds. In forest areas, these compounds may play a role in restricting the number of species in a given population. Considering the significance of the possible role of these naturally occurring toxicants--alantolactone and usnic acid--it was decided to study their action on seed germination which might prove an effective way of screening such toxic compounds.

Mechanism of action of germination inhibitors

It is clear from the foregoing review that there is a wealth of knowledge on the inhibition of seed germination by a variety of pesticides. However,

from the relevant literature it appears that there is a paucity of information about the mode-of-action studies on the pesticides that inhibit germination or seedling growth. According to Chopra and Nandra (1969), Thiometon an organophosphate insecticide, inhibited germination of sarson seeds (Brassica campestris L.) by limiting the activity of lipase enzyme. Thus the overall process of germination as indicated by actual sprouting was considered of more interest than specific reactions within the seed because of the complexity of the germination process and the variety of possible mechanisms of actions of toxic compounds. The two insecticides, menazon and disulfoton, and a herbicide, GS-14254, were therefore used in this study to investigate their toxic effects on metabolic changes in germinating seeds of wheat and mung beans.

The possible role of alantolactone and usnic acid as our environmental contaminants has already been discussed. But, little is known regarding the effects of alantolactone in plant systems although it shows antihelminthic action to Fasciola hepatica (Kim, Suh, and Park, 1961), bactericidal activity (Yudovich, 1962), and inhibits growth of several pathogenic fungi (Olechnowicz-Stepien and Stepien, 1963). As already mentioned it has allergenic properties too.

Usnic acid is also allergenic and causes contact dermatitis in sensitive humans (Mitchell, 1966). It occurs in the d- and l-sterioisomeric forms in nature and is a well known constituent of many lichens, for instance, Usnea, Alectoria, Ramalina, Evernia, Citraria, Parmela, Cladonia, Lecanoda, and

Haematomma sp. (Ashahina and Shibata, 1954). This compound may comprise up to 6 per cent by weight of the species of Alectoria and Usnea (Bandoni and Towers, 1967). The antibiotic properties of usnic acid were discovered by Burkholder and Evans (1945), but scattered reports on its pharmacology preceded this discovery. Later on, several workers (Marshak, 1947; Marshak, Barry, and Graig, 1947; Shaw, 1967; Stoll, Brack, and Renz, 1947) reported more studies on its antibiotic properties. Bandoni and Towers (1967) noted that usnic acid inhibited the growth of 6 species of bacteria and 10 of fungi isolated from soil. Usnic acid appears to uncouple oxidative phosphorylation (Johnson, Feldott, and Lardy, 1950; Marshak and Harting, 1948; Whitehouse and Dean, 1965). Pressman (1963) attempted to locate the site of uncoupling. It has been reported that usnic acid blocks the synthesis of certain bacterial proteins (Brock, 1963; Creaser, 1955) and there is some evidence that this antibiotic disrupts nucleic acid metabolism (Brachet, 1951; Steinert, 1953). However, studies on the effects of usnic acid on plants are few (Kinraide and Ahmadjian, 1970).

Therefore, studies were undertaken to investigate the toxic effects of menazon, disulfoton, GS-14254, alantolactone, and usnic acid on such parameters as germination response, respiration, degradation of some food reserves and development of amylase, ATPase, and protease activities in the germinating seeds of wheat and mung beans. These enzymes were studied because they are synthesized de novo in the germinating seeds. The data thus obtained were then correlated to histochemical and ultrastructural

changes in the treated seeds to offer biologic explanations for the mechanism of action of these chemicals.

MATERIALS AND METHODS

Chemicals

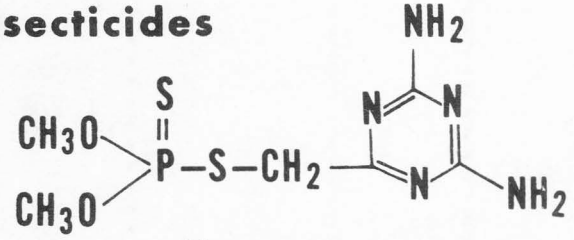
Menazon, S-(4, 6-diamino-s-triazin-2-ylmethyl)O, O-dimethyl phosphorodithioate, was obtained from Imperial Chemical Industries Ltd., Haslemere, Surrey, England and the wettable powder contained 70 percent active ingredient. Disulfoton, O, O-diethyl S-2-(ethylthio) ethyl phosphorodithioate, (technical grade containing 98 percent active ingredient) was procured from Chemagro Corporation, Kansas City, Missouri. GS-14254, 2-methoxy-4-isopropylamino-6-butyl-amino-s-triazine, was received from Geigy Agricultural Chemicals, Ardsley, New York. The wettable powder (80W) contained 80 percent active ingredient. Helenin, a mixture of alantolactone and isoalantolactone, was purchased from the Sigma Chemical Company, St. Louis, Missouri. To separate the two lactones, the mixture was run on a comumn of silica gel containing 10 percent $AgNO_3$ with ether-petroleum ether (Mitchell et al., 1970). Usnic acid was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The structural formulas of these chemicals are shown in Figure 3. ^{14}C-L-leucine (U) was purchased from New England Nuclear, Boston, Massachusetts. Triton B-1956 was from Rohm and Haas, Philadelphia, Pennsylvania.

Seeds

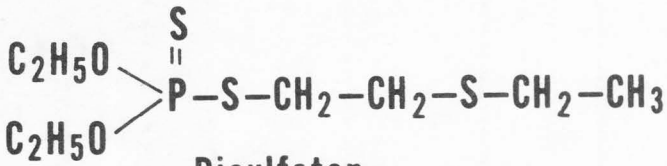
Mung beans, Phaseolus mungo L., and wheat, Triticum aestivum L., seeds were purchased from a local market.

A: Pesticide chemicals

1. Insecticides

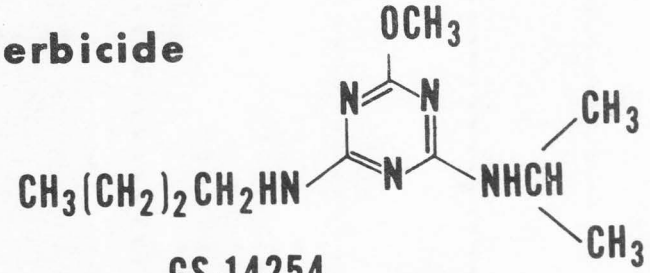


Menazon



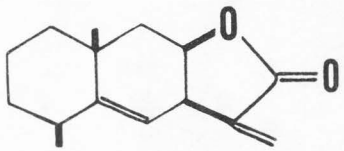
Disulfoton

2. Herbicide

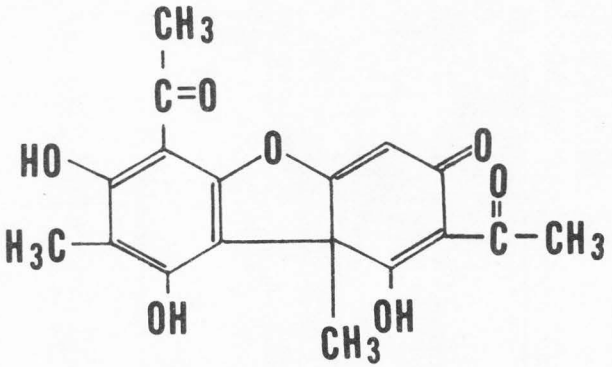


GS-14254

B: Allergenic compounds



Alantolactone



Usnic acid

Figure 3. Structural formulas of the pesticide chemicals and allergenic compounds.

Germination procedure

Uniform-sized seeds were soaked in running tap water for 1-2 h and surface-sterilized by immersion in a 10 per cent (v/v) water-diluted solution of Clorox for about 10-15 min. The seeds were stirred occasionally, rinsed thoroughly in distilled water, and then placed (10 seeds per petri dish of 5 cm diameter) on a double layer of filter paper. Menazon (0 - 250 ppm), disulfoton (0 - 100 ppm), GS-14254 (0 - 100 ppm), or alantolactone (0 - 100 mg/1) in 0.02 per cent Triton B-1956 or 2 per cent DMSO (dimethyl sulfoxide) solution in water was added to each petri dish. The aqueous solution of usnic acid (0 - 250 mg/1) was made by adding 2N KOH in small increments (0.01 ml) to the suspension of crystalline usnic acid in water until the pH was 7.0-7.5 (Johnson, Feldott and Lardy, 1950). The petri dishes containing the seeds were placed in the dark in a humid (85 - 90 per cent) germinator at 30 C. There were three replicates in each treatment.

The per cent germination and length of root and shoot were determined after a period of 120 h. Those seeds which did not germinate after the treatments were not included in the measurement of root and shoot growth.

Respiration

After 24, 48, and 72 h germination periods, both treated and untreated seeds were placed in Warburg flasks with 1 or 2 ml of distilled water to keep the respiring seeds moist. The well in the flask contained 0.1ml of 20N KOH to absorb released CO_2. Each manometric flask contained five or ten seeds. The oxygen uptake by the germinating seeds was measured on a Gilson

respirometer. The readings were taken every ten or five minutes and the respiratory rates were expressed as µl per hour per gram on a dry-weight basis.

Free amino acids

Previously weighed 20 seeds, treated and untreated, at various stages of germination were ground with a mortar and pestle and extracted with 80 per cent boiling ethanol. The extraction was repeated four times by centrifuging at 5000 r.p.m. for 15 min. The combined ethanolic extract was then evaporated to about 5 ml and the contents were dissolved in a known volume of water. This aqueous extract was used for the determination of free amino acids and reducing sugars. The washed residue in the centrifuge tubes was used for starch determination.

The amino acid content in the aqueous solution was determined by the method of Rosen (1957). One ml of the sample was heated for 15 min in a boiling water bath after the addition of 0.5 ml of cyanide-acetate buffer (pH 5.4) and 0.5 ml of 3 per cent ninhydrin in methyl cellosolve. Immediately after removing the sample tubes from the water bath 5 ml of isoprophyl alcohol-water (1:1) diluent was added and the tubes were shaken vigorously and allowed to cool to room temperature. The intensity of color was read at 570 mµ in a Bausch and Lomb spectrophotometer. The concentration of amino acids was calculated from a standard curve prepared from L-leucine.

Reducing sugars

The aqueous extract prepared for the determination of free amino acids was cleared and deleaded (Loomis and Shull, 1937) before determining reducing sugars by the arsenomolybdate reagent method of Nelson (1944). To 1 ml of the sugar solution was added 1 ml of copper (sulfate) reagent which was a mixture of 25 parts of reagent A and 1 part of reagent B. Reagent A was prepared by dissolving 25 g of Na_2CO_3 (anhydrous), 25 g of Rochelle salt, 20 g of $NaHCO_3$ and 200 g of Na_2SO_4 (anhydrous) in about 800 ml of water which was made to one liter. Reagent B was 15 per cent $CuSO_4$ containing 1-2 drops of concentrated sulfuric acid per 100 ml. The solutions were mixed and heated for 20 min in a boiling water bath. At the end the tubes were cooled and 1 ml of arsenomolybdate reagent was added. The mixture was diluted to 8 ml and the optical density was measured at 520 mμ.

The arsenomolybdate reagent was prepared by dissolving 25 g of ammonium molybdate in 450 ml of distilled water. To this was added 21 ml of conc. sulfuric acid and 3 g of sodium arsenate dissolved in 25 ml of water. The contents were mixed and placed in an incubator at 37 C for 48 h.

The reducing sugar was calculated from a standard curve and expressed as mg per gram of dry seeds.

Starch

The sugar-free residue in the preparation of the ethanolic extract of the germinating seeds was suspended in 5 ml of water and while stirring 6.5 ml of diluted (52 per cent) perchloric acid were added (McCready et al., 1950).

Stirring was continued for 20 min and the contents were centrifuged after adding 20 ml of water. The aqueous starch solution was poured into a 100-ml volumetric flask and 5 ml of water were added to the residue, and stirred while adding 6.5 ml diluted perchloric acid reagent. The residue was solubilized as before for 30 min with occasional stirring and the contents were washed into the 100-ml flask containing the first extract. Combined solutions were diluted to 100 ml, filtered and the filtrate was used for starch determination essentially following the method of McCready et al., (1950). Five ml of the appropriately diluted starch solution were pipetted into a 25 x 250 mm borosilicate glass tube, cooled in an ice water bath and 10 ml of fresh anthrone reagent (prepared by dissolving 2 g of anthrone in 1 liter of cold 95 per cent sulfuric acid) were added. After the anthrone had been added to all of a series of sample tubes, each tube was mixed thoroughly and then all were heated together for 7.5 min at 100 C. The tubes were cooled rapidly to 25 C in a water bath and the color intensities were determined at 630 mμ. A standard curve was prepared using 0 - 100 μg of glucose containing the same amount of perchloric acid as that in the starch aliquots and this calibration curve was used to obtain the yield of glucose from starch.

Enzyme assays

The method of preparation of enzyme extracts was essentially that of Young and Varner (1959). Twenty seeds from each treatment at 1-4 days germination period were thoroughly washed with deionized water and blended in an appropriate amount (40 ml for mung beans and 20 ml for wheat seeds)

of cold Tris buffer, pH 7.3, in a Sorvall Omnimixer. The homogenate was centrifuged at 20,000 x g for 1 h and the supernatant fluid was collected for enzyme assays. The above procedures were done at 0 to 4 C.

Amylase activity. Amylase activity was measured by adding 1.0 ml of enzyme preparation to 1.0 ml of 1 per cent potato starch solution in 0.016 M acetate buffer (pH 4.8) and incubating the mixture at 25 C for 5 to 10 min. The increase in reducing power was determined by addition of dinitrosalicylic acid (Bernfeld, 1955). The specific activity was defined as mg of maltose produced per mg of protein in 10 min.

ATPase activity. ATP-hydrolyzing phosphatase activity was estimated by incubating 1.0 ml of the enzyme preparation with 0.033 M Tris buffer (pH 7.3), 0.001 M $MgCl_2$, and 0.004 M ATP in a total volume of 3.0 ml at 38 C for 15 min. The reaction was stopped by the addition of 1.0 ml of 15 per cent TCA, and the phosphate released was determined by the method of Sumner (1944). A known volume of the phosphate-containing samples were pipetted into corresponding tubes graduated at 50 ml to which was added 5 ml amount of 6.6 per cent ammonium molybdate and distilled water to about 40 ml. Then 5 ml of 7.5 N sulfuric acid were added and mixed gently by rotating the tubes. Four ml of the ferrous sulfate (5 g $FeSO_4 . 7H_20$ in 50 ml water and 1 ml of 7.5 N sulfuric acid) were then added to each of the tubes. The tube contents were diluted to 50-ml mark, stoppered with clean rubber stoppers, and inverted 4 or 5 times. A blank was also prepared simultaneously in the same manner. The optical density was measured at 660 mμ and the concentration of

phosphate was calculated from a standard curve prepared by using a standard solution of pure KH_2PO_4.

The specific activity was defined as μg of Pi formed per mg of protein in 15 min.

Proteolytic activity. Proteolytic activity was determined by adding 2 ml of enzyme preparation to 3 ml of 2 per cent casein adjusted to pH 7.1 in 0.067 M phosphate buffer. The incubation was done at 38 C for 1 h. For each sample, duplicate tubes plus a zero time control were prepared. A 2.0 ml aliquot was pipetted into 2.0 ml of 15 per cent TCA, centrifuged at 1000 x g for 10 min, filtered, and the increase in optical density (O.D.) was measured at 280 mμ in a 1-cm cuvette. The proteolytic activity was defined as that amount of enzyme which in 1 h caused an increase of one unit in O.D. of the filtrate at 280 mμ. The specific activity was expressed as units of proteolytic activity per mg of protein in the enzyme preparation.

Protein determination. In all cases, the activities of enzymes were based on the protein content of the enzyme extracts. Protein was estimated by the procedure of Lowry et al., (1951).

In vitro effect of the chemicals on amylase activity. Seeds without any treatment were germinated in water for 3 days and the enzyme extract was prepared as described in the beginning of this section. One ml starch solution, 0.5 ml enzyme preparation and 0.5 ml of a pesticide or an allergenic compound were incubated in triplicate for 5 or 10 min. In each tube the final concentration of menazon, disulfoton, GS-14254, alantolactone, or usnic acid was 250, 100, 100, 100, and 250 ppm, respectively. Thus, including the control

there were six treatments each having three replicates. The reducing power was determined by addition of dinitrosalicylic acid and the results were expressed as the amylase activity per cent of control.

Studies on ^{14}C-L-leucine incorporation

With a view to studying the effects of the chemicals used in the present work on in vivo protein synthesis, two separate experiments were carried out. Since potato tuber slices are known to be able to synthesize protein (Click and Hackett, 1963), these as well as germinating seeds were used to explore the effect of the pesticides and allergenic compounds on uptake and incorporation of radioactive L-leucine in new proteins.

Potato tuber slices. Cylinders (1 cm diameter) of storage tissue were removed from a potato tuber (var. Idaho russet) with a cork borer and 1 mm thick discs were cut using a hand microtome. After thoroughly washing the discs in sterile deionized water, ten discs were placed in a 250-ml Erlenmeyer flask that contained 8.0 ml of the sterile medium (Click and Hackett, 1963) including 0.5 μCi (specific activity 262 mCi/mM) of ^{14}C-L-leucine (U). In each of the five flasks menazon, usnic acid (250 mg/l final concentration of each chemical), disulfoton, GS-14254, or alantolactone (100 mg/l final concentration of each chemical) was individually added and the sixth being kept as control. For each of the six treatments triplicate flasks were kept. The flasks were then placed on a rotary shaker set at 50 r.p.m. After incubating the samples at 25 C for 24 h each set of 10 aged discs was removed from the radioactive medium, washed in carrier L-leucine solution (0.2 mg/ml)

and extracted in ethanol according to the procedure of White and Taniguchi (1969). Radioactivity was measured with a Unilux II-A Scintillation counting system, Nuclear-Chicago, after adding 2 ml of the ethanol extract to 15 ml of the scintillation liquid (5 g PPO, 0.3 g dimethyl POPOP, and 333 ml Triton X-100 in toluene made to 1000 ml). Dried potato discs were powdered, weighed and the radioactivity was measured in 10 ml of INSTA-GEL formulation.

Germinating mung beans. Mung bean seeds of uniform size were soaked in water under aseptic conditions for 24 h. Eighteen test tubes each containing 8 soaked seeds (without coats) and 0.5 ml sterile water with 0.1 μCi of ^{14}C-L-leucine were divided into 6 batches. To one batch, 0.2 ml sterile water was added and to each of the rest, 0.2 ml of menazon, disulfoton, GS-14254, alantolactone or usnic acid was added so that the final concentration of each chemical in each batch was the same as for potato tuber discs. After the incubation period of 24 h at 35 C (Mandal and Biswas, 1970) seeds were removed from the tubes and washed repeatedly with water and then with carrier L-leucine until washings were free of radioactivity. The samples were homogenized in ethanol, centrifuged at 1000 x g for 10 min, and extracted with ethanol essentially following the same procedure used for potato tuber discs and the radioactivity was determined. The pellet was dissolved in a known volume of 0.01 N NaOH, centrifuged, and a portion of the supernatant was used for measuring radioactivity incorporated in protein.

Histochemical studies

Mung bean seeds were treated separately with 250 ppm of menazon or usnic acid and allowed to germinate along with control seeds for 72 h as described earlier. Whole cotyledons of the treated and untreated seeds were washed thoroughly in distilled water and were fixed in FAA (formalin-acetic-alcohol) fixative under vacuum (12 psi) at room temperature for 24 h. Then the cotyledons were dehydrated through an ETOH:TBA (ethanol-tertiary butyl alcohol) series and embedded in Paraplast. Cross sections were cut at 10 μ with an AO-801 microtome and then the following procedures were adopted for nucleic acids, RNA, insoluble polysaccharides, and protein localization in the storage cells.

Nucleic acids. The methylene blue staining procedure of Dietch (1966) was used for the detection of nucleic acids in the cotyledons. The rehydrated sections were first stained in freshly-made solution of methylene blue chloride for 30-60 min. at room temperature. The slides were rinsed in distilled water for 30-60 sec., blotted lightly and passed them through 3 changes (10 min. each) of tert-butanol in staining jars. The sections were dehydrated in 2 changes of xylene and mounted in a synthetic resin. Photographs were taken with a Zeiss light microscope.

RNA. Ribonucleic acid in the treated and untreated seeds was localized by the pyronin Y method of Tepper and Gifford (1962). After removing the paraffin and rehydrating in water, the sections were stained in 2 per cent aqueous solution of pyronin Y for 6-30 min. The sections were then

differentiated in two changes (5 min. each) of n-butanol followed by two changes of xylene and finally mounted in a synthetic resin.

Carbohydrates. Total insoluble polysaccharides were localized by periodic acid-Schiff's (PAS) reaction as described by Hotchkiss (1948). The slides were placed in a 0.5 per cent periodic acid solution for 5-30 min., washed in running tap water for 10 min., and then stained in Schiff's reagent for 10-15 min. After rinsing the sections in water, they were placed in 2 per cent sodium bisulfite solution for 1-2 min., washed in running tap water, and mounted.

Protein. Protein distribution was viewed in Hg-BPB (meruric-bromophenol blue) stain (West and Gunckel, 1968). The sections were stained in the mixture of 0.1 per cent $HgCl_2$, 0.05 per cent bromophenol blue, and 2 per cent acetic acid for 2 h. The stained sections were immediately rinsed in 0.5 per cent acetic acid for 5 min., immersed in water (pH 7.5) for 3 min. and then transferred to absolute TBA (2 changes, 1 h each), passed through xylene:TBA (1:1) and then in only xylene and mounted in a synthetic resin.

Electron microscopy

For ultrastructural examination, transversely cut slices of the cotyledons were fixed in Karnovsky's fixative (1965) for 5 h at room temperature under vacuum (12 psi). The slices were rinsed in 0.2 M cacodylate buffer (pH 7.4) and then post-fixed in 2 per cent osmium tetroxide for 2 h at 2-4 C. The fixation was followed by four half-hour rinses in the cacodylate buffer. Dehydration was carried out in graded ethanol. The tissue was then rinsed

in propylene oxide and embedded in Epon 812 resin. The properly oriented blocks were trimmed and sectioned with a glass knife on a Sorvall MT-2 ultramicrotome; sections showing silver and grey interference were selected and double stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). The sections on the copper grids (200 mesh) were examined in a Zeiss EM-9A electron microscope.

Statistical analysis

All the experiments were arranged in a completely randomized block design with two or three replicates in each treatment. Analyses of variance were computed and the means were compared according to the least significant difference (LSD) procedure (Steel and Torrie, 1960).

RESULTS

Inhibition of seed germination and seedling growth

Each value in the tables is a mean of triplicate determinations within a given experiment. Repeated experiments gave essentially similar data. It is evident from Tables 1 and 2 that at various concentrations menazon, disulfoton, GS-14254, alantolactone, and usnic acid were not only able to inhibit the germination of mung beans and wheat seeds but also were considerably effective against the seedling growth. Figures 4, 5, 6, and 7 exhibit the toxic effects with the highest concentrations of these chemicals on germination of mung beans and wheat seeds. It is seen from the Figures 8, 9, and 10 that among the pesticide chemicals disulfoton seems to be relatively less toxic to seed germination at the given concentrations. Thus, the degree of inhibition of germination and seedlings development depended on the concentration of the chemicals. When solution of the herbicide GS-14254 (100 ppm) was added to either of the insecticides at their maximum concentrations (Table 1) the inhibitory effect of the combined pesticides on seed germination and seedling growth was more marked, especially with wheat. It was also noted that the seedlings surviving the pesticide treatment were distorted and weak.

Among the allergenic compounds, usnic acid when applied to the seeds at concentrations ranging from 50 to 250 mg/1 resulted in a

Table 1. Effect of pesticides on germination of seeds and seedling growth during 120 h

Pesticides	Concentration (ppm)	Germination %		Root Growth cm		Shoot Growth cm	
		Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
Menazon	0	100	90	2.87	3.97	6.72	4.51
	50	90	87	2.47	3.47	5.09**	3.89
	100	67**	83	2.18	3.10*	4.00**	3.64*
	200	33**	50**	1.02**	2.15**	1.54**	3.56*
	250	30**	23**	1.26**	1.36**	1.44**	2.47**
	S.E.[a]	±5.57	±5.16	±.33	±.28	±.23	±.26
Disulfoton	0	100	100	3.75	4.53	4.28	4.88
	20	100	87	3.07	3.46	3.88	4.07
	40	90	93	3.35	3.82	3.53	4.18
	80	90	90	3.27	2.83*	2.86**	3.47**
	100	80**	90	2.94*	2.92*	3.03*	3.38**
	S.E.	±3.65	±4.21	±.22	±.47	±.31	±.27
GS-14254	0	100	90	4.95	4.23	7.78	4.48
	10	90	63	3.75*	2.18**	6.96	2.94**
	20	100	60*	4.03	1.26**	6.45*	1.75**
	50	93	50*	2.60**	1.04**	4.00**	1.73**
	100	63**	23**	2.09**	0.87**	2.45**	0.92**
	S.E.	±5.58	±9.18	±.32	±.27	±.35	±.26
GS-14254 + Menazon	100 + 250	44**	10**	1.27**	1.00**	1.23**	0.50**
	S.E.	±3.27	±2.08	±.25	±.10	±.35	±.21
GS-14254 + Disulfoton	100 + 100	71**	13**	2.30**	0.50**	2.10**	0.50**
	S.E.	±1.63	±2.16	±.27	±.07	±.35	±.21

*Significantly different at 0.05 level.

**Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 21.

Table 2. Effect of allergenic compounds on germination of seeds and seeding growth during 120 h

Allergenic Compounds	Concentration (mg/l)	Germination %		Root Growth cm		Shoot Growth cm	
		Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
Alantolactone	0	93	90	1.71	3.78	3.08	4.39
	10	80	70	1.23	2.00**	2.41*	2.36**
	25	70	73	1.05*	1.72**	1.95**	2.55**
	50	70	73	0.86**	1.72**	1.46**	2.33**
	100	13**	33**	0.50**	0.88**	0.50**	1.37**
S.E.[a]		±3.54	±7.75	±.04	±.25	±.05	±.20
Usnic Acid	0	93	100	2.83	5.06	5.20	5.23
	25	90	96	2.82	1.57**	3.64**	2.60**
	50	70*	86	1.37**	1.10**	2.50**	2.19**
	100	40**	63**	1.26**	0.84**	2.03**	2.22**
	250	30**	43**	0.82**	0.61**	1.17**	1.92**
S.E.		±6.50	±5.37	±.21	±.27	±.25	±.30

* Significantly different at 0.05 level

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 22.

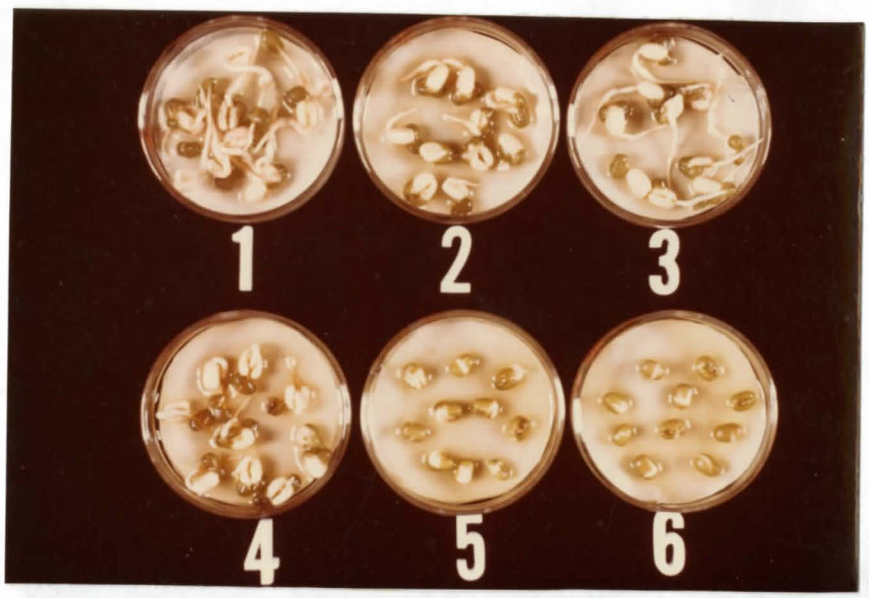


Figure 4. Three-day germinated control and treated mung beans.

1 = control, 2 = menazon (250 ppm), 3 = disulfoton (100 ppm), 4 = GS-14254 (100 ppm), 5 = alantolactone (100 mg/1), 6 = usnic acid (250 mg/1).
Compared to the control seeds menazon-treated seeds have short radicles, GS-14254-treated seeds show necrosis of the radicles while alantolactone and usnic acid completely inhibit emergence of the radicles.

Figure 5. Three-day germinated control and treated wheat seeds.

1 = control, 2 = menazon (250 ppm), 3 = disulfoton (100 ppm), 4 = GS-14254 (100 ppm), 5 = alantolactone (100 mg/1 i.e. 100 ppm), 6 = usnic acid (250 mg/1 i.e. 250 ppm).
Note the normal germination of the control seeds; some seeds treated with pesticides are germinating whereas seeds treated with allergens do not germinate.

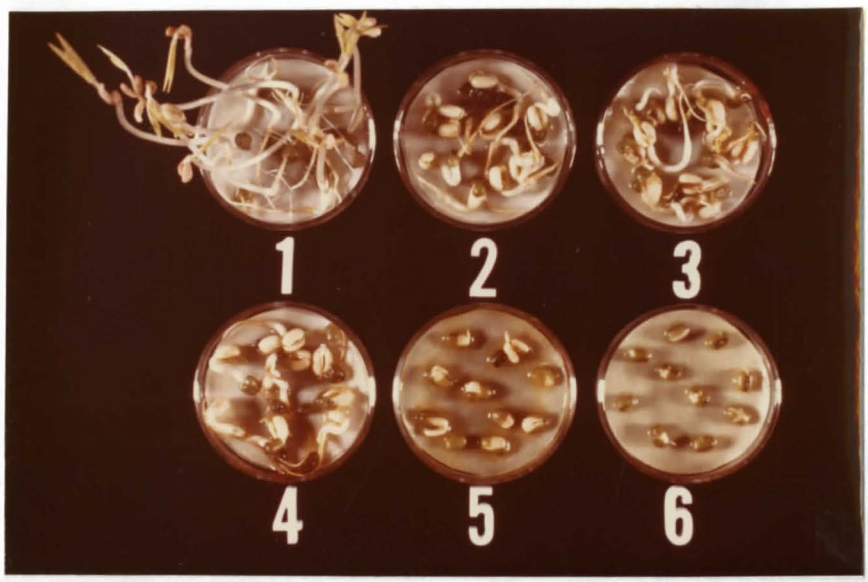


Figure 6. Five-day germinated control and treated mung beans.

1 = control, 2 = menazon (250 ppm), 3 = disulfoton (100 ppm), 4 =GS-14254 (100 ppm), 5 = alantolactone (100 mg/1), 6 = usnic acid (250 mg/1).
Note the healthy growth of mung bean seedlings without treatment. Germinating mung beans treated with menazon or disulfoton do not show further growth. Their shoots start thickening and roots are becoming brown due to necrosis. These symptoms are also seen in mung beans treated with GS-14254. No germination is observed with mung beans treated with allergens even after 5 days.

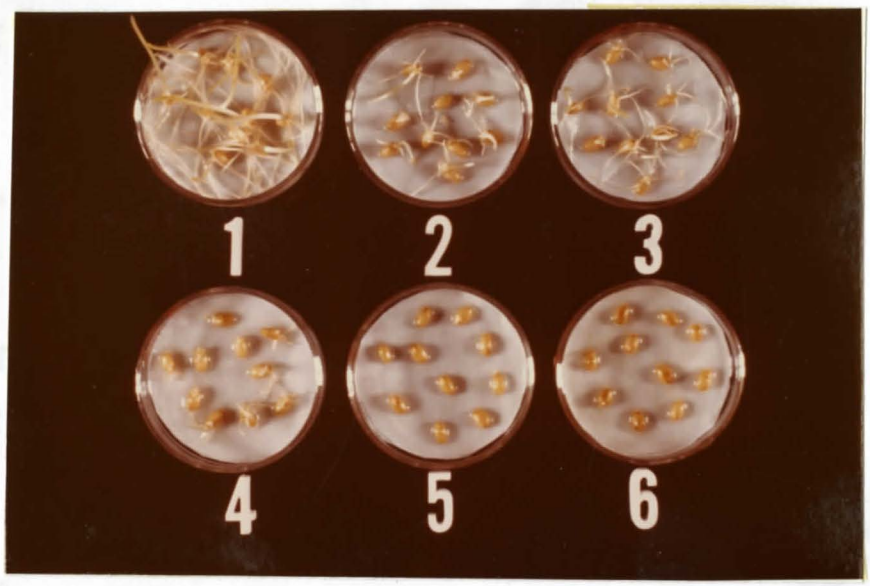


Figure 7. Five-day germinated control and treated wheat seeds.

1 =control, 2 = menazon (250 ppm), 3 = disulfoton (100 ppm),
4 =GS-14254 (100 ppm), 5 = alantolactone (100 mg/1),
6 =usnic acid (250 mg/1).
Note the healthy growth of wheat seedlings without treatment. Germinating wheat seeds treated with menazon or disulfoton show comparatively poor growth. The shoots and roots appear to be weak. These symptoms are severe in wheat seeds treated with GS-14254. No germination is observed with wheat seeds treated with allergens even after 5 days.

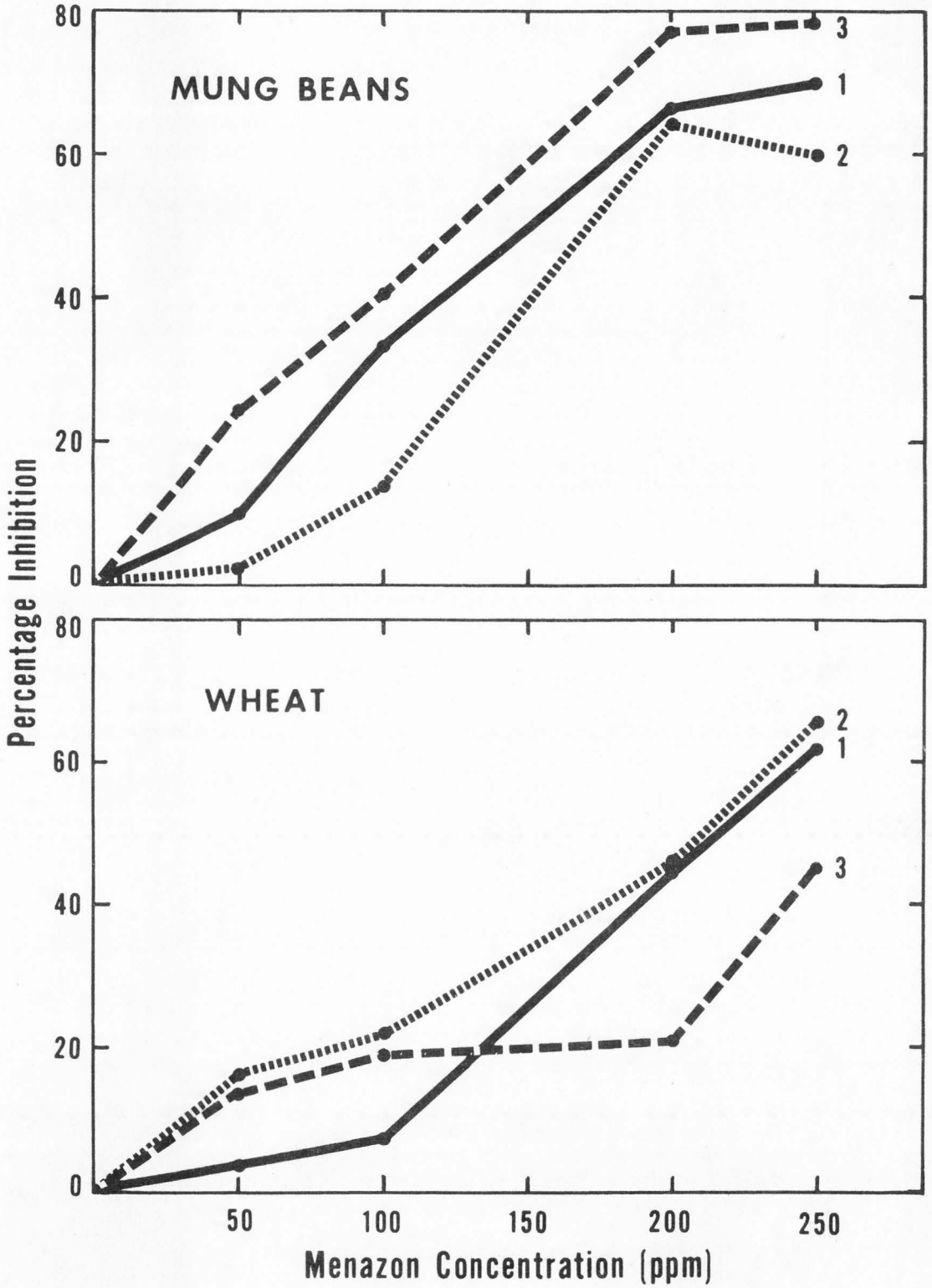


Figure 8. Effect of menazon on percentage inhibition of germination (1), and root (2) and shoot (3) growth of mung bean and wheat seeds.

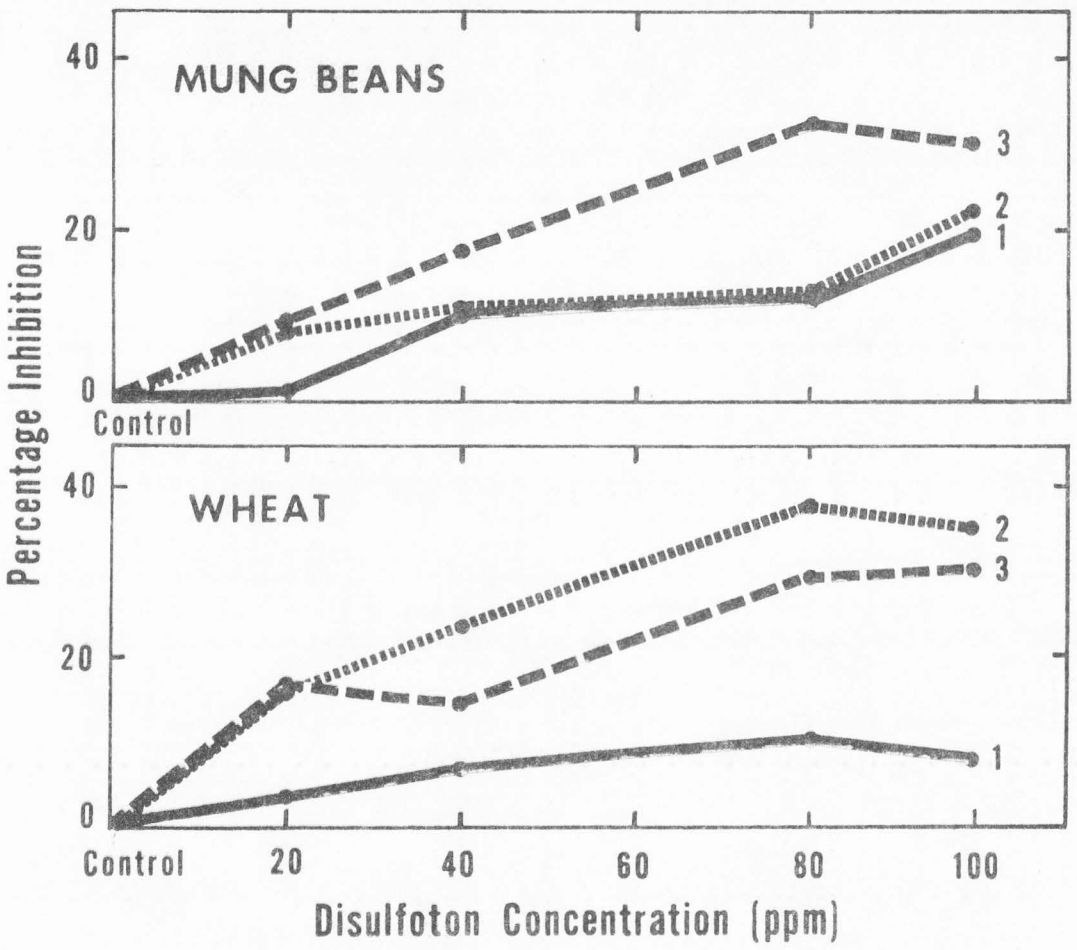


Figure 9. Effect of disulfoton on percentage inhibition of germination (1), and root (2) and shoot (3) growth of mung bean and wheat seeds.

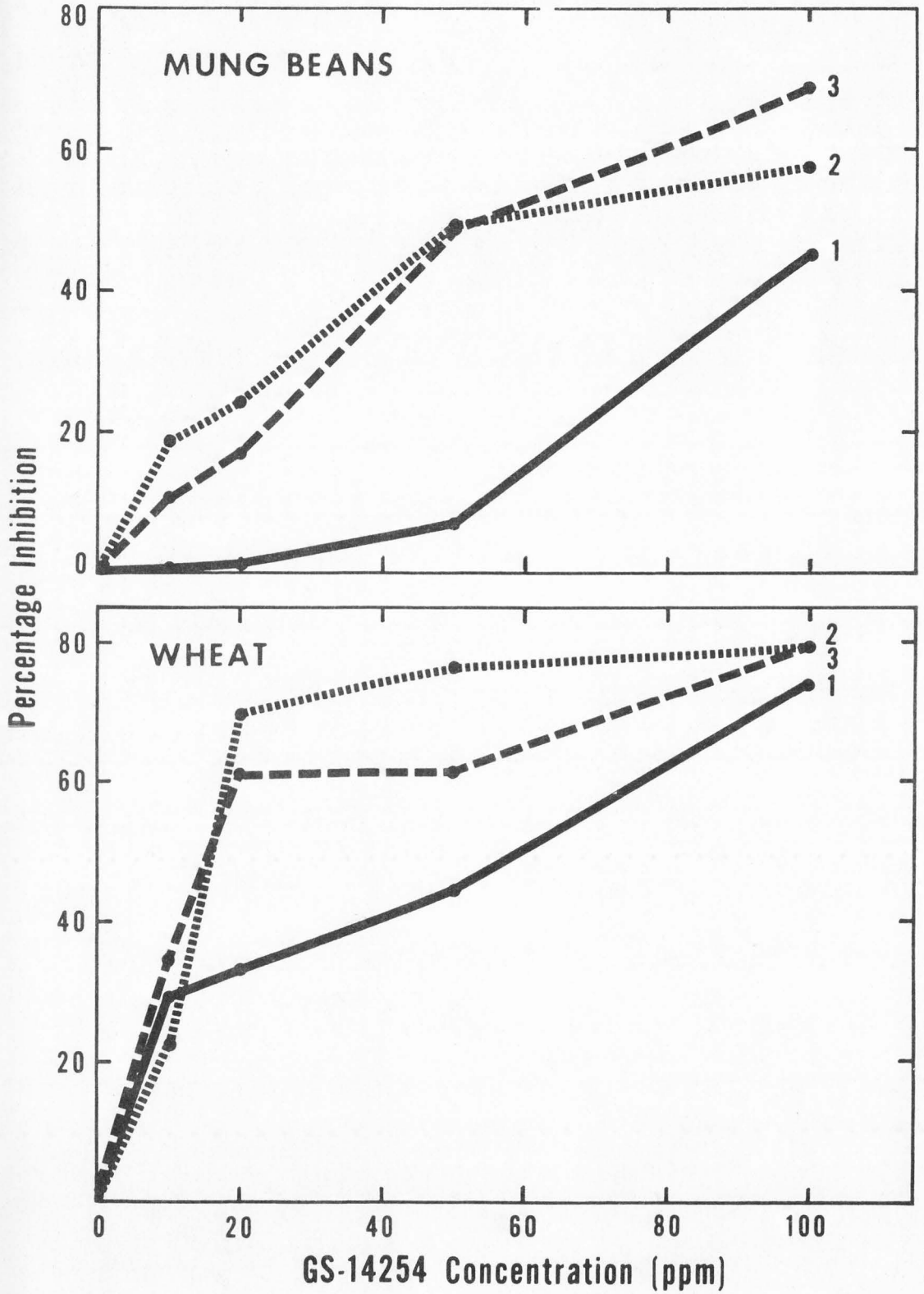


Figure 10. Effect of GS-14254 on percentage inhibition of germination (1), and root (2), and shoot (3) growth of mung bean and wheat seeds.

significant inhibition of germination and root and shoot growth in both mung bean and wheat seeds (Figure 12). The concentration at 25 mg/1 of usnic acid did not inhibit the seed germination significantly. On the other hand, alantolactone concentration at 100 mg/1 inhibited seed germination and seedling growth nearly in an identical manner in both species (Figure 11). The growth of mung bean and wheat seeds was almost completely inhibited at the highest concentrations of usnic acid and alantolactone. A slight radicle growth was seen after 24 h, perhaps primarily by cell expansion. Later on, however, no further growth was visible.

Reversal of growth inhibition

The ability of gibberellic acid to counteract the inhibitory effect of the pesticide chemicals and allergenic compounds was studied. However, only a partial reversal of the inhibitory effects was noticed in all cases (Table 3).

Measurement of respiration

At their maximum concentrations, menazon, disulfoton, and GS-14254 significantly blocked the respiration of the germinating seeds at the end of 72 h after treatment (Tables 4, 5, and 6). However, it is interesting to note that the respiration of germinating wheat seeds was considerably more affected than that of the mung beans in all cases (Figures 13, 14 and 15).

Alantolactone as well as usnic acid inhibited the rate of respiration of the germinating seeds of mung bean and wheat (Tables 7 and 8). It is

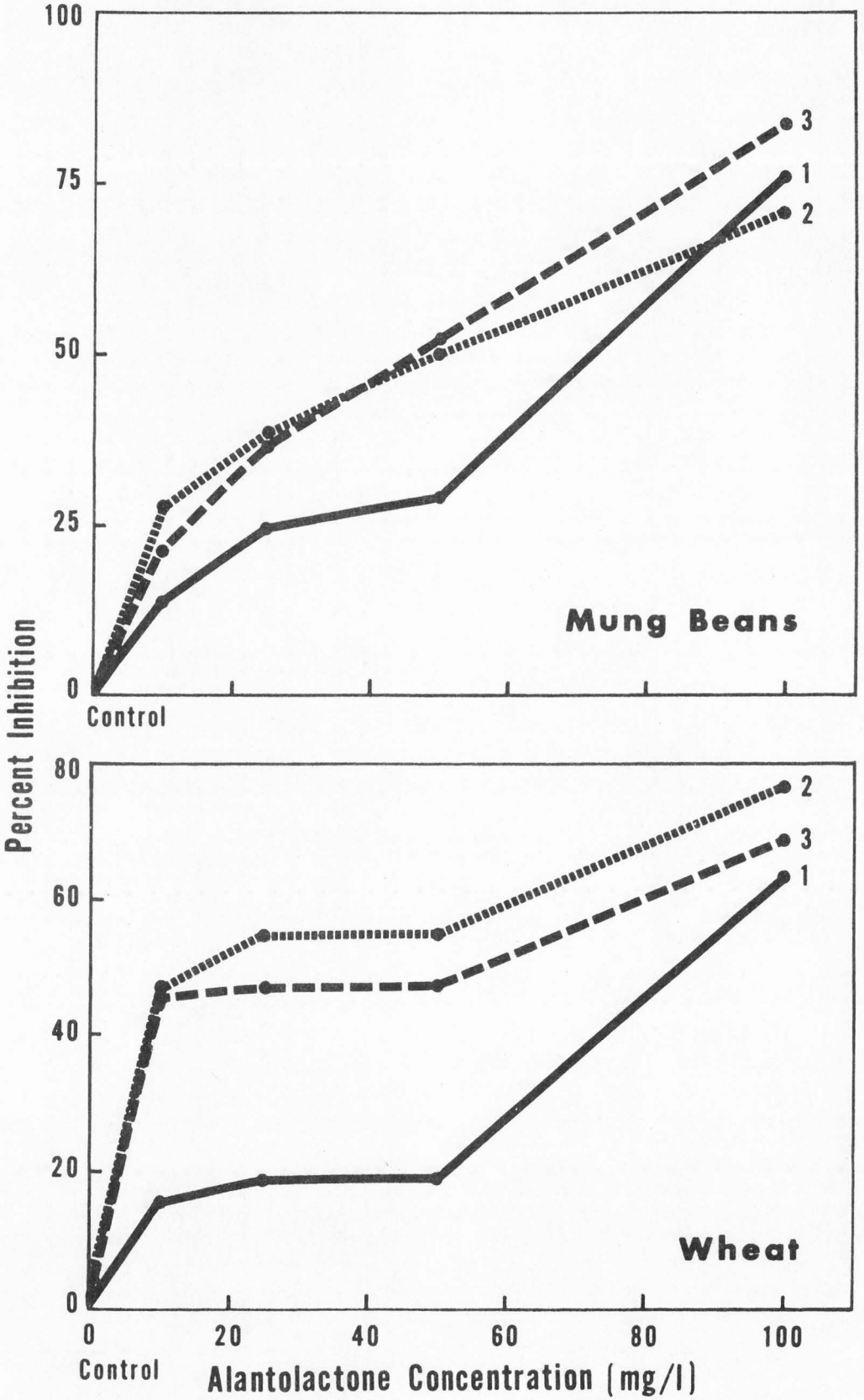


Figure 11. Effect of alantolactone on percentage inhibition of germination (1), and root (2) and shoot (3) growth of mung bean and wheat seeds.

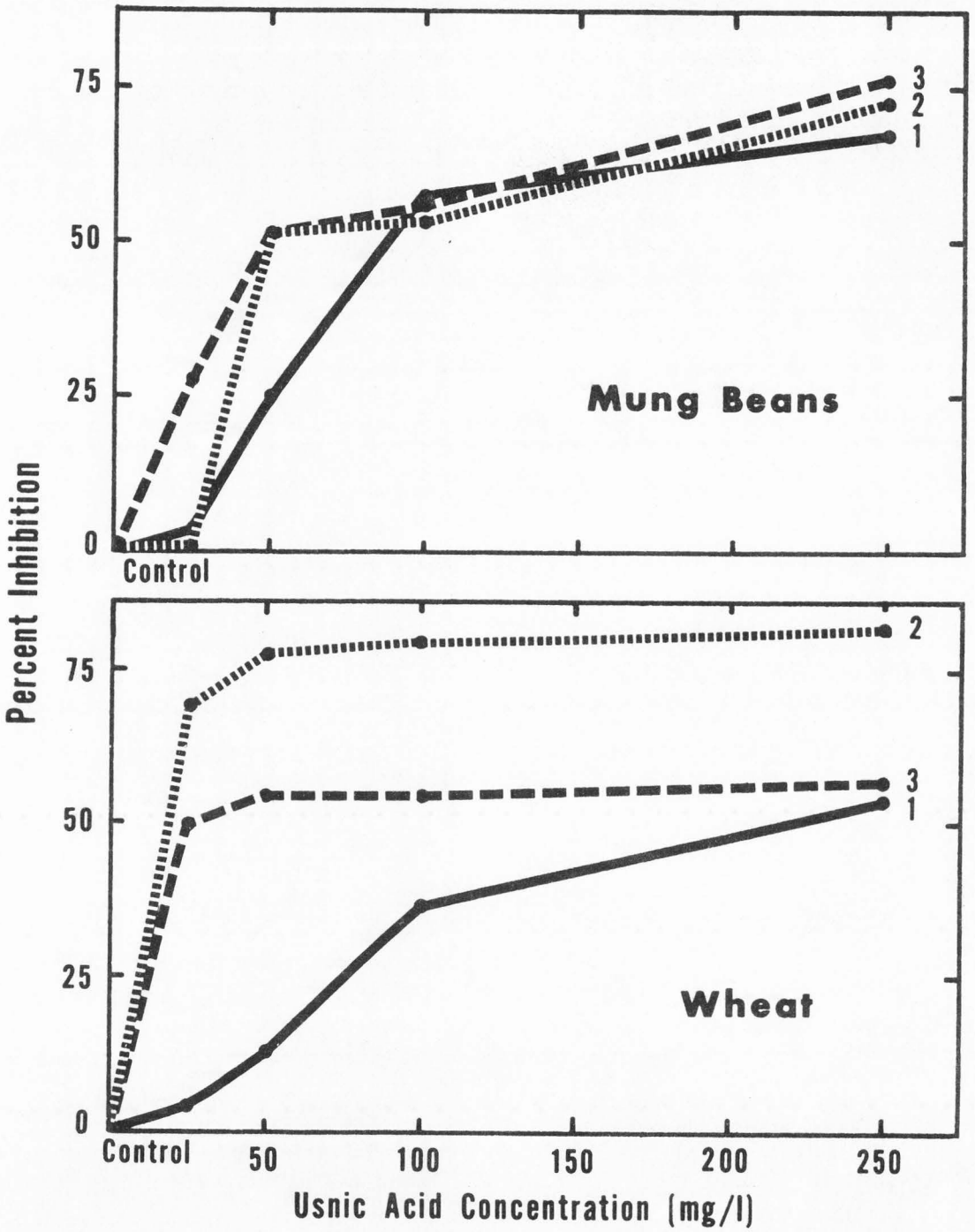


Figure 12. Effect of usnic acid on percentage inhibition of germination (1), and root (2) and shoot (3) growth of mung bean and wheat seeds.

Table 3. Effect of gibberellic acid (10^{-5}M) on the reversal of inhibition of seedling growth by the pesticides and allergens

Treatment	Root Growth cm		Shoot Growth cm	
	Mung beans	Wheat	Mung beans	Wheat
Menazon (250 ppm)				
Control	3.91	1.00	9.03	1.90
Menazon	1.46	0.33	2.21	0.59
Menazon + GA_3	1.70	0.35	3.43	0.84
Disulfoton (100 ppm)				
Control	4.30	5.10	7.70	5.11
Disulfoton	3.93	1.46	4.30	1.75
Disulfoton + GA_3	4.10	1.56	5.76	3.06
GS-14254 (100 ppm)				
Control	4.80	1.72	6.70	3.73
GS-14254	2.63	0.41	2.63	0.66
GS-14254 + GA_3	2.53	0.55	3.00	1.76
Alantolactone (100 ppm)				
Control	1.80	3.10	3.80	2.98
Alantolactone	0.34	1.16	0.80	0.73
Alantolactone + GA_3	0.39	1.25	0.97	2.02
Usnic acid (250 ppm)				
Control	2.60	1.95	4.20	3.10
Usnic acid	1.06	0.90	1.38	1.55
Usnic acid + GA_3	1.50	1.11	2.10	1.95

Table 4. Respiration of the control and menazon-treated mung bean and wheat seeds during germination

Menazon (ppm)	Oxygen uptake (μl/g hr)		
	24 h	48 h	72 h
	Mung beans		
0	549.03	1741.41	2483.30
50	590.15	1162.92**	2251.63*
100	489.62*	1083.23**	1761.53**
200	466.19*	1012.72**	1512.31**
250	455.09*	955.52**	929.19**
S.E.[a]	±23.51	±81.23	±77.66
	Wheat		
0	124.25	718.57	1758.01
50	119.67	559.61*	634.16**
100	118.97	415.15**	435.97**
200	133.69	272.48**	450.12**
250	117.23	242.52**	233.35**
S.E.	±7.29	±43.59	±77.95

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 23.

Table 5. Respiration of the control and disulfoton-treated mung bean and wheat seeds during germination

Disulfoton (ppm)	Oxygen uptake (μl/g hr)		
	24 h	48 h	72 h
	Mung beans		
0	335.28	1831.53	2278.97
20	307.14	1381.89*	2056.29
40	284.76	1357.87*	1999.31
80	280.27	1418.64*	1912.18
100	284.16	1398.04*	1497.52**
S.E.[a]	±27.84	±115.38	±133.50
	Wheat		
0	103.24	357.07	1323.14
20	113.67	263.99	768.05**
40	132.42	300.00	691.83**
80	136.56	301.68	631.59**
100	103.78	234.22*	535.28**
S.E.	±5.81	±29.45	±70.04

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 24.

Table 6. Respiration of the control and GS-14254-treated mung bean and wheat seeds during germination

GS-14254 (ppm)	Oxygen uptake (µl/g hr)		
	24 h	48 h	72 h
	Mung beans		
0	591.17	1748.88	2720.00
10	531.63	1607.14**	2265.75**
20	549.73	1137.05**	1937.44**
50	535.10	981.66**	1711.63**
100	459.11	975.57**	1246.67**
S.E.[a]	±36.67	±33.70	±56.64
	Wheat		
0	72.98	497.48	1224.32
10	90.41	244.49**	590.62**
20	97.35	235.83**	488.35**
50	91.98	260.19**	401.65**
100	74.75	226.07**	403.78**
S.E.	±9.61	±35.05	±29.11

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 25.

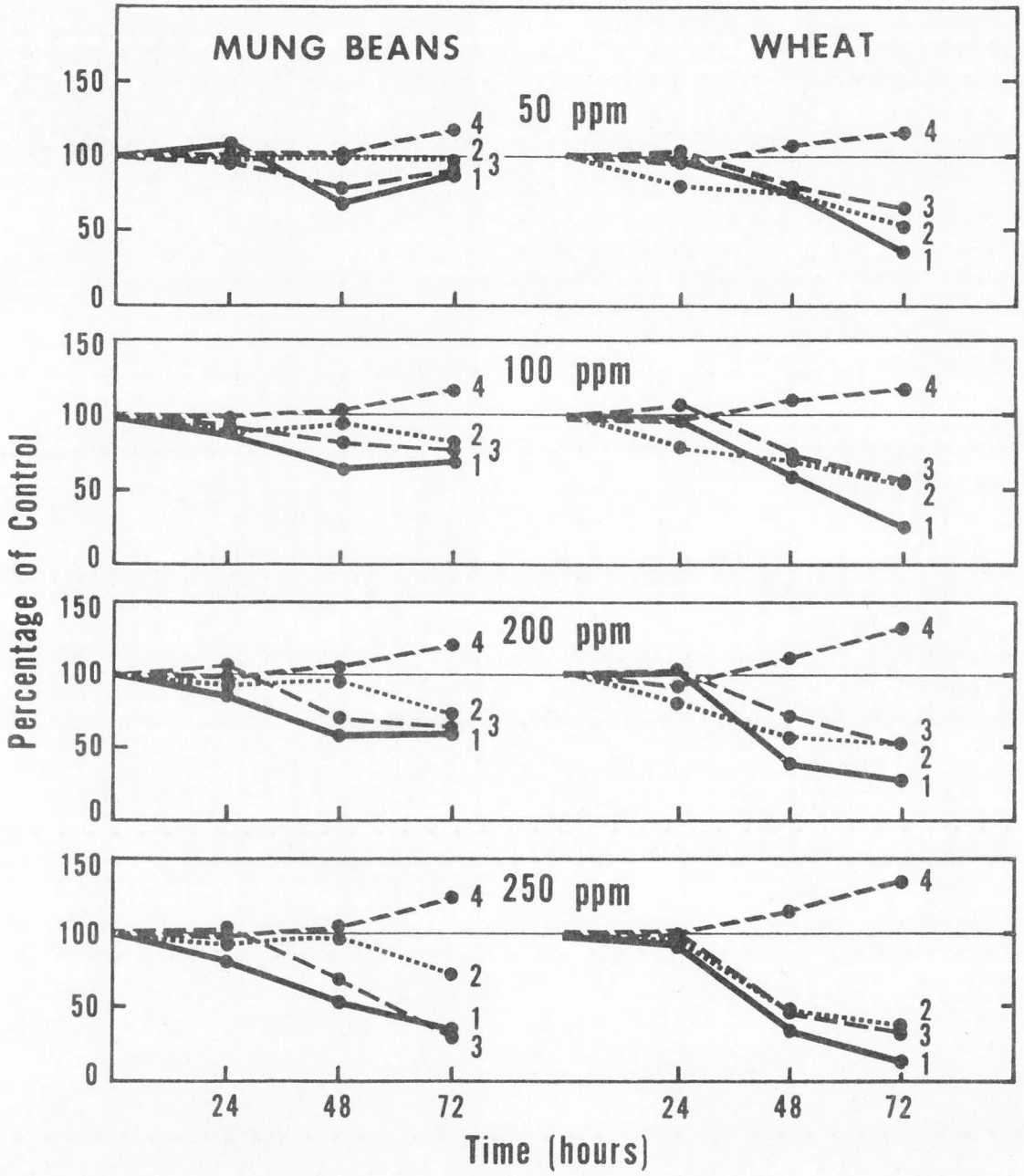


Figure 13. Effect of menazon on respiration (1), and content of free amino acids (2), reducing sugars (3), and starch (4) of the germinating seeds of mung bean and wheat.

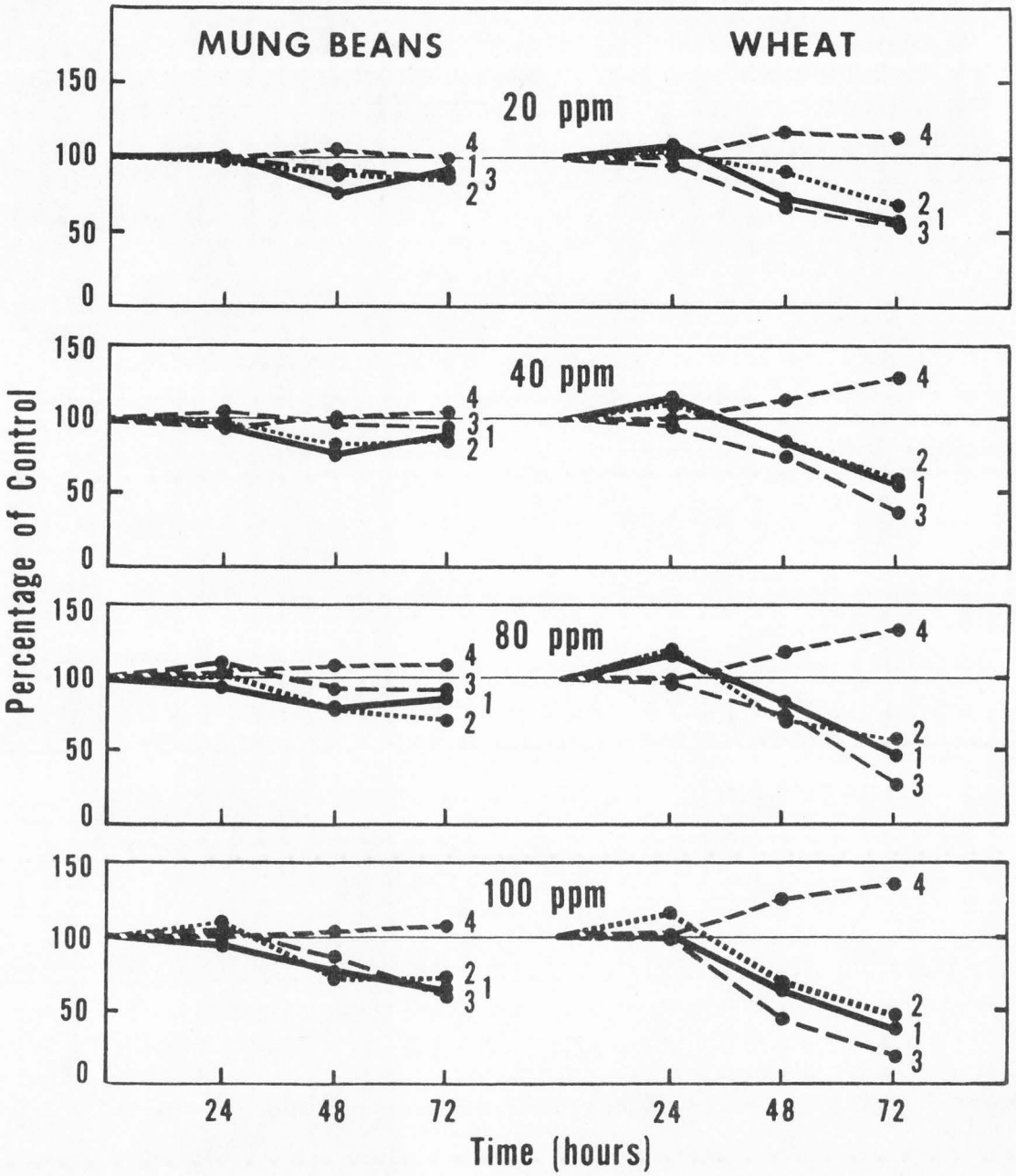


Figure 14. Effect of disulfoton on respiration (1), and content of free amino acids (2), reducing sugars (3), and starch (4) of the germinating seeds of mung bean and wheat.

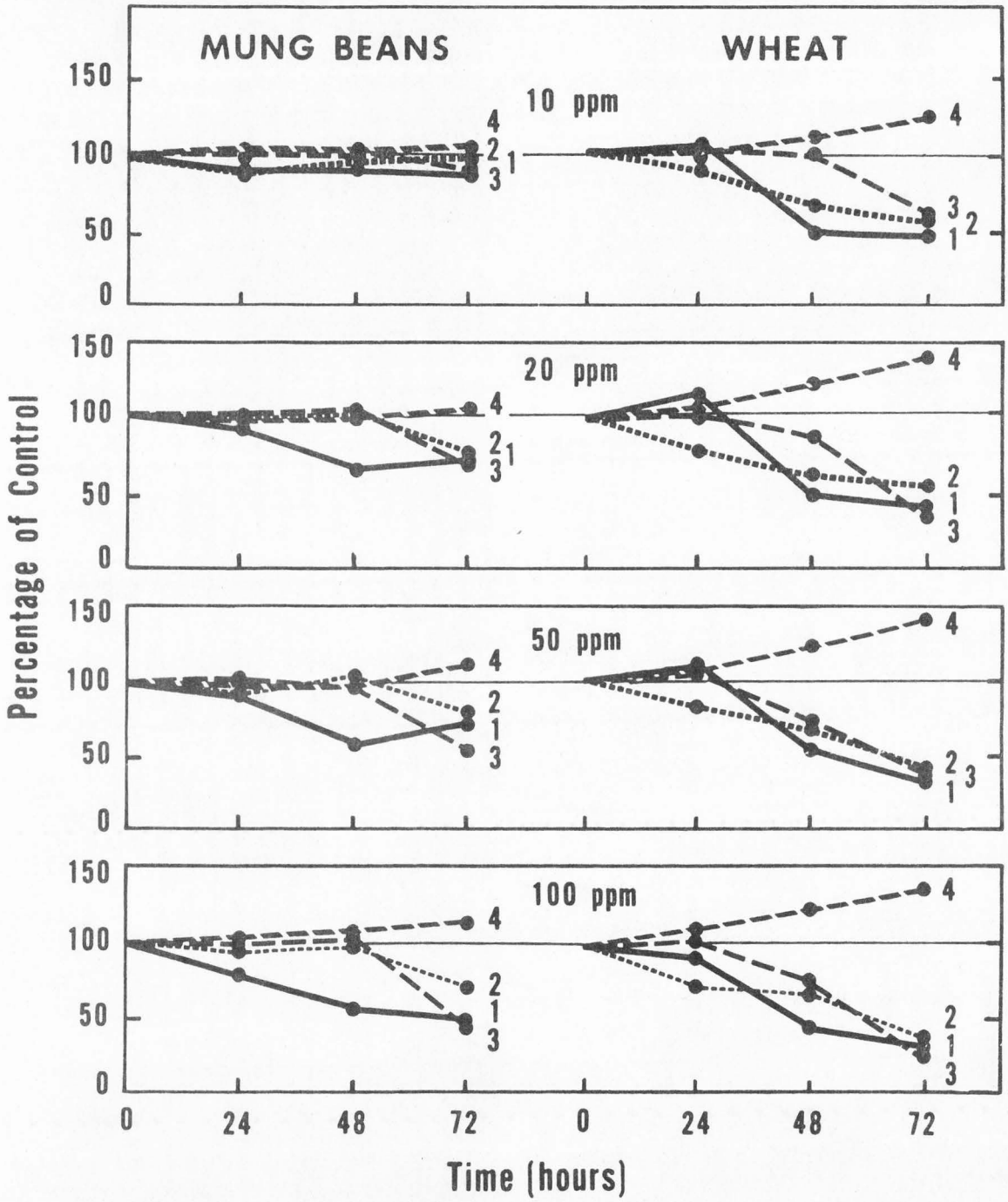


Figure 15. Effect of GS-14254 on respiration (1), and content of free amino acids (2), reducing sugars (3), and starch (4) of the germinating seeds of mung bean and wheat.

Table 7. Respiration of the control and alantolactone-treated mung bean and wheat seeds during germination

Alantolactone (mg/l)	Oxygen uptake (μl/g hr)		
	24 h	48 h	72 h
	Mung beans		
0	417.08	1490.73	2464.42
10	429.40	1448.03	2191.26**
25	411.26	1063.91**	1997.35**
50	407.40	729.78**	1665.29**
100	396.12	456.30**	791.29**
S.E.[a]	±8.63	±66.41	±28.34
	Wheat		
0	137.87	363.00	1537.27
10	131.63	194.73*	1240.60**
25	145.02	206.77*	1131.96**
50	144.99	222.97*	1072.34**
100	129.08	172.20*	493.07**
S.E.	±14.70	±34.70	±42.25

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 26.

Table 8. Respiration of the control and usnic acid-treated mung bean and wheat seeds during germination

Usnic acid (mg/l)	Oxygen uptake (µl/g hr)		
	24 h	48 h	72 h
	Mung beans		
0	609.66	1518.71	2785.53
25	521.72	1065.80**	1726.98**
50	616.76	899.61**	1332.05**
100	506.09	900.33**	1178.05**
250	424.59*	774.08**	1098.99**
S.E.[a]	±36.66	±59.19	±53.34
	Wheat		
0	72.31	699.13	2012.13
25	62.51	230.90**	996.05**
50	63.57	243.90**	726.79**
100	64.93	192.54**	765.66**
250	65.72	255.54**	430.82**
S.E.	±3.34	±38.55	±34.44

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 27.

apparent from the Figures 16 and 17 that the effects were more pronounced between 48 and 72 h after the treatment than at 24 h.

Changes in chemical composition of the seeds

Compared to the control seeds, the pesticide chemicals in the treated seeds caused significant reductions in the amounts of soluble sugars and amino acids after 72 h of germination period (Tables 9, 10 and 11). It is also seen from the data that the rate of starch degradation in seeds treated with the pesticides was significantly lower than in the controls. Figures 13, 14 and 15 indicate that the rate of starch degradation was diminished as the concentration of the chemicals increased. Consequently with time, content of reducing sugars was more in the controls than in the treated seeds. Similarly, free amino acid content was lower in the treated than in the untreated seeds. However, among the species of seeds, considerably less amounts of reducing sugars and amino acids were formed in the treated wheat seeds than in the mung beans as compared to their respective controls.

A quantitative analysis was similarly made for reducing sugars, free amino acids, and starch in the control and the alantolactone-and usnic acid-treated seeds at various stages of germination. The results (Tables 12 and 13) showed a significant reduction in the amount of sugars and amino acids at 72 h after the treatment with maximum concentration of these compounds. Also, the rate of starch degradation in the treated seeds was significantly lower than that in the controls (Figures 16 and 17). Nevertheless, like

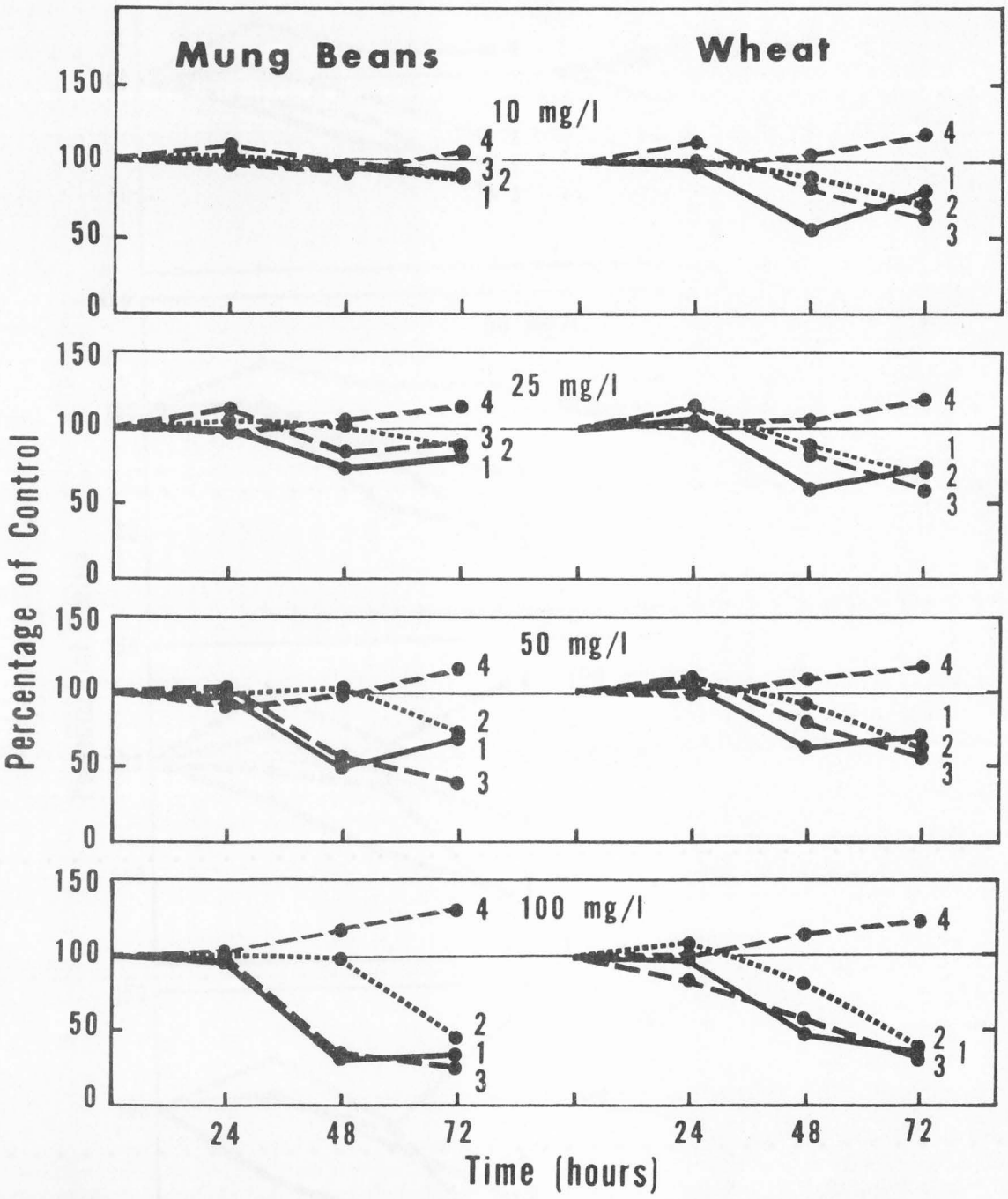


Figure 16. Effect of alantolactone on respiration (1), and content of free amino acids (2), reducing sugars (3), and starch (4) of the germinating seeds of mung bean and wheat.

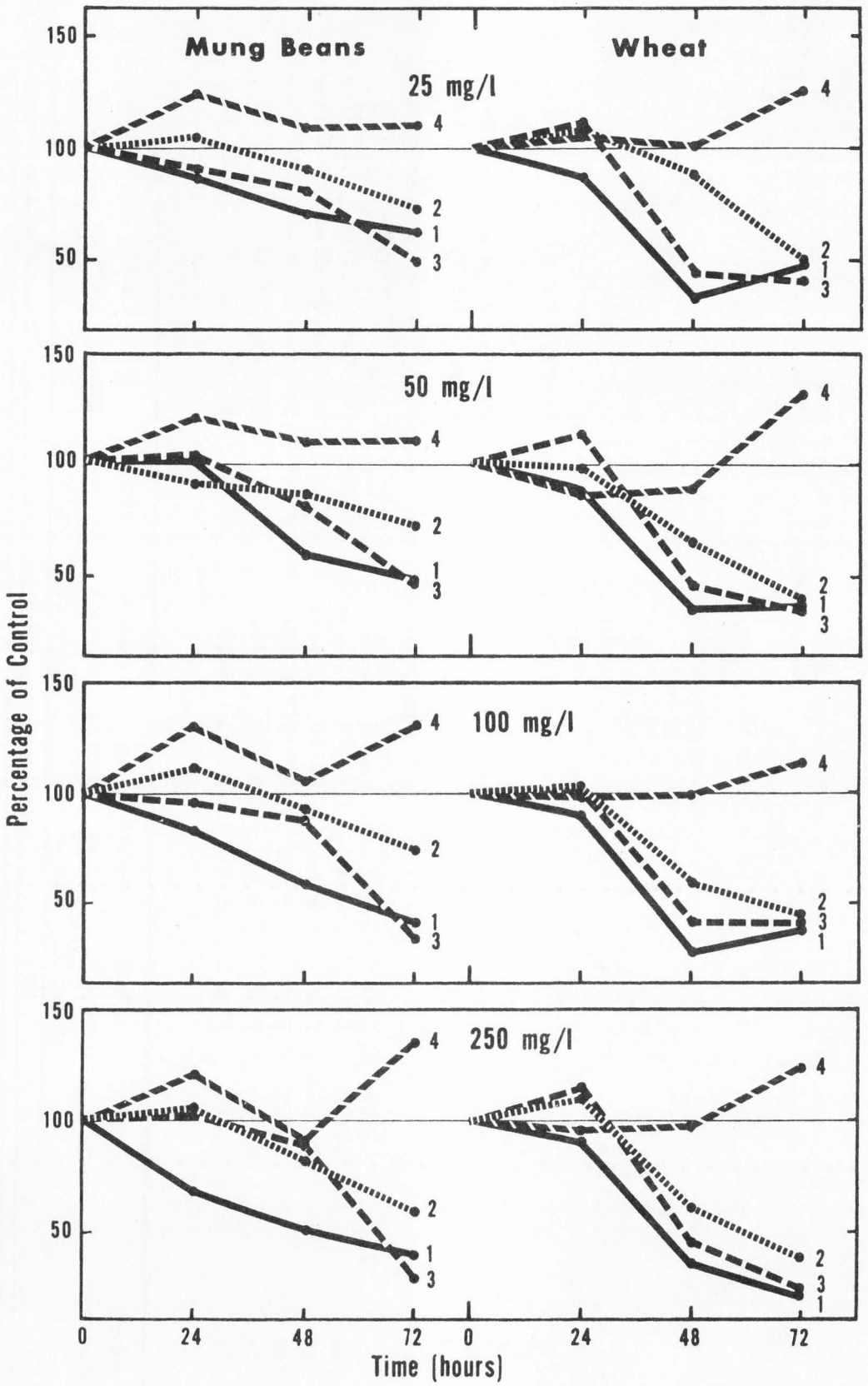


Figure 17. Effect of usnic acid on respiration (1), and content of free amino acids (2), reducing sugars (3), and starch (4) of the germinating seeds of mung bean and wheat.

Table 9. Starch, reducing sugars, and amino acid content of menazon treated and untreated mung bean and wheat seeds during germination

Menazon (ppm)		24 h			48 h			72 h		
		Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g
					Mung beans					
0		38.96	1.66	9.12	35.57	9.66	16.39	22.65	38.52	44.17
50		37.38	1.59	8.63	35.59	7.46*	16.31	26.90**	33.79**	42.20
100		37.12*	1.56	8.23*	36.72	8.04*	15.89	26.18**	30.14**	35.72**
200		37.14*	1.79	8.41	36.36	6.77**	15.64	26.24**	24.04**	32.22**
250		38.35	1.78	8.53	37.59	6.70**	16.30	29.54**	11.64**	31.64**
	S.E.[a]	±.44	±.08	±.24	±.68	±.39	±.42	±.23	±.82	±1.16
					Wheat					
0		63.47	7.30	3.43	54.09	18.34	6.81	47.62	54.96	14.58
50		58.99**	7.56	2.74**	57.08	12.64**	5.16**	55.31**	35.82**	7.66**
100		58.77**	7.92	2.90*	59.33*	13.59**	4.70**	55.61**	30.31**	7.98**
200		56.59**	7.54	2.73**	59.75*	13.05**	3.79**	63.21**	28.16**	7.71**
250		61.09*	6.63	3.16*	61.80**	8.65**	3.26**	63.18**	19.74**	5.44**
	S.E.	±.56	±.18	±.10	±1.26	±.71	±.28	±1.11	±1.48	±.25

*Significantly different at 0.05 level.

**Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 28.

Table 10. Starch, reducing sugars, and amino acid content of disulfoton-treated and untreated mung bean and wheat seeds during germination

Disulfoton (ppm)	24 h			48 h			72 h		
	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g
				Mung beans					
0	39.39	1.65	6.84	36.69	8.33	16.63	33.32	33.69	36.94
20	37.73	1.65	6.63	38.27	7.54	14.59	32.48	30.34*	31.93*
40	35.19	1.70	6.54	36.21	7.97	12.70*	34.06*	30.28*	31.00*
80	39.72	1.80	6.70	36.61	7.39	12.76*	35.33**	29.84**	25.72**
100	38.98	1.73	7.80	37.50	7.12*	11.81**	34.45**	22.08**	26.98**
S.E.[a]	±.61	±.05	±.25	±1.17	±.39	±.94	±.21	±.79	±1.54
				Wheat					
0	63.14	8.40	2.73	49.70	22.19	5.78	43.08	48.70	11.36
20	64.47	8.04*	2.92	59.83**	14.99**	5.41*	48.62**	25.87**	7.64
40	60.58	7.95*	2.94	55.85**	16.39**	4.78**	54.19**	17.22**	6.46
80	61.78	8.07*	3.38	58.68**	16.39**	4.08**	57.45**	12.76**	6.46
100	61.89	8.53	3.26	63.11**	9.99**	4.08**	60.35**	10.04**	5.46*
S.E.	±.84	±.01	±.03	±.64	±.22	±.13	±.45	±1.33	±.17

* Significantly different at .05 level.

** Significantly different at .01 level.

a. The detailed analysis of variance is presented in Appendix Table 29.

Table 11. Starch, reducing sugars, and amino acid content of GS-14254-treated and untreated mung bean and wheat seeds during germination

GS-14254 (ppm)	24 h			48 h			72 h		
	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g
				Mung beans					
0	38.02	1.05	9.99	33.42	7.25	15.63	25.22	30.26	31.56
10	39.61	1.04	8.78	34.74	7. 55	15.59	26. 62	24.33**	30.99
25	37.17	1.07	9.61	32.63	7.60	15.66	26.47	20.68**	24.22**
50	37.98	1.60	9.07	32.05	7.07	16.09	27.94**	15.87**	24.02**
100	39.42	1.24	8.44	35.32	7.51	15.52	28.32	13.49**	19.73**
S.E.[a]	±.11	±.01	±.60	±1.14	±.17	±.66	±.43	±.28	±1.02
				Wheat					
0	57.29	7.91	2.02	51.47	12.55	6.33	44.98	48.79	11.40
10	56.09	8.12	1.82	59.05*	13.14	4.21**	56.94**	34.74**	6.44**
25	59.86	8.26	1.53*	62.95**	10.98**	3.93**	63.00**	21.71**	6.29**
50	60.69	8.48	1.69	63.52**	9.19**	4.27**	63.61**	22.59**	4.47**
100	61.91	8.99	1.43*	63.23**	9.51**	4.20**	60.57**	14.76**	4.29**
S.E.	±1.35	±.19	±.14	±1.69	±.21	±.19	±.67	±.51	±.21

*Significantly different at 0.05 level.

**Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 30.

Table 12. Starch, reducing sugars, and amino acid content of alantolactone-treated and untreated mung bean and wheat seeds during germination

Alantolactone (mg/l)	24 h			48 h			72 h		
	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g
					Mung beans				
0	39.06	1.93	9.11	32.56	7.53	12.20	28.90	36.12	48.23
10	35.50*	2.16	9.51	30.84	6.84*	11.68	30.20	33.36**	43.31
25	36.16*	2.21	9.66*	33.04	6.33**	12.44	32.60**	31.86**	40.49**
50	34.88*	1.98	8.95**	31.88	4.20**	13.03	33.28**	14.04**	35.55**
100	36.24*	1.97	8.84**	37.40**	3.34**	11.86	37.34**	9.00**	21.50**
S.E.[a]	± .62	±.14	±.36	±.63	±.20	±.46	±.58	±.90	±1.87
					Wheat				
0	60.86	5.62	2.69	58.14	24.00	4.94	46.18	80.00	14.46
10	59.37	6.42	2.76	60.49**	19.59**	4.41	54.29**	29.17**	10.21**
25	62.38*	6.32	3.32**	60.79**	19.53**	4.26	54.79**	47.06**	10.31**
50	59.63	5.78	2.94	63.12**	19.11**	4.52	53.60**	44.46**	8.99**
100	60.51	4.59	2.95	65.21**	13.76**	3.99	56.02**	25.34**	5.59
S.E.	±4.7	±.48	±.10	±3.81	±.59	±.47	±1.20	±1.16	±.42

*Significantly different at 0.05 level.

**Significantly different at 0.01 level.

a. The detailed analysis of variance is presented in Appendix Table 31.

Table 13. Starch, reducing sugars, and amino acid content of usnic acid-treated and untreated mung bean and wheat seeds during germination

Usnic acid (mg/l)	24 h			48 h			72 h		
	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g	Starch %	Reducing sugars mg/g	Amino acids mg/g
				Mung beans					
0	34.7	1.9	7.8	25.5	7.8	16.2	20.1	36.1	34.6
25	34.6	1.8	8.1	27.8	6.4**	14.6	22.5**	17.6**	25.2**
50	33.6	2.0	7.1	28.0	6.4**	14.0	22.1**	16.7**	25.4**
100	36.0	1.9	8.7	26.5	6.9**	15.1	26.0**	12.4**	25.6**
250	33.6	2.0	8.2	23.0	6.9**	13.3	27.2**	10.2**	20.6**
S.E.[a]	±3.5	±.05	±.41	±.89	±.10	±2.41	±1.09	±.85	±1.37
				Wheat					
0	49.4	5.5	2.9	49.5	33.5	10.6	38.1	74.6	17.1
25	52.1*	6.2	3.1	49.5	15.0**	9.2	49.4**	31.9**	8.0**
50	45.0*	6.3	2.9	42.8**	15.6**	6.9**	50.6**	25.9**	6.6**
100	48.0*	5.7	2.9	49.5	14.0**	6.2**	42.8**	30.9**	7.4**
250	47.3*	6.4	3.2	48.9	15.3**	6.6**	47.4**	17.3**	6.6**
S.E.	±6.47	±.19	±.08	±5.26	±.73	±.49	±1.37	±1.95	±.32

* Significantly different at 0.05 level.

** Significantly different at 0.01 level.

[a]. The detailed analysis of variance is presented in Appendix Table 32.

pesticides there was no difference in the inhibitory effects on the chemical composition of the germinating mung beans and wheat seeds treated with these allergenic compounds.

Inhibitory effect on enzymes development

The effects of menazon, disulfoton, and GS-14254 on amylase, ATPase, and protease development in the germinating seeds are illustrated in Figures 18, 19, and 20 respectively. It is evident that the development of amylase activity in the seeds treated with the maximum concentrations of the pesticides tended to be lower than that in the control seeds (Tables 14, 15, and 16). At higher concentrations the enzyme activity was lower. It was observed that the adverse effect of menazon on the development of amylase activity was more pronounced than that of disulfoton or GS-14254.

As germination progressed after 24 h, it was observed that the increase in ATPase activity was significantly lower in the treated seeds than in the untreated controls at the maximum concentrations of the pesticides. In contrast, the development of proteolytic activity in control and disulfoton-and menazon-treated seeds was not significantly different during the germination period. However, in the seeds treated with GS-14254 it was considerably lower at 72 h germination period.

It is apparent from Figure 22, Table 18, that the development of amylase activity in the control seeds increased rapidly during 24 to 96 h germination period. In contrast, amylase activity in the mung beans treated with the maximum concentration of usnic acid remained almost unchanged over the

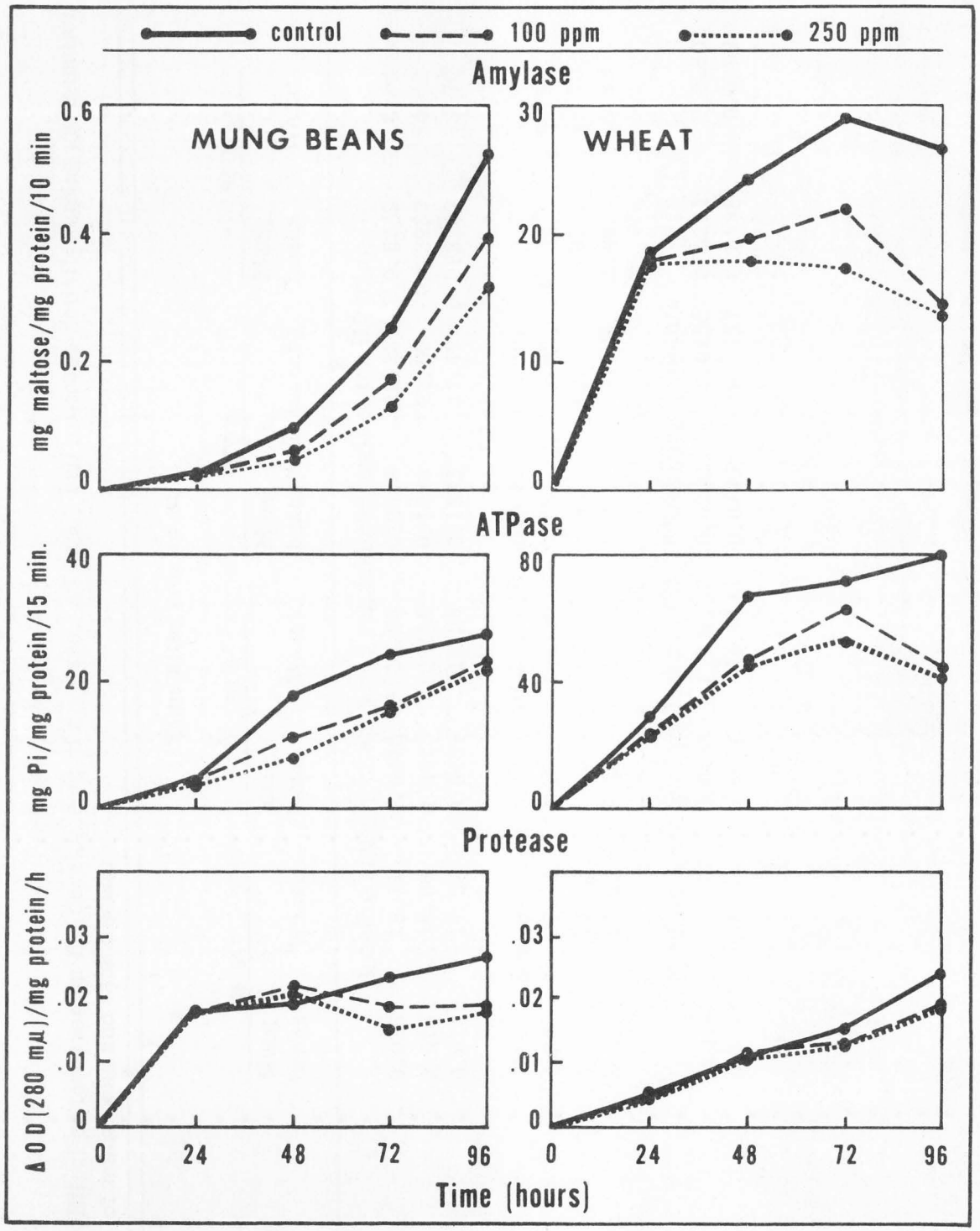


Figure 18. Effect of menazon on the amylase, ATPase, and protease activities during germination of mung bean and wheat seeds.

Table 14. Effect of menazon on the development of amylase, ATPase, and protease activity during germination of mung bean and wheat seeds

Treatment (ppm)	Time after treatment: 24 h		48 h		72 h		96 h	
	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
	Specific activity of amylase, mg maltose/mg protein/10 min.							
Control	0.0267	18.34	0.0948	24.27	0.2528	29.33	0.5259	26.33
100	0.0241	18.06	0.0615	19.56	0.1738	22.02	0.3987	14.43
250	0.0206	17.54	0.0455	15.28	0.1333	17.49	0.3262	13.70
	Specific activity of ATPase, μg Pi/mg protein/15 min.							
Control	4.60	27.13	17.55	64.60	23.99	71.50	27.64	80.84
100	4.51	21.83	11.30	46.84	16.05	62.58	23.91	43.70
250	3.55	22.23	7.88	44.86	15.39	53.17	22.48	40.92
	Specific activity of protease, OD (280 mμ)/mg protein/h							
Control	0.0186	0.0051	0.0188	0.0103	0.0232	0.0150	0.0261	0.0240
100	0.0182	0.0040	0.0216	0.0111	0.0189	0.0137	0.0186	0.0193
250	0.0184	0.0041	0.0204	0.0100	0.0151	0.0132	0.0189	0.0187

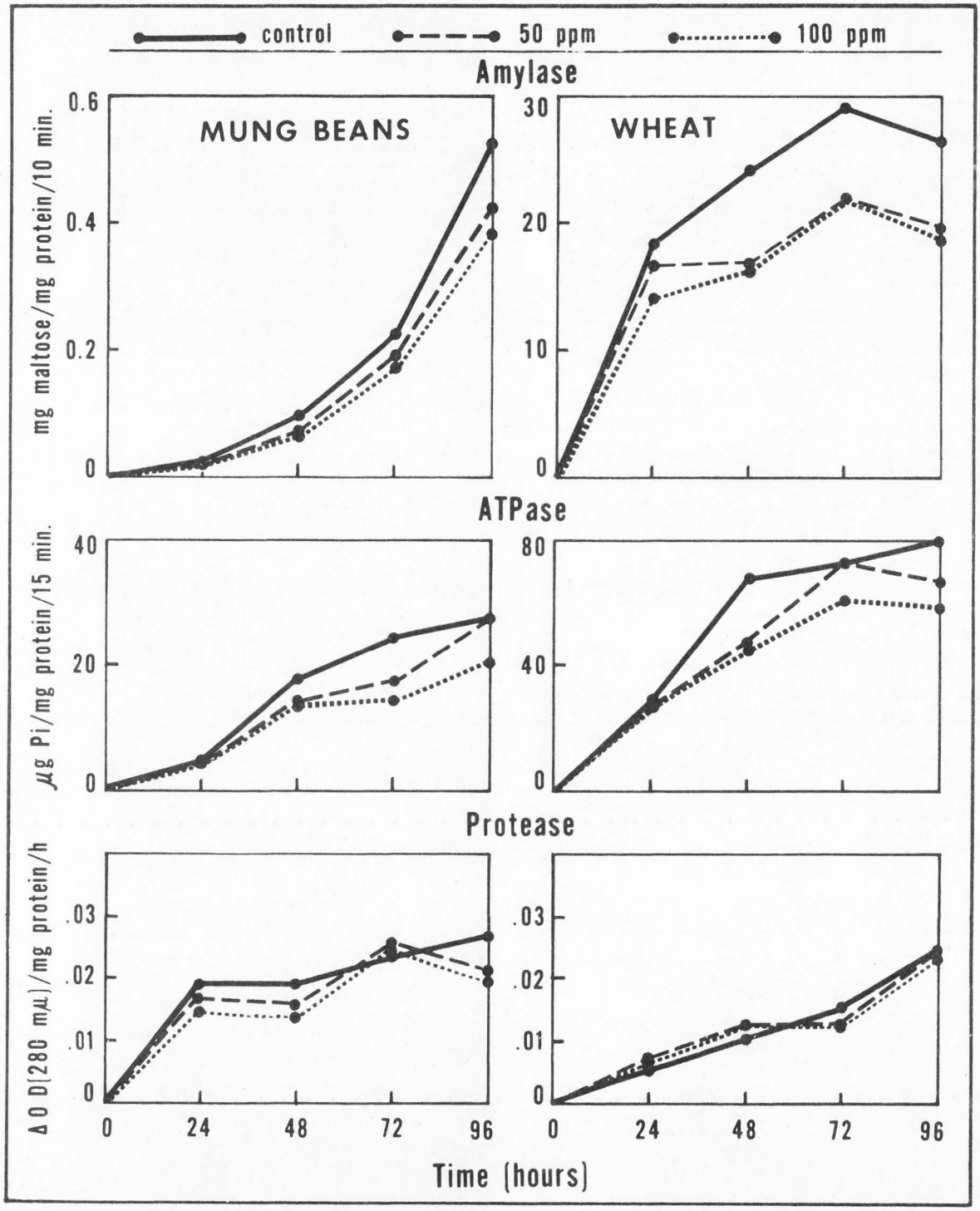


Figure 19. Effect of disulfoton on the amylase, ATPase, and protease activities during germination of mung bean and wheat seeds.

Table 15. Effect of disulfoton on the development of amylase, ATPase, and protease activity during germination of mung bean and wheat seeds

Treatment (ppm)	Time after treatment: 24 h		48 h		72 h		96 h	
	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
	Specific activity of amylase, mg maltose/mg protein/10 min.							
Control	0.0267	18.38	0.0948	24.27	0.2528	29.33	0.5259	26.33
50	0.0268	16.52	0.0814	16.42	0.1907	22.09	0.4240	19.47
100	0.0261	14.77	0.0692	16.25	0.1711	22.18	0.3824	18.73
	Specific activity of ATPase, μg Pi/mg protein/15 min.							
Control	4.60	27.13	17.55	67.60	23.99	71.50	27.64	80.84
50	4.74	26.45	13.86	46.23	17.24	71.58	27.97	65.98
100	4.12	26.47	13.20	44.71	13.99	59.93	20.31	57.71
	Specific activity of protease, OD (280 mμ)/mg protein/h							
Control	0.0186	0.0051	0.0186	0.0103	0.0233	0.0150	0.0260	0.0244
50	0.0163	0.0071	0.0151	0.0121	0.0248	0.0123	0.0209	0.0247
100	0.0146	0.0062	0.0131	0.0121	0.0242	0.0123	0.0188	0.0211

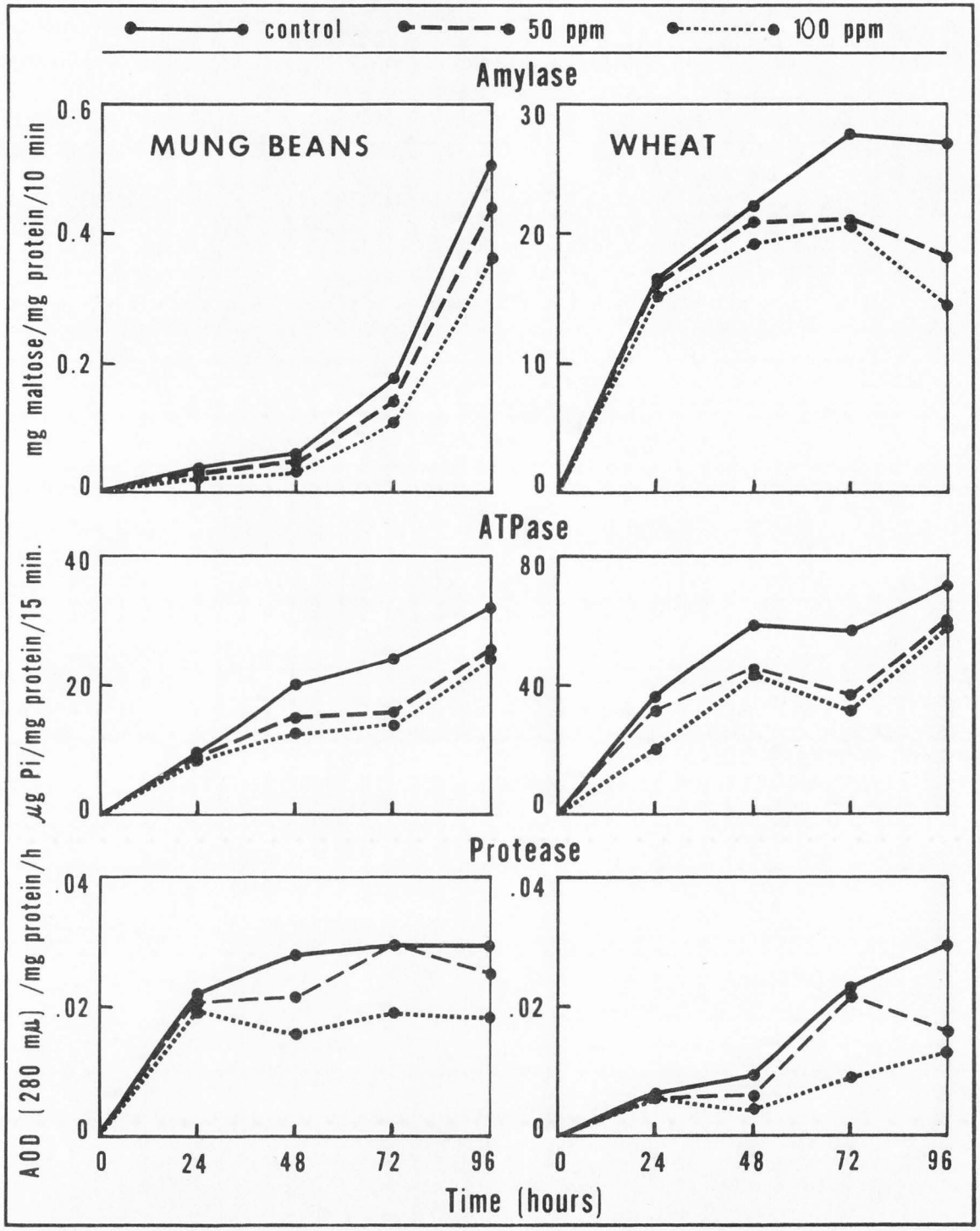


Figure 20. Effect of GS-14254 on the amylase, ATPase, and protease activities during germination of mung bean and wheat seeds.

Table 16. Effect of GS-14254 on the development of amylase, ATPase, and protease activity during germination of mung bean and wheat seeds

Treatment (ppm)	Time after treatment: 24 h		48 h		72 h		96 h	
	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
	Specific activity of amylase, mg maltose/mg protein/10 min.							
Control	0.0406	16.51	0.0550	21.94	0.1761	26.66	0.5184	25.94
50	0.0223	16.61	0.0434	20.27	0.1431	22.05	0.4394	18.17
100	0.0209	15.39	0.0350	19.06	0.1089	22.28	0.3655	14.55
	Specific activity of ATPase, μg Pi/mg protein/15 min.							
Control	9.04	36.16	20.16	59.23	24.08	56.73	32.09	71.17
50	9.12	32.58	14.96	44.94	15.36	37.00	25.76	61.42
100	9.17	20.30	12.40	45.93	13.76	32.13	25.16	60.75
	Specific activity of protease, OD (280 mμ)/mg protein/h							
Control	0.0225	0.0067	0.0280	0.0934	0.0295	0.0229	0.0291	0.0295
50	0.0206	0.0061	0.0217	0.0059	0.0308	0.0224	0.0253	0.0165
100	0.0188	0.0069	0.0154	0.0039	0.0189	0.0128	0.0184	0.0126

germination period. However, in case of alantolactone-treated mung bean seeds (Figure 21, Table 17) it was significantly lower at the highest concentration but was not severely inhibited. The amylase activity in the wheat seeds treated with usnic acid steadily declined after 48 h germination while it was sharply decreased after 72 h germination in alantolactone-treated wheat seeds. As germination progressed after 24 h it was observed that increase in ATPase activity was significantly lower at the maximum concentration of usnic acid in both species. In the seeds treated with alantolactone it was markedly lower in both species of seeds. The development of protease activity in control and alantolactone- and usnic acid-treated seeds showed more or less the same trend during the germination period.

In vitro effect of pesticides and allergenic compounds on amylase activity

The activity per se of amylase isolated from mung bean and wheat seeds germinated for 3 days was not significantly inhibited by the presence of the pesticide chemicals or allergenic compounds (Table 19) in the reaction mixture.

Uptake and incorporation of ^{14}C-L-leucine

Results of the effect of the pesticide chemicals on uptake and incorporation of radioactive L-leucine into protein of potato tissue and germinating mung beans are shown in Table 20. Observations with potato tissue and germinating mung beans indicated that both total uptake and incorporation of the

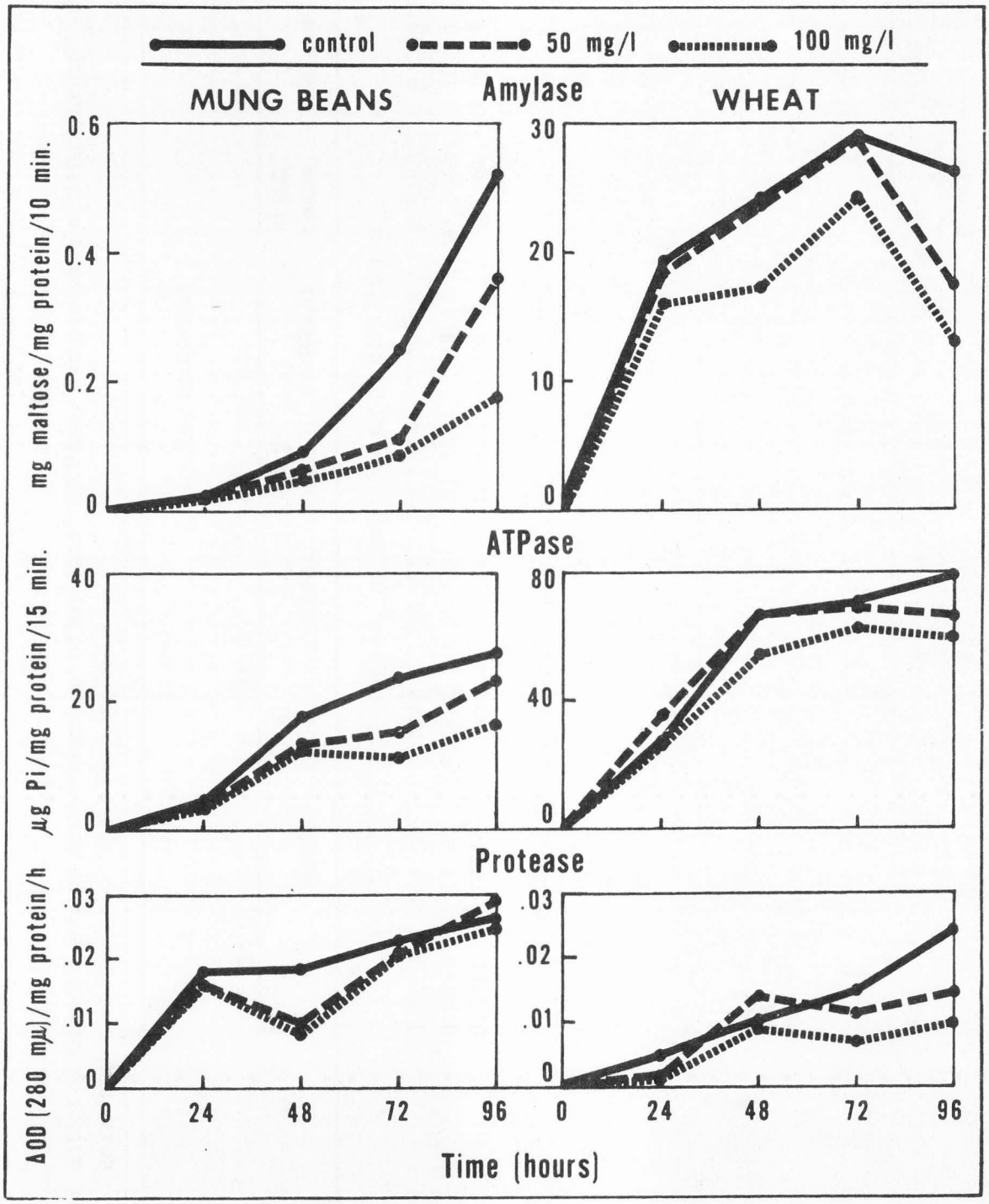


Figure 21. Effect of alantolactone on the amylase, ATPase, and protease activities during germination of mung bean and wheat seeds.

Table 17. Effect of alantolactone on the development of amylase, ATPase, and protease activity during germination of mung bean and wheat seeds

Treatment (mg/l)	Time after treatment: 24 h		48 h		72 h		96 h	
	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
	Specific activity of amylase, mg maltose/mg protein/10 min.							
Control	0.0267	18.38	0.0948	24.27	0.2528	29.33	0.5259	26.33
50	0.0249	18.66	0.0622	23.85	0.1107	29.49	0.3645	17.64
100	0.0250	16.10	0.0482	17.37	0.0845	24.65	0.1766	13.11
	Specific activity of ATPase, μg Pi/mg protein/15 min.							
Control	4.6	29.10	17.55	67.60	23.99	71.50	27.64	80.84
50	4.03	27.73	13.72	68.14	15.34	71.57	23.94	66.65
100	3.53	27.67	12.99	55.16	11.41	63.85	17.06	64.11
	Specific activity of protease, OD (280 mμ)/mg protein/h							
Control	0.0185	0.0051	0.0186	0.0103	0.0233	0.0150	0.0261	0.0244
50	0.0173	0.0015	0.0100	0.0149	0.0212	0.0155	0.0264	0.0150
100	0.0168	0.0012	0.0089	0.0099	0.0211	0.0068	0.0256	0.0104

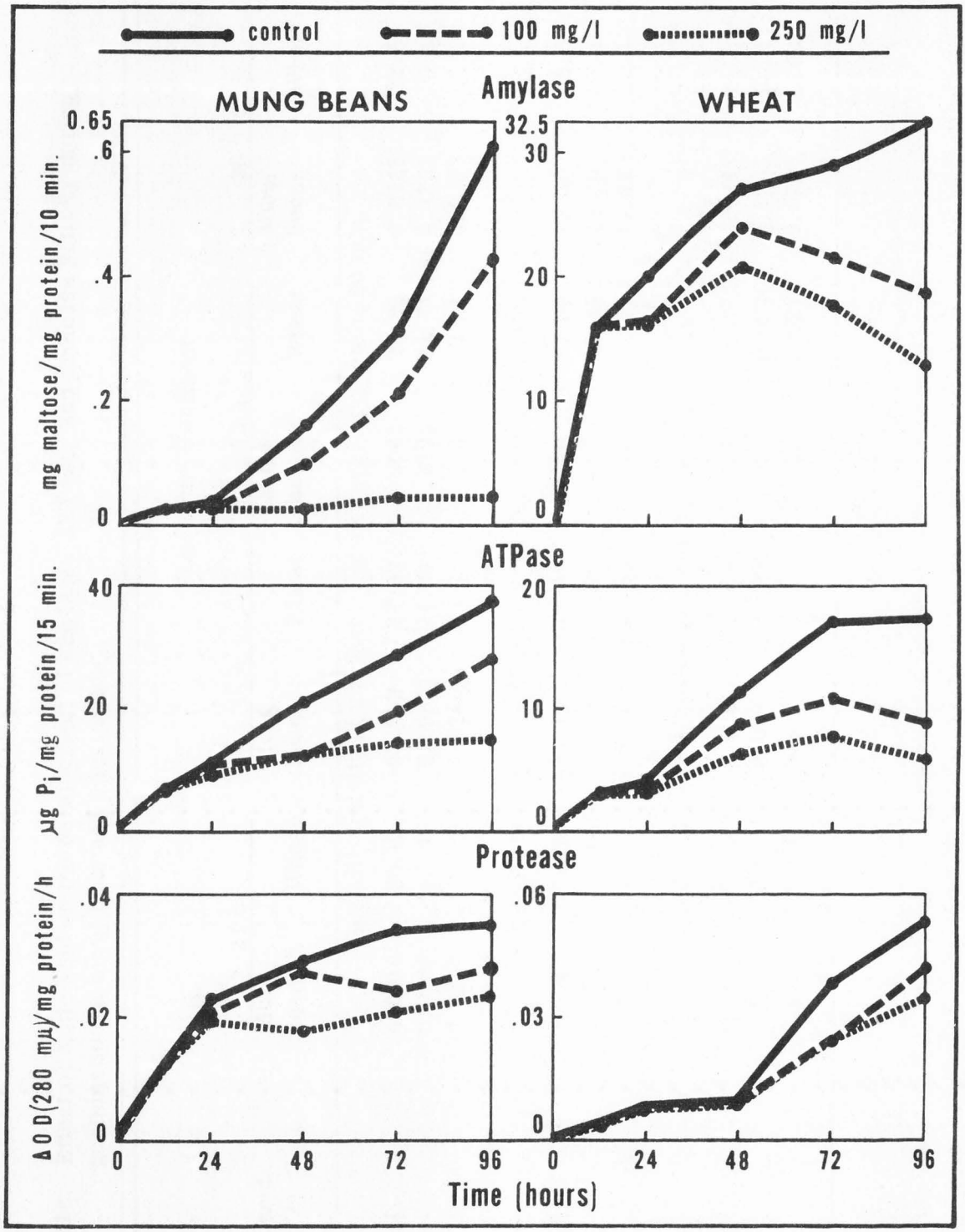


Figure 22. Effect of usnic acid on the amylase, ATPase, and protease activities during germination of mung bean and wheat seeds.

Table 18. Effect of usnic acid on the development of amylase, ATPase, and protease activity during germination of mung bean and wheat seeds

Treatment (mg/l)	Time after treatment: 24 h		48 h		72 h		96 h	
	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat	Mung beans	Wheat
	Specific activity of amylase, mg maltose/mg protein/10 min.							
Control	0.0310	20.02	0.1592	27.93	0.3082	28.84	0.6145	32.54
100	0.0296	15.62	0.0992	24.04	0.2105	21.71	0.4372	18.47
250	0.0260	15.88	0.0248	20.98	0.0407	17.70	0.0435	12.71
	Specific activity of ATPase, μg Pi/mg protein/15 min.							
Control	10.70	16.76	21.17	45.48	28.70	67.88	37.30	68.60
100	10.61	13.64	12.10	34.76	19,90	43.48	28.00	37.46
250	9.20	11.32	12.80	25.80	14.60	31.53	14.71	23.12
	Specific activity of protease, OD (280 mμ)/mg protein/h							
Control	0.0232	0.0088	0.0298	0.0090	0.0341	0.0411	0.0343	0.0538
100	0.0214	0.0070	0.0274	0.0072	0.0241	0.0276	0.0283	0.0458
250	0.0198	0.0077	0.0175	0.0093	0.0208	0.0274	0.0236	0.0325

Table 19. Amylase activity with the pesticide chemicals and allergenic compounds present in the reaction mixture

Treatment	Concentration ppm	Mung beans	Wheat
		Amylase activity (per cent of control)	
Control	--	100	100
Menazon	250	115	110
Disulfoton	100	113	97
GS-14254	100	105	93
Alantolactone	100	109	91
Usnic acid	250	124	111

Table 20. Effect of pesticides and allergenic compounds on the uptake and incorporation of ^{14}C-L-leucine into protein of white potato tissue and germinating mung beans

Treatment	Radioactivity			
	Potato discs		Mung beans	
	Alcohol-soluble (d.p.m./mg dry wt.)	Alcohol-insoluble (d.p.m./mg dry wt.)	Alcohol-soluble (d.p.m./8 seeds)	Alcohol-insoluble (d.p.m./8 seeds)
Control	2472	2459	96450	53245
Menazon	207**	133**	82900**	31935*
Disulfoton	453**	307**	88700**	34455*
GS-14254	489**	193**	86200**	32280*
Alantolactone	91**	135**	81100**	34200**
Usnic Acid	160**	108**	107849**	24228**
S.E.[a]	± 44.43	± 94.20	± 2402.00	± 1259.33

*Significantly different at 0.05 level.
**Significantly different at 0.01 level.
a. The detailed analysis of variance is presented in Appendix Table 33.

isotope were significantly inhibited by menazon, disulfoton, and GS-14254. On the other hand, uptake of ^{14}C-L-leucine in germinating mung beans treated with usnic acid was not affected although both uptake and incorporation were inhibited in potato tissue (Table 20). Table 20 shows that both uptake and incorporation of the radioactive amino acid were inhibited in the potato tissue and mung beans treated with alantolactone. It should be noted that alantolactone-treated potato tissue displayed lowest uptake of the precursor of proteins.

Histochemistry and electron microscopy

Figure 23a shows the intensity of methylene blue-stained nucleic acid material in the storage parenchyma cells of control mung bean seeds at day 3 of germination. Staining of the corresponding tissue cells from the cotyledons treated with menazon (Figure 23b) or usnic acid (Figure 23c) appears to be considerably less than did the control. It is to be noted that this tissue is not meristematic where sharp differences in the staining of nucleic acid content of the control and treated cotyledonary tissue might have been clearly visible, of course, depending on the extent of germination inhibition. However, these observations indicate that the nucleic acid content in mung beans increases as germination progresses. Secondly, menazon and usnic acid treatments of the seeds prevent such nucleic acid synthesis during germination.

The distribution of ribonucleic acid (RNA) as revealed by pyronin Y staining can be observed in the storage parenchyma cells of control

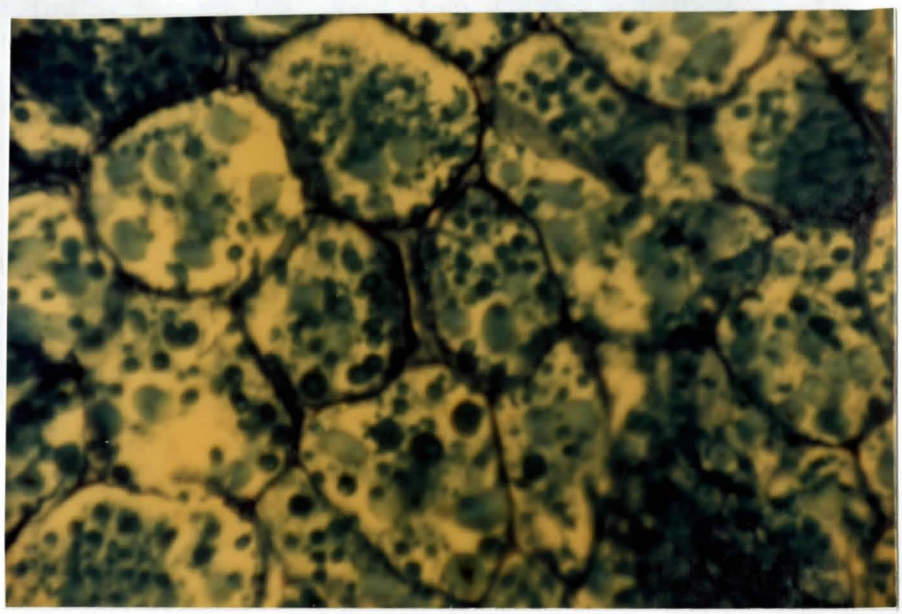
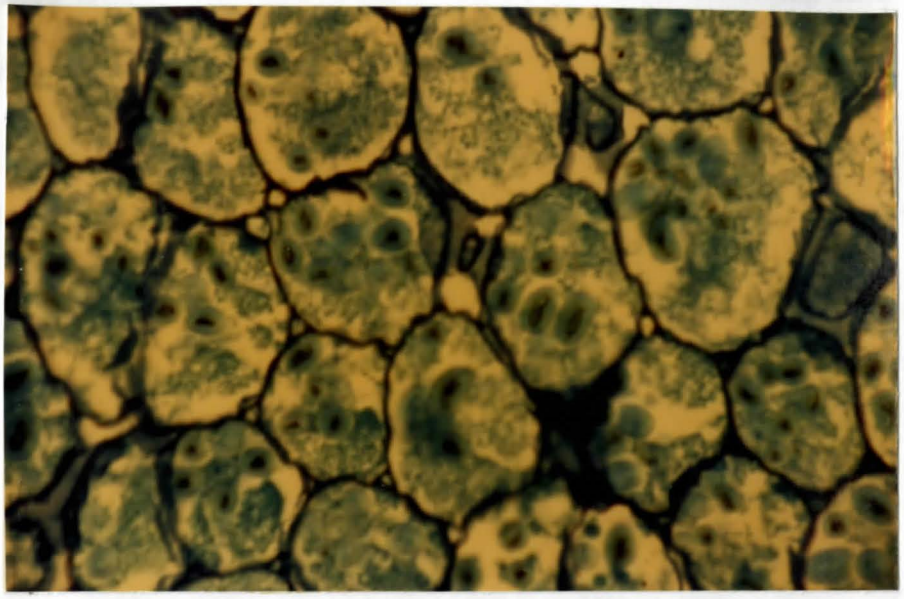


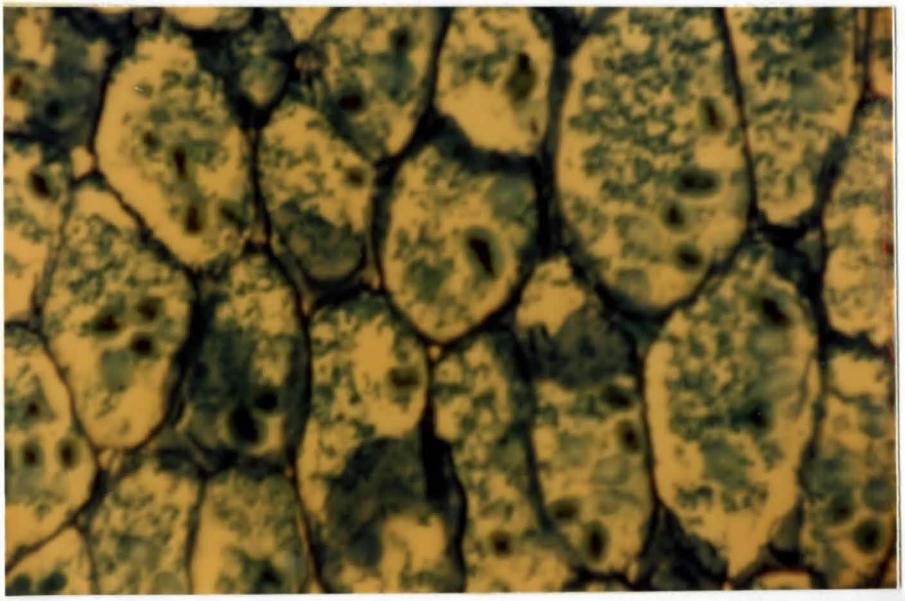
Figure 23. Photomicrographs of storage parenchyma cells of control (a), menazon-(b), and usnic acid-(c) treated mung beans at day 3 of germination showing nucleic acid content, (x 249)

a. Note the concentration of nucleic acids stained with methylene blue. Intensity of staining is heavy in the vascular tissue region where more nucleic acid synthesis occurs. Cytoplasm seems to be rich in nucleic acids which in turn initiate new enzyme and protein syntheses in the control tissue cells, thus rationalizing the heavy staining.

Figure 23b. Note the concentration of nucleic acids stained with methylene blue. Treatment of menazon appears to affect nucleic acid synthesis. Compared to the control parenchyma cells, menazon-treated tissue has less staining intensity because of the impaired germination process.

Figure 23c. Note the concentration of nucleic acids stained with methylene blue. Treatment of usnic acid appears to affect nucleic acid synthesis. Compared to the control parenchyma cells, usnic acid-treated tissue has less staining intensity because of the impaired germination process.





(Figure 24a), menazon- (Figure 24b), and usnic acid-treated (Figure 24c) mung bean cottyledons. Evidently, RNA concentration in the untreated tissue is more intense than that in the treated cotyledons indicating less RNA synthesis and consequently less metabolic activity in the latter.

Figure 25a indicates that the control tissue is steadily losing its stainable insoluble polysaccaride material as is apparent from most of the storage cells depleted of starch grains. In contrast, Figures 25b and 25c illustrate the sites of insoluble polysaccharides including those of cell walls and inclusions such as starch grains stained bright red with PAS. There is a difference in the intensity of carbohydrate staining among the menazon- and usnic acid-treated tissues. Thus, it is interesting to observe that the storage cells of the treated seeds contain abundant starch grains stained heavily with PAS.

Menazon- or usnic acid-treated cotyledons (Figures 26b and 26c) appear to contain more protein bodies (spherical in shape) per unit volume than do the cells of the control tissue (Figure 26a). Large protein bodies in the treated tissue cells are seen; this might be because of the swelling and subsequent coalescence of the adjacent protein bodies to form larger ones. The presence of large or small protein bodies in the control tissue is scarce since the storage cells soon become depleted of these due to protein digestion.

The transverse sections near the embryo of the cotyledon tissue of the control, menazon- and usnic acid-treated mung beans were examined with an electron microscope and the corresponding micrographs have been presented

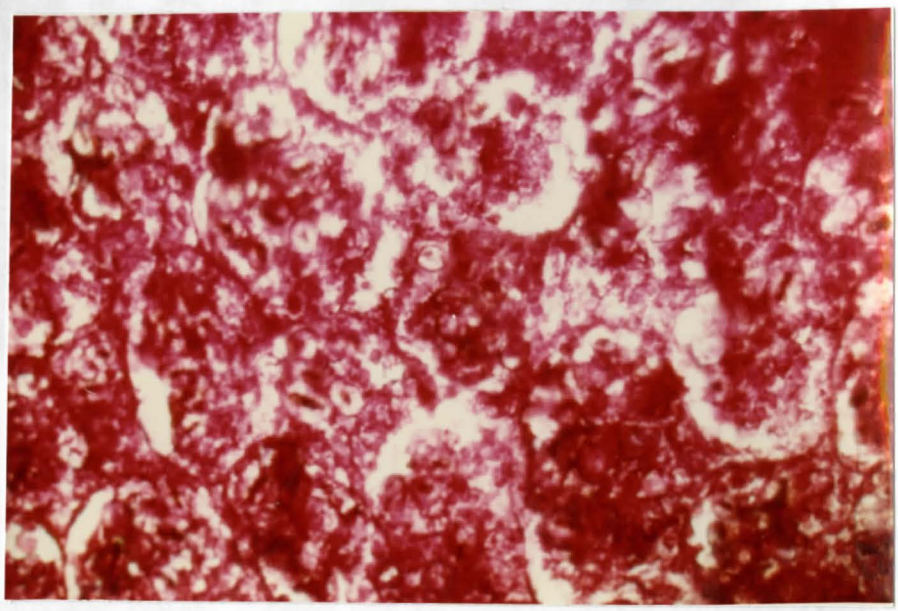
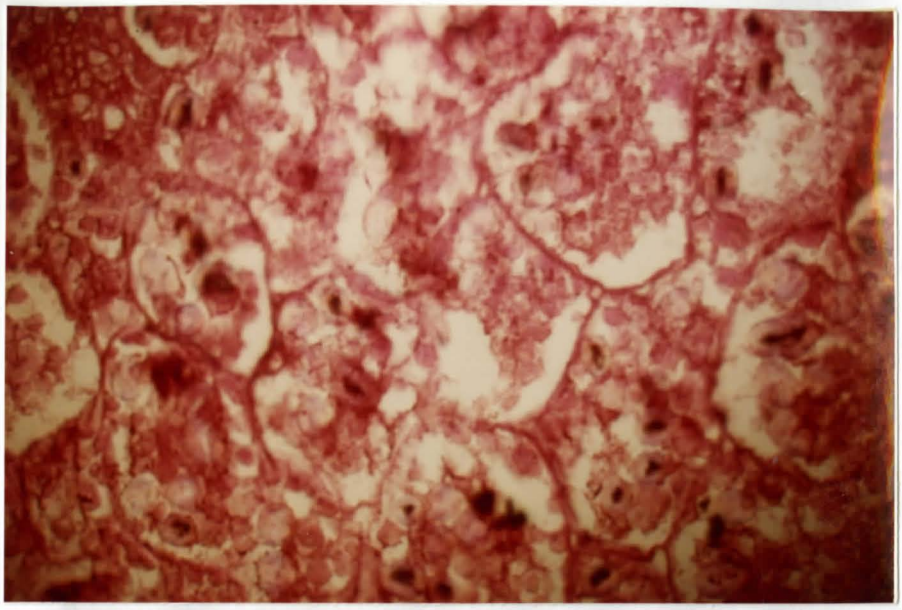


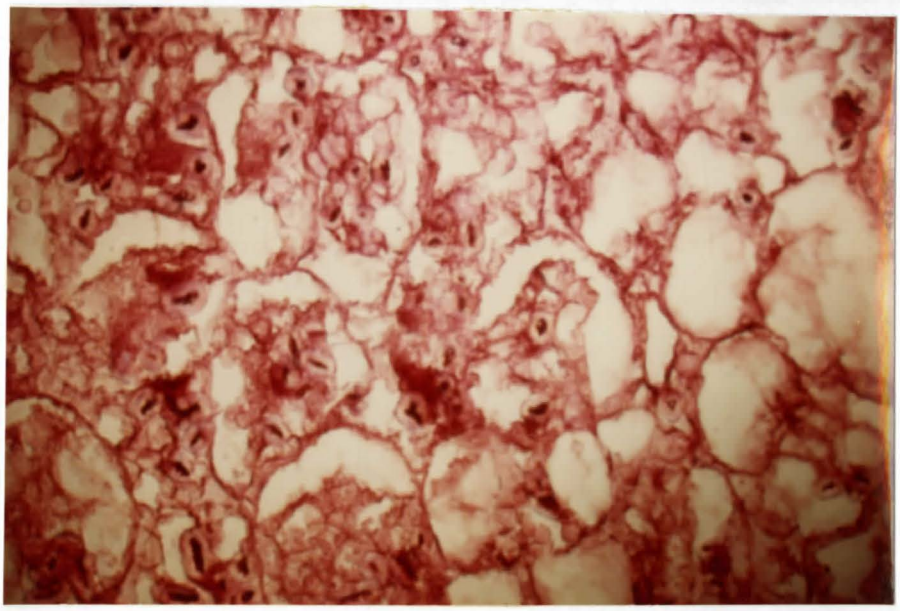
Figure 24. Photomicrographs of storage parenchyma cells of control (a), menazon-(b), and usnic acid-(c) treated mung beans at day 3 of germination showing RNA content, (x 249).

a. Note the concentration of RNA stained with pyronin Y. Intensity of staining is heavy in the parenchyma cells. Important role of RNA in α-amylase production during normal seed germination is thus evident from this photomicrograph of the control tissue.

Figure 24b. Note the concentration of RNA stained with pyronin Y. Compared to the control parenchyma cells intensity of staining is light. Cytoplasm appears to have low rate of RNA synthesis due to the inhibitory action of menazon.

Figure 24c. Note the concentration of RNA stained with pyronin Y. Compared to the control parenchyma cells intensity of staining is light. Some of the cells are even blank and cell walls appear to be broken due to the usnic acid treatment.





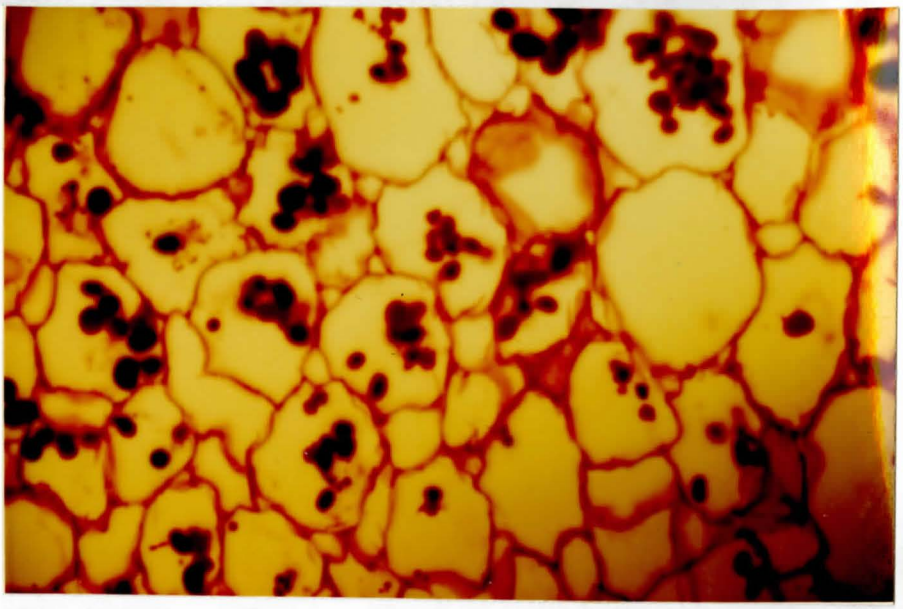
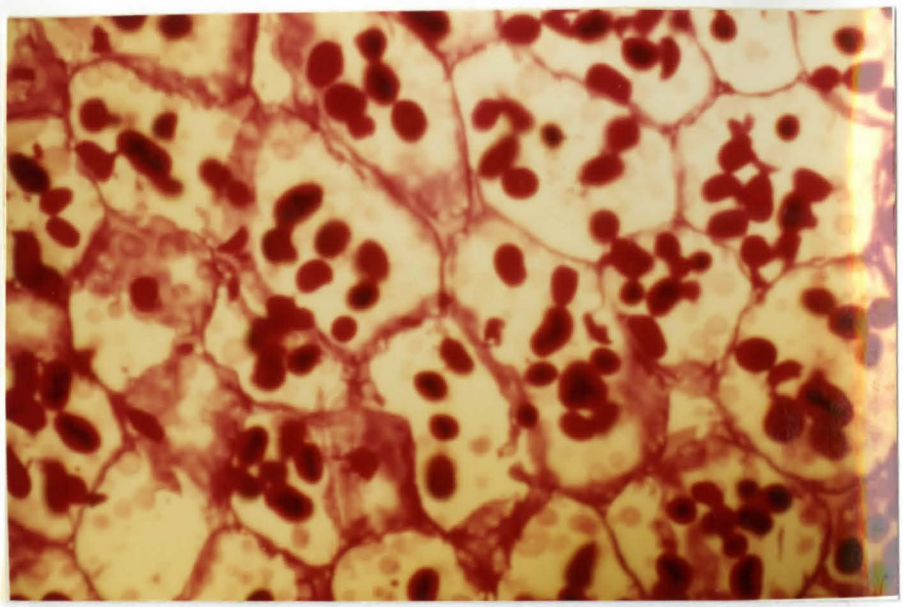


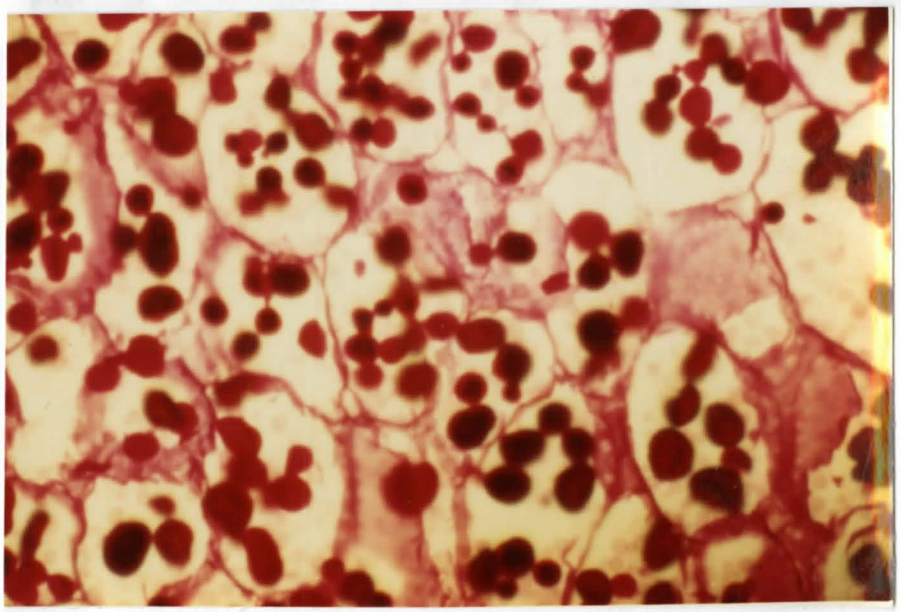
Figure 25. Photomicrographs of storage parenchyma cells of control (a), menazon-(b), and usnic acid-(c) treated mung beans at day 3 of germination showing starch grains, (x 249).

a. Note the presence of starch grains stained bright red with PAS reagent. Most of the parenchyma cells appear empty and some have very small starch grains. These storage cells seem to be shrunken as they become depleted of food reserves.

Figure 25b. Note the number and size of starch grains stained bright red with PAS reagent. Compared to the control cells menazon-treated cells contain large number of starch grains and heavily stained cell walls.

Figure 25c. Note the number and size of starch grains stained bright red with PAS reagent. Compared to the control cells usnic acid-treated cells contain larger starch grains with heavy staining and also cell walls are stained intensely.





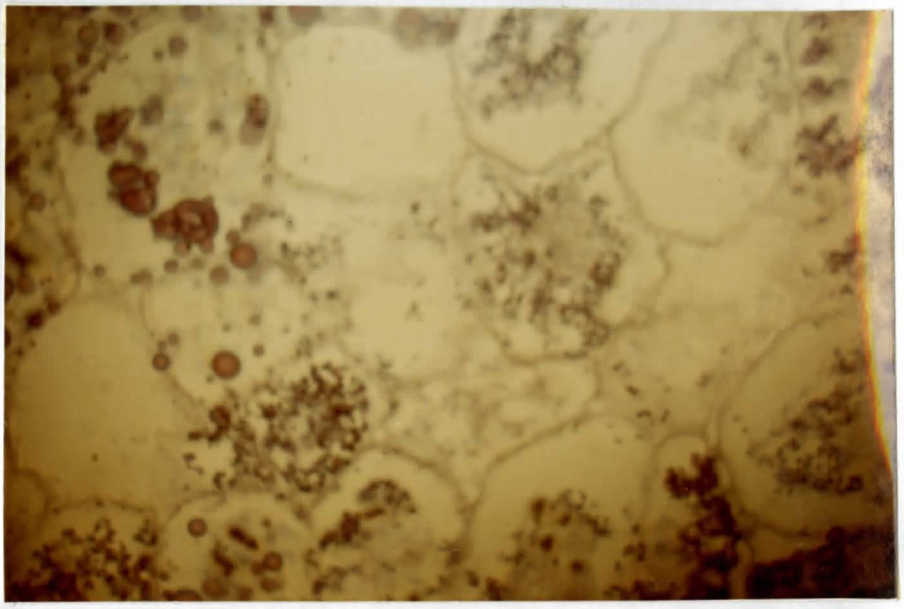
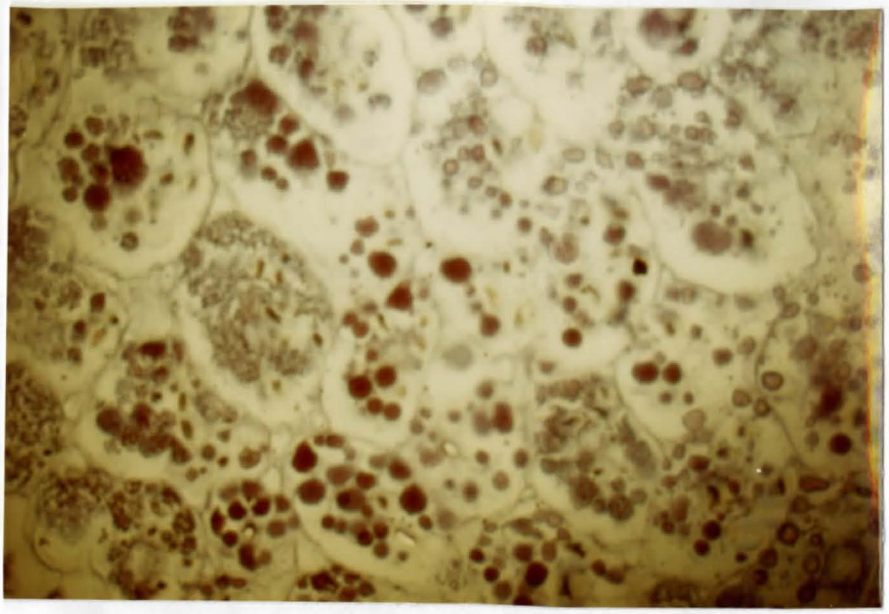
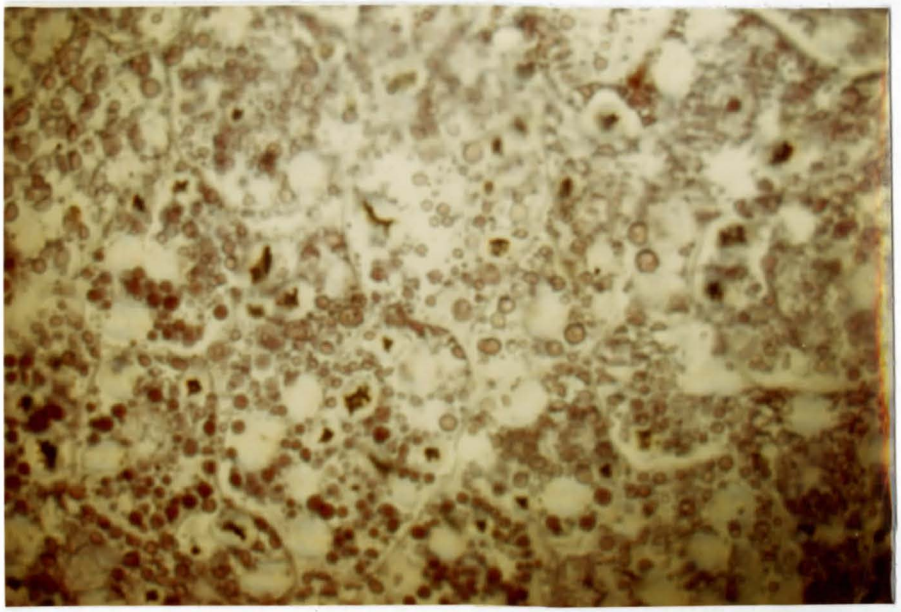


Figure 26. Photomicrographs of storage parenchyma cells of control (a), menazon-(b), and usnic acid-(c) treated mung beans at day 3 of germination showing spherical protein bodies, (x 249).

a. Note the presence of spherical protein bodies stained brown with Hg-BPB reagent. Most of the parenchyma cells appear empty and some have very small amount of protein bodies.

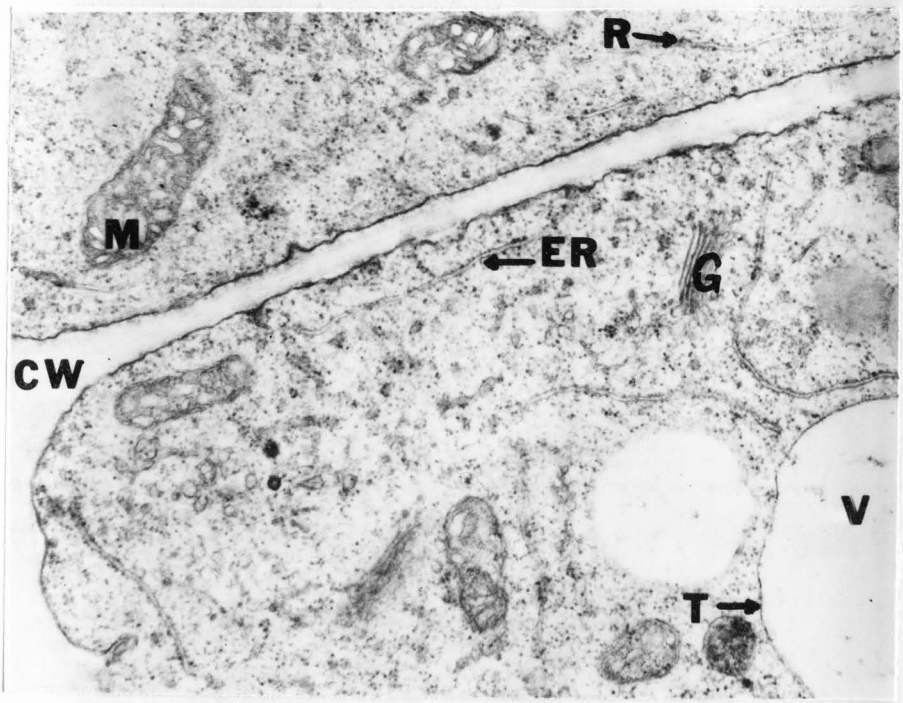




in Figures 27a, 27b, and 27c. The fine structure of the untreated cotyledons shows the presence of developed mitochondria, extensive endoplasmic reticulum with bound and unbound ribosomes, intact cell wall and tonoplast. In both cases of treated cotyledons there are no vacuoles but undigested protein bodies are present indicating no major loss of protein content during germination. This is further evident from the electron micrographs that there is less amount of endoplasmic reticulum in the treated cotyledons compared to that in the control tissue. Golgi bodies are not seen in the treated cotyledons but their presence is noticed in the control tissue. No obvious changes were observed in other cell organelles.

Figure 27. Electron micrographs of transverse sections near the embryo of the cotyledonary tissue of the control (a), menazon-(b), and usnic acid-(c) treated mung beans at day 3 of germination. M, mitochondrion: ER, endoplasmic reticulum: R, ribosome: CW, cell wall: V, vacuole: T, tonoplast: PB, protein body: S, starch granule: G, golgi body.

Figure 27a. Electron micrographs of cotyledonary tissue of control mung beans, (x 14,400). Note the conspicuous cellular organelles.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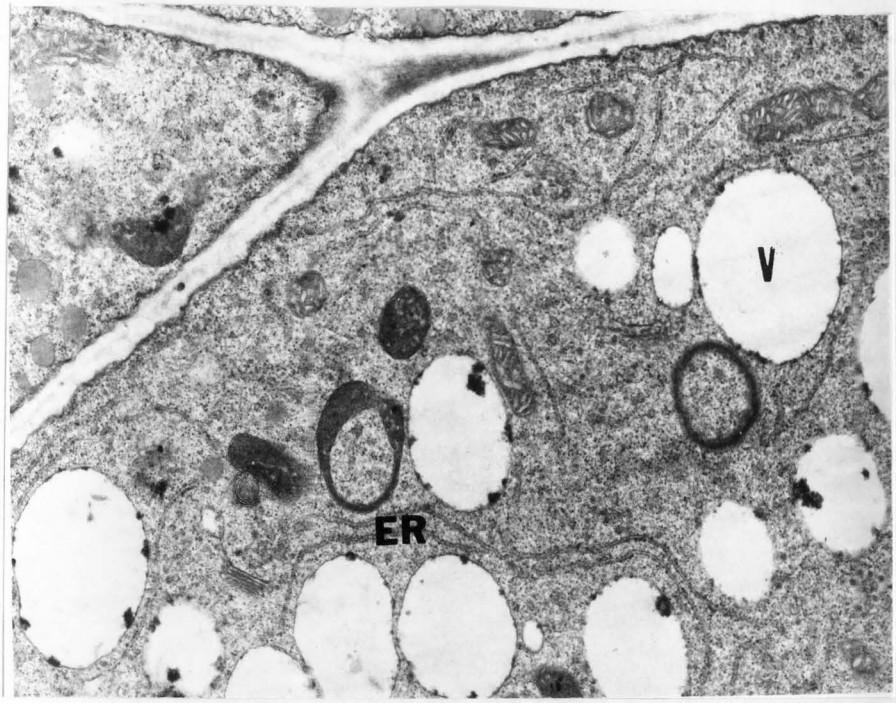
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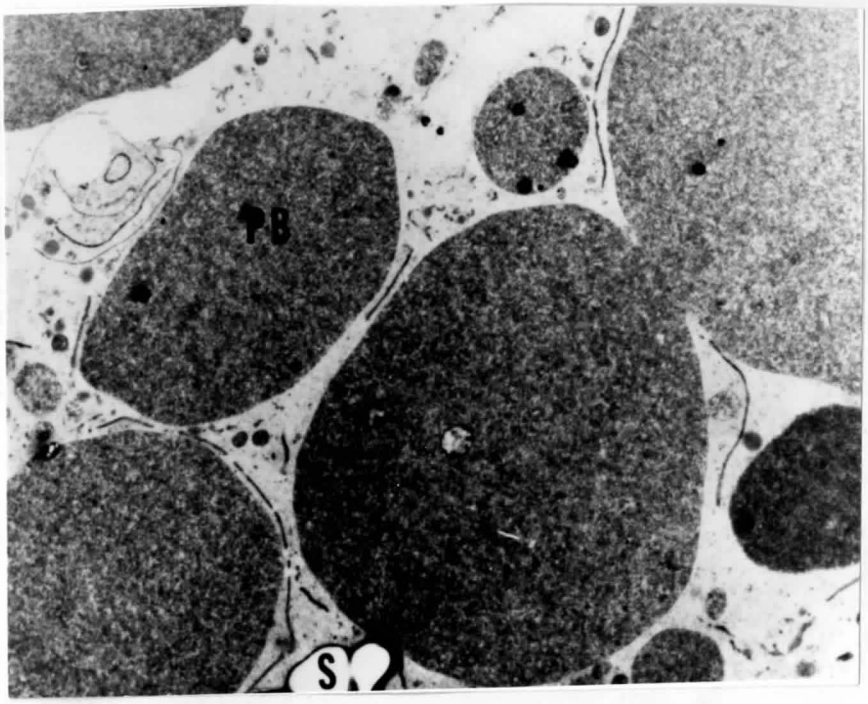
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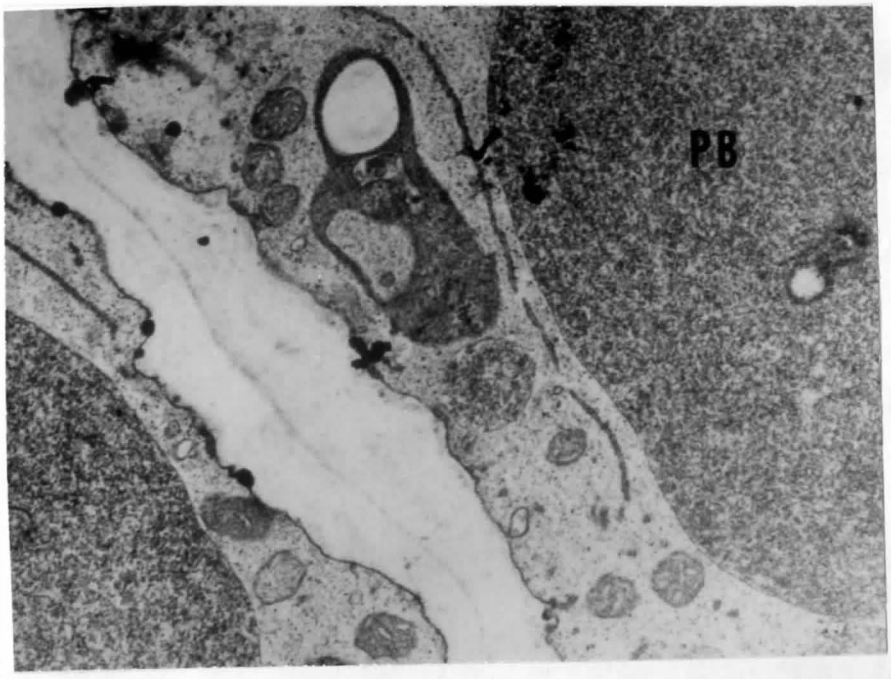
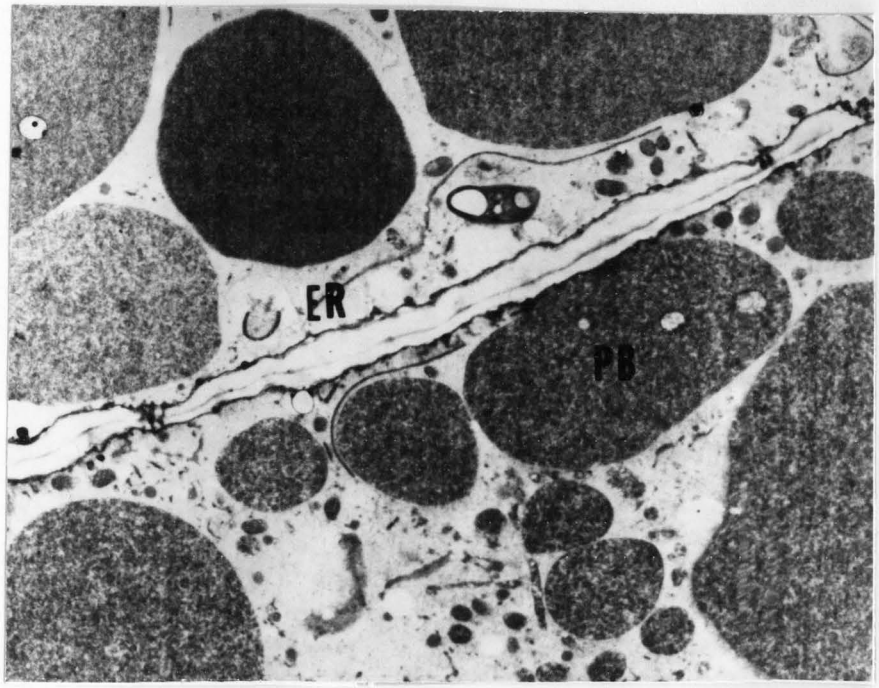
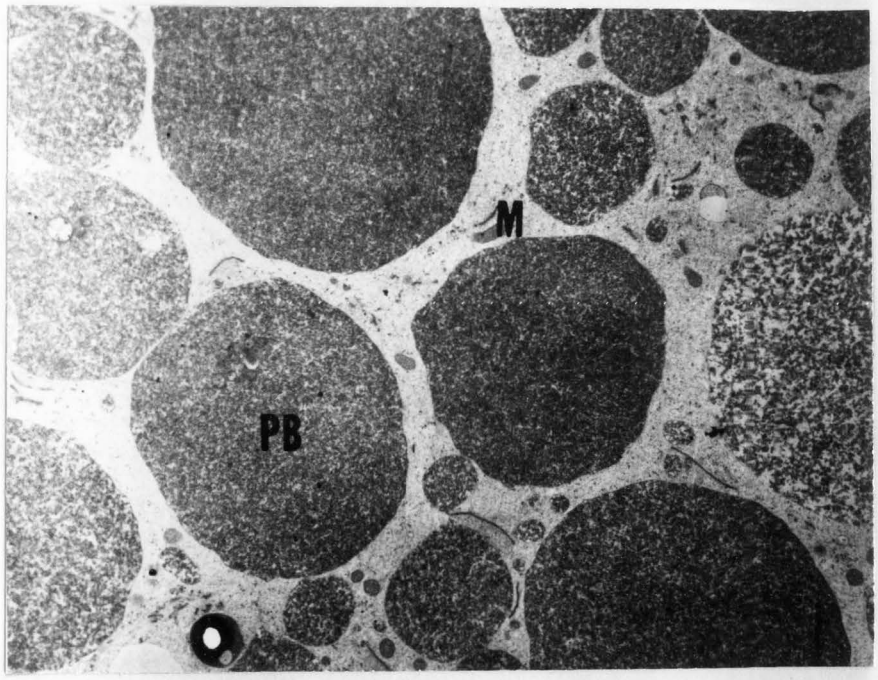

PB

Figure 27c. Electron micrographs of cotyledonary tissue of usnic acid-treated mung beans, (x 14,400). Note the presence of undigested protein bodies and fairly small amount of endoplasmic reticulum compared to that in the control tissue (Figure 27a).


ER
PB


M
PB

DISCUSSION

Pesticide chemicals

Suppression of germination and subsequent seedling growth by the pesticidal treatments indicates that some of the biochemical processes taking place during germination are impaired. Adverse effects of pesticides on seed germination have been demonstrated by several workers (Scopes, 1969; Gifford et al., 1959; Tas, 1961; Sund and Nomura, 1963; Penner, 1968). Contrary to the results reported by Scopes (1969) we found that menazon is more toxic than disulfoton to seed germination. Our results also indicated that GS-14254 in combination with menazon or disulfoton caused a severe damage to wheat germination. However, mung bean germination was not severely impaired. The increased toxicity to wheat germination might have occurred due to the inhibition of detoxification of either of the pesticides at the active site or sites. Kaufman et al., (1970) have observed several such interactions for pesticide combinations. Severe injury or death of cotton seedlings was reported by Hacskaylo, Walker, and Pires (1964) when systemic phosphate insecticides were applied to the soil with the herbicide monuron or diuron. Nash (1967, 1968) also observed that combinations of diuron with some phosphate insecticides showed synergistic phytotoxicity to oats and corn. Thus, simultaneous presence of insecticides and herbicides may result in severe phytotoxic properties.

An experiment was designed to study the effect of gibberellic acid (GA_3) on the reversal of the inhibition of germination by these pesticides. However, no complete reversal of the inhibitory effect was seen. This apparently indicates that these pesticides do not have antigibberellin activity.

In fact there are very few reports pertaining to the pesticidal effects on chemical constituents quantitatively formed during the germination process. Such analysis of the pesticide-treated seeds in this study indicated that there was significantly less formation of reducing sugars and free amino acids at the end of a 3 day germination period. These results are in agreement with the findings of Chopra and Nandra (1969) who reported a decrease in reducing sugars in germinating mustard seeds treated with Thiometon (O, O-dimethyl-S-ethylmercapto ethyldithiophosphate) and they attributed it to the inhibition of lipase activity in the seeds. In our results this might be related to the less starch degradation in the treated seeds.

The reduction in the rate of respiration of germinating seeds treated with the pesticides evidently suggests the blocking of biochemical processes essential for the supply of energy to the growing embryo. Similar results were noted by Wassink and van Elk (Sweeney and Marsh, 1971) where isopropyl N-3-chlorophenyl carbamate inhibited the rate of respiration of germinating pea seeds. Many other herbicides have also been reported to inhibit respiration when applied to the plants (Bishop, 1958; Simon, 1953).

The data concerning the effect of the pesticides--menazon, disulfoton, and GS-14254--on development of the hydrolytic enzymes activity show that these pesticides inhibit amylase and ATP-hydrolyzing phosphatase activities at varying degree in germinating mung beans and wheat seeds. Since hydrolytic enzymes such as amylase, protease, and phosphatase are produced during germination (Briggs, 1963) pesticides may have adverse effect on their syntheses. According to Penner (1968) the inhibition of barley germination and seedling development in culture solutions containing herbicide (amiben or bromoxynil) was due to the effect of the herbicide on enzyme development or synthesis during germination. Inhibition of GA_3-enhanced synthesis of α-amylase by several herbicides has been reported (Jones and Foy, 1971). It seems to be likely then that the pesticides studied herein might have inhibited seed germination in a similar manner as they did not inhibit in vitro amylase activity.

More pronounced inhibition of amylase activity in treated wheat seeds compared to that in mung beans may suggest the possibility that these pesticides inhibit germination by impairing degradation of carbohydrate reserves during germination since wheat seeds are dependent on starch for their energy supply. Penner (1968) noted that amiben and bromoxynil herbicides inhibited barley germination by inhibiting the degradation of storage carbohydrates but not in squash seeds which were tolerant to the same concentrations of the herbicides. Chopra and Nandra (1969) also concluded that Thiometon insecticide slowed the breakdown of carbohydrates in germinating mustard seeds. Less inhibition of mung bean germination than that of the

wheat seeds may be attributed to the comparatively large protein reserves of mung beans since (sulfhydryl groups of) proteins are known to detoxify foreign chemicals in the biological systems. Alternatively, certain detoxifying enzyme systems might have been more active in mung beans as a defense mechanism rather than in wheat.

In contrast to amylase and phosphatase activities in the germinating seeds treated with the pesticides, the activity of casein-hydrolyzing protease, with the exception of GS-14254, was not significantly different from that of the controls. Ashton et al., (1968) reported that different herbicides varied widely in their ability to inhibit increase in proteolytic activity in germinating squash seeds. Young and Varner (1959) found that the inhibitors of amylase and phosphatase syntheses did not have significant effects on proteolytic activity although de novo synthesis of proteolytic enzymes (Penner and Ashton, 1966) occurs in the cotyledons during germination. But the former group of workers demonstrated only small changes in proteolytic activity as normal germination proceeded. Therefore, it is difficult to draw any conclusion regarding the effect on these pesticides on proteases of the germinating seeds.

The effect of these pesticides on the uptake and incorporation of ^{14}C-L-leucine into proteins of potato tuber tissue and germinating mung beans was studied. Results of this experiment revealed that menazon, disulfoton, and GS-14254 not only inhibited uptake of the radioactive amino acid by plant tissues but also significantly inhibited protein synthesis in the potato tuber tissue and in the 24 h germinated mung bean seeds. Litterst, Lichtenstein,

and Kajiwara (1969) reported that disulfoton affected protein synthesis in HeLa cells. Similarly, the inhibition of protein synthesis by some herbicides was reported by Mann, Jordan, and Day (1965). Synthesis of amylase in barley seeds was inhibited by inhibitors of RNA and protein synthesis (Chrispeels and Varner, 1967); therefore, these pesticides may perhaps be inhibitors of RNA and protein synthesis.

Thus numerous factors may be involved in the inhibition of germination process by a pesticide treatment. Since the growth of both radicle and plumule in the pesticide-treated seeds was impaired it can be predicted that these pesticides may inhibit cell division like phenylcarbamates (Sweeney and Marsh, 1971). The phytotoxic effects of these chemicals on oxidative phosphorylation (Ashton et al., 1968) or other energy-rich compounds required for the synthesis of hydrolytic enzymes may not be ruled out in the treated seeds.

Allergenic compounds

From the results it is evident that both alantolactone and usnic acid are highly phytotoxic since at various concentrations they inhibit germination and seedling growth to different extents. The reduction in reducing sugars and free amino acids with a concomitant reduction in the rate of starch utilization during germination of the seeds indicates that these antibiotics inhibit the enzymes associated with the degradation of starch and protein. These steps are essential for the supply of energy and synthesis of new proteins from free amino acids. Perhaps these compounds may bind one or more

enzyme proteins to inhibit their activities. Working with alantolactone and other sesquiterpene lactones derived from plants, Mitchell et al., (1970) suggested that the allergenic reaction of sesquiterpene lactones may be the result of binding of these compounds with one or more proteins in sensitive humans. They found only one common structural requirement for allergenic activity, viz., the presence of the conjugated lactone group. When the exocyclic carbon-carbon double bond conjugated with the carbonyl is reduced (e.g., in dihydro-alantolactone), there is a loss of allergenic activity. Usnic acid is an allergenic compound and has free hydroxyl groups which might be responsible for protein binding in allergic reactions. It needs to be investigated whether a similar phenomenon exists with respect to the phytotoxic action of alantolactone and usnic acid.

Both these compounds inhibited the rate of respiration in germinating seeds. Kinraide and Ahmadjian (1970) also found a reduction in oxygen uptake resulting in complete inhibition of growth of Trebouxia from Acarospora fuscata in 100 μg/ml sodium usnate. On the other hand Marshak and Harting (1948) did not observe any reduction in oxygen uptake by Arbacia eggs treated with usnic acid. Nevertheless, the observed effect on oxygen uptake in this study indirectly reflects some fundamental disruption in the cells. In this respect, the action of these antibiotics resembles that of antimycin A which inhibits respiration in barley seeds (Pollard, 1968).

Partial reversal of inhibition of germination by alantolactone or usnic acid was achieved when GA_3 was added to the medium. This suggests that unavailability of GA_3 is not the primary cause of reduction in seed germination.

The inhibitory action of alantolactone and usnic acid may be compared with that of antimycin A, which has been reported to inhibit respiration, protein synthesis, and excretion of inorganic phosphate by GA_3-treated barley seeds (Pollard, 1968). On the contrary, compounds like 6-methylpurine and barban, 4-chloro-2 butynyl-N-(3-chlorophenyl) carbamate have been found to be ineffective or less effective when added after GA_3 (Chrispeels and Varner, 1967; Yung and Mann, 1967).

It is now well established that as germination proceeds de novo synthesis of amylase, phosphatase (Young and Varner, 1959), protease (Cohen, Leshem, and Pinsky, 1969), and phytase (Mandal and Biswas, 1970) occurs in the cotyledon and endosperm tissue. Our results indicate that both alantolactone and usnic acid inhibit the development of amylase and ATP-hydrolyzing phosphatase activities in mung bean and wheat seeds. Young and Varner (1959) have also reported similar action of chloramphenicol, DNP, and p-fluorophenylalanine on de novo synthesis of these enzymes in germinating peas. On the other hand, azetidine-2-carboxylic acid, a potent analogue of proline and effective growth inhibitor, did not inhibit acid phosphatase and isocitritase activities in germinating seeds since these enzymes are assumed to arise in cotyledons by the activation of zymogens (Presley and Fowden, 1965). According to our results GA_3 did not reverse the alantolactone- and usnic acid-induced inhibition of seed germination. Apparently, then, these antibiotics do not have antiauxin properties to inhibit amylase synthesis. Other antibiotics such as actinomycin D (Yung and Mann, 1967) and streptomycin (Drury and Khan,

1969) have been reported to exhibit similar action. In a striking contrast to amylase and phosphatase activities there was no considerable inhibitory influence of either alantolactone or usnic acid on proteolytic activity. In this regard our results concur with the findings of Young and Varner (1959). These workers also did not observe any inhibitory effect of chloramphenicol, DNP, and p-fluorophenylalanine on proteolytic activity in germinating pea cotyledons.

Alantolactone and usnic acid inhibited both the uptake by the tissue and incorporation into protein of ^{14}C-L-leucine in potato discs. Although alantolactone did, similar concentration of usnic acid, however, did not influence the uptake of the radioactive leucine in mung bean seeds but significantly inhibited the incorporation into protein. The variation in the rate of uptake of ^{14}C-L-leucine by the tissue has been noted in the case of other antibiotics like chloramphenicol. Nooden and Thimann (1963) showed that chloramphenicol did not influence the amount of radioactivity in ethanol-soluble fraction of pea stem sections but in Avena coleoptile sections it produced substantial decrease in the ethanolextractable radioactivity (Nooden and Thimann, 1965). Our experiments do not permit us to conclude whether alantolactone or usnic acid inhibits protein synthesis directly (for example, inhibition of aminoacyl-RNA or M-RNA attachment to ribosomes) or indirectly (for example, ATP synthesis). Nevertheless, we have demonstrated that alantolactone and usnic acid inhibit the rate of O_2 uptake by the germinating wheat and mung bean seeds. Chrispeels and Varner (1967) have shown that phosphorylation uncouplers rapidly stop the midcourse synthesis of amylase.

The slow but eventually increased inhibition of starch degradation accompanying the inhibition of amylase activity may be the result of more inhibition of respiration. This also concurs with the findings that the synthesis of protein and ribonucleic acid involved in protein formation is retarded by the inhibition of energy forming steps in cells where amylase and protease are synthesized de novo (Varner, 1964; Jacobsen and Varner, 1967).

Histochemistry and electron microscopy

It is apparent from the results on histochemical changes in the treated and untreated mung beans that considerably more nucleic acid synthesis occurred in the control cotyledons than that in the menazon- or usnic acid-treated cotyledons during 3 day of germination and growth. It is known that new cell constitutents are synthesized in the embryonic axis utilizing the solubilized and mobilized material from the storage tissue of seeds. Participation of nucleic acids in these metabolic changes is vitally important. In the storage tissue of some seeds it goes on diminishing (Olsson and Boulter, 1968) and in some cases it increases as much as two or three fold within the first few days of germination and then decreases as the depletion of storage reserves increases (Cherry, 1963). Evidently, due to the treatment of menazon or usnic acid the nucleic acid synthesis in germinating mung beans appears to be impaired.

RNA content of the untreated cotyledons was markedly higher than that of the treated cotyledons, suggesting its important and essential role in

metabolic activities during germination, particularly in protein synthesis. Oota and Takata (1959) separated the RNA from Vigna beans electrophoretically and concluded that two of the separated components can be identified as functional and transportable RNA. The functional RNA is linked to protein and is involved in protein synthesis. The transportable RNA seems to be separate from the proteins and is transported to the seedling. The other components observed by Oota and Takata are regarded by them as intermediaries in the conversion of functional and transportable RNA. The significance of RNA in protein synthesis and subsequent normal growth is thus evident. Our biochemical data on decreased enzyme and mitochondrial (low respiration) activities closely correlate with the decreased nucleic acid synthesis in the mung bean cotyledons treated with menazon or usnic acid.

Histological and histochemical evidence shows that during normal course of germination of seeds and seedling growth there occurs a large loss of starch and proteins from the cotyledon or endosperm tissue (Smith and Flinn, 1967). However, histochemical tests in this study revealed that these were not depleted from the mung bean cotyledons treated with menazon or usnic acid. This suggests that the biochemical mechanism connected with starch and protein degradation is impaired as a result of toxic action of menazon and usnic acid to germination process. Thus, the biochemical evidence already reported regarding the effect of menazon and usnic acid on the inhibition of hydrolytic enzyme development, incorporation of labelled L-leucine into proteins during germination, formation of free amino acids,

reducing sugars, and mitochondrial activity is in agreement with the histochemical observations.

At the ultrastructural level, the close association of rough-surfaced endoplasmic reticulum (ER) and unbound ribosomes in the cytoplasm of the storage cells of the control cotyledons is noticeable. This ribosome-endoplasmic reticulum complex could secrete the hydrolytic enzymes responsible for digestion of the food reserves (Opik, 1966). The abundance of ribosomes in the cotyledons can be related to the observations that protein (enzyme) snythesis (Young et al., 1960) is active in cotyledonary tissues during germination. In contrast, both in menazon- and usnic acid-treated mung bean cotyledons relatively less amount of ER is seen. Presumably, this might have resulted in less enzyme synthesis to consequently affect the digestion of the storage material in the treated seeds. As a result, protein bodies were conspicuous in the cotyledons of the treated mung beans whereas the presence of vacuoles in the control was seen perhaps due to the replacement of fused and digested protein bodies (Opik, 1966). In this process protein membranes become part of the vacuolar membranes (tonoplasts). Singh, Campbell, and Salunkhe (1972) have also ultrastructurally shown the presence of protein bodies in ungerminated bean cotyledons. Likewise, the golgi vesicles appear to form and move through the control tissue as normal germination proceeds. This is an essential process observed during unimparied seed germination. Electron microscopic investigations on barley by Jones (1969) have shown that numerous golgi vesicles derived from the ER were found in the aleurone cells after 16 h germination and it was suggested that these may be continuously secreted into

the endosperm for the digestion of food reserves. Absence of golgi apparati in the menazon- and usnic acid-treated cotyledons indicates an impairment of germination process.

Furthermore, from the electron micrographs it appears that mitochondria are fully developed in the storage cells during normal germination to supply energy needed for metabolic activites. Similar observation was made by Lott and Castelfranco (1970) in the fine structure of germinating cotyledons of Cucurbita maxima. On the other hand, mitochordria in the menazon- and usnic acid-treated tissue appear to be smaller in size. This may suggest mitochondria as a possible site of action of menazon and usnic acid as in the case of picloram (Baur and Bowman, 1973). In attempting to compare the biochemical characteristics of an organ and cellular fine structure it is emphasized that the former concern the entire organ and the latter are restricted to a small portion of it. Although tissues were always handled in the same way and at the same time one should also point out, as did Shaw and Manocha (1965), that degenerating organelles are more easily damaged than normal ones during preparation for electron microscopy.

SUMMARY AND CONCLUSIONS

Phytotoxic actions of selected pesticides--menazon, disulfoton, and GS-14254--and allergens--alantolactone and usnic acid--on germination of mung bean (Phaseolus mungo L.) and wheat (Triticum aestivum L.) seeds were studied. Effect of these toxicants on subsequent metabolic changes during early germination process of both mung bean and wheat seeds was investigated with regard to biochemical, physiological, histochemical, and ultrastructural alterations in the storage tissue of the seeds.

Among the insecticides, menazon (250 ppm) was found to be more toxic to both species than did disulfoton. The herbicide, GS-14254 (100 ppm), also was equally inhibitory to seed germination and seedling growth. Simultaneous presence of the herbicide with either of the insecticides caused almost complete inhibition of germination of wheat in particular. Both alantolactone and usnic acid significantly inhibited germination and seedling growth at their maximum concentrations. These two compounds appear to be more phytotoxic than the pesticides.

At their maximum concentraions, pesticides as well as allergens significantly blocked the respiration of germinating seeds at the end of 72 h after the treatment. In all cases except alantolactone, respiration of wheat seeds was considerably more affected than that of the mung beans.

The highest concentrations of pesticide chemicals or allergenic compounds impaired the carbohydrate and protein metabolism in the treated seeds as was evident from the significantly less formation of reducing sugars and free amino acids. Among the species of seeds, considerably lesser amounts of reducing sugars and free amino acids were found in the pesticide-treated wheat seeds than in the mung beans as compared to their respective controls. Such variation was not observed in seeds treated with the allergens.

All pesticide chemicals inhibited the development of amylase and ATPase activities in the treated seeds. However, inhibition of amylase activity in menazon-treated seeds was more than that of disulfoton or GS-14254. Proteolytic activity was not significantly inhibited. In case of allergens, usnic acid was more effective than alantolactone in inhibiting the amylase activity in mung beans. Both compounds inhibited the development of amylase activity in a similar manner in wheat seeds. ATPase inhibition in seeds treated with usnic acid was more severe than that in the alantolactone-treated seeds. However, proteolytic activity in control and treated seeds showed almost the same trend.

In vitro activity of amylase isolated from mung bean and wheat seeds was not significantly inhibited by any of these toxicants.

Observations with potato tissue and germinating mung beans indicated that both uptake and incorporation of ^{14}C-L-leucine into protein were significantly inhibited by the pesticides and alantolactone. Uptake of the amino acid in germinating mung beans treated with usnic acid was not affected although both uptake and incorporation were inhibited in potato tissue.

To study the histochemical changes in the treated mung beans menazon and usnic acid were selected as the representatives of the pesticides and allergens, respectively. At the end of a 3 day germination period, it was observed that content of total nucleic acids and RNA was less in parenchymous cells of the storage tissue of the treated seeds than that in the controls. On the contrary, presence of starch grains and protein bodies was considerably more in the treated seeds compared to the untreated controls indicating normal occurrence of the digestion of food reserves in the latter.

Ultrastructurally, close association of endoplasmic reticulum of bound and unbound ribosomes in the cotyledons of the storage cells of 3 day germination control seeds was noticed. Vacuoles were observed in these cells perhaps due to the replacement of digested protein bodies. This and the presence of normal mitochondria might account for protein (enzyme) synthesis in germinating control seeds. On the other hand, protein bodies in the storage cells of menazon- and usnic acid-treated seeds were conspicuous, reflecting their impaired digestion.

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APPENDIX

Table 21. Analysis of variance for the effect of pesticides on germination of seeds and seedling growth during 120 h*

Pesticides	Source	df	Germination MS	Germination F	Root Growth MS	Root Growth F	Shoot Growth MS	Shoot Growth F
					Mung beans			
Menazon	Total	14						
	Treatments	4	3056.7	32.75	1.867	5.48	15.672	103.03
	Error	10	93.3		0.341		0.152	
					Wheat			
	Total	14						
	Treatments	4	2533.3	31.67	3.312	14.04	1.648	8.03
	Error	10	80.0		0.236		0.205	
Disulfoton					Mung beans			
	Total	14						
	Treatments	4	210.0	5.25	0.287	1.98	1.034	3.54
	Error	10	40.0		0.145		0.292	
					Wheat			
	Total	14						
	Treatments	4	3856.7	72.31	1.467	2.123	1.102	4.88
	Error	10	53.33		0.691		0.226	
GS-14254					Mung beans			
	Total	14						
	Treatments	4	690.00	7.39	3.942	13.06	14.852	41.12
	Error	10	93.32		0.302		0.361	
					Wheat			
	Total	14						
	Treatments	4	174.0	6.87	5.790	24.86	5.761	26.86
	Error	10	253.3		0.233		0.215	

Table 21. Continued

Pesticides	Source	df	Germination MS	Germination F	Root Growth MS	Root Growth F	Shoot Growth MS	Shoot Growth F
GS-14254					Mung beans			
+Menazon	Total	5						
	Treatments	1	4704.0	147.0	20.3	108.5	65.6	171.5
	Error	4	32.0		0.187		0.378	
					Wheat			
	Total	5						
	Treatments	1	960.0	73.85	15.65	55.0	27.82	213.9
	Error	4	13.0		0.285		0.130	
+Disulfoton					Mung beans			
	Total	5						
	Treatments	1	1261.5	157.7	10.53	46.8	48.39	129.12
	Error	4	8.0		.225		.375	
					Wheat			
	Total	5						
	Treatments	1	889.4	63.53	20.89	155.2	27.74	203.6
	Error	4	14.0		0.135		0.136	

*Reference to Table 1.

Table 22. Analysis of variance for the effect of allergenic compounds on germination of seeds and seedling growth during germination*

Allergenic Compounds	Source	df	Germination MS	Germination F	Root Growth MS	Root Growth F	Shoot Growth MS	Shoot Growth F
					Mung beans			
Alanto-lactone	Total	14						
	Treatments	4	1838.3	49.02	0.907	2.22	4.479	5.82
	Error	10	37.5		0.480		0.769	
					Wheat			
	Total	14						
	Treatments	4	1310.0	7.28	3.439	18.69	3.223	26.15
	Error	10	180.0		0.184		0.123	
Usnic Acid					Mung Beans			
	Total	14						
	Treatments	4	2476.7	19.55	2.577	19.91	7.351	38.17
	Error	10	126.7		0.129		0.193	
					Wheat			
	Total	14						
	Treatments	4	1743.3	20.12	10.254	47.26	5.587	2.04
	Error	10	86.7		0.217		0.273	

*Reference to Table 2.

Table 23. Analysis of variance for the respiration of the control and menazon-treated mung bean and wheat seeds during germination*

Source	df	Oxygen uptake					
		24 h		48 h		72 h	
		MS	F	MS	F	MS	F
		Mung beans					
Total	9						
Treatments	4	6648.82	6.011	201291.10	15.25	829895.00	68.80
Error	5	1106.16		13199.90		12062.35	
		Wheat					
Total	9						
Treatments	4	87.86	0.825	79785.12	20.99	736813.00	60.62
Error	5	106.49		3800.85		12154.81	

*Reference to Table 4.

Table 24. Analysis of variance for the respiration of the control and disulfoton treated mung bean and wheat seeds during germination*

Source	df	Oxygen uptake					
		24 h		48 h		72 h	
		MS	F	MS	F	MS	F
		Mung beans					
Total	9						
Treatments	4	1077.19	0.6947	79284.70	2.977	163152.90	4.576
Error	5	1550.60		26628.97		35646.90	
		Wheat					
Total	9						
Treatments	4	495.50	7.3370	4256.24	2.453	192164.91	19.590
Error	5	67.53		1735.19		9810.85	

Reference to Table 5

Table 25. Analysis of variance for the respiration of the control and GS-14254 treated mung bean and wheat seeds during germinaton*

		Oxygen uptake					
		24 h		48 h		72 h	
Source	df	MS	F	MS	F	MS	F
				Mung beans			
Total	9						
Treatments	4	4564.45	1.6970	264241.90	116.340	620426.59	96.68
Error	5	2689.38		2271.39		6417.33	
				Wheat			
Total	9						
Treatments	4	239.39	1.2960	26494.25	10.780	238904.00	141.00
Error	5	184.71		2457.05		1694.35	

*Reference to Table 6.

Table 26. Analysis of variance for the respiration of the control and alantolactone treated mung bean and wheat seeds during germination*

Source	df	Oxygen uptake					
		24 h		48 h		72 h	
		MS	F	MS	F	MS	F
				Mung beans			
Total	9						
Treatments	4	752.22	0.5045	403666.40	45.759	833364.40	518.94
Error	5	1490.80		8821.53		1605.88	
				Wheat			
Total	9						
Treatments	4	407.89	0.9327	11422.52	4.717	290498.60	65.06
Error	5	437.32		2421.56		4465.02	

*Reference to Table 7.

Table 27. Analysis of variance for the respiration of the control and usnic acid treated mung bean and wheat seeds during germination*

Source	df	Oxygen uptake					
		24 h		48 h		72 h	
		MS	F	MS	F	MS	F
				Mung beans			
Total	9						
Treatments	4	12729.06	4.734	169710.70	24.22	959776.10	168.62
Error	5	2688.29		7007.96		5692.04	
				Wheat			
Total	9						
Treatments	4	27.260	1.225	104245.59	35.06	739019.70	311.52
Error	5	22.247		2973.18		2372.23	

*Reference to Table 8

Table 28. Analysis of variance for the starch, reducing sugars, and amino acid content of menazon treated and untreated mung bean and wheat seeds during germination*

Parameter	Source	df	24 h		48 h		72 h	
			MS	F	MS	F	MS	F
			Mung beans					
Starch	Total	9						
	Treatments	4	1.3630	3.513	1.429	1.529	12.404	112.817
	Error	5	0.3880		0.9350		00.1099	
Reducing	Total	14						
sugars	Treatments	4	0.0341	1.858	4.3860	9.794	323.5040	161.370
	Error	10	0.0183		0.4478		2.0047	
Amino	Total	14						
acids	Treatments	4	0.3275	1.926	0.3132	0.602	98.644	24.299
	Error	10	0.1700		0.5207		4.059	
			Wheat					
Starch	Total	9						
	Treatments	4	13.560	21.478	17.275	5.450	84.779	34.410
	Error	5	00.631		3.169		2.463	
Reducing	Total	14						
sugars	Treatments	4	0.6965	6.932	35.714	23.822	520.284	79.575
	Error	10	0.1004		1.499		6.538	
Amino	Total	14						
acids	Treatments	4	0.2737	8.902	5.678	24.764	35.867	195.461
	Error	10	0.0307		0.229		0.1835	

*Reference to Table 9.

Table 29. Analysis of variance for the starch, reducing sugars, and amino acid content of disulfoton treated and untreated mung bean and wheat seeds during germination*

Parameter	Source	df	24 h MS	24 h F	48 h MS	48 h F	72 h MS	72 h F
					Mung beans			
Starch	Total	9						
	Treatments	4	6.815	9.099	2.083	0.7567	2.3670	27.684
	Error	5	0.7489		2.753		0.0855	
Reducing	Total	14						
sugars	Treatments	4	0.0123	1.435	0.6946	1.5411	55.3191	29.527
	Error	10	0.0086		0.4507		1.8735	
Amino	Total	14						
acids	Treatments	4	0.7898	4.0714	10.326	3.9325	59.1806	8.368
	Error	10	0.1940		2.657		7.0720	
					Wheat			
Starch	Total	9						
	Treatments	4	4.383	3.228	50.917	62.41	96.196	238.64
	Error	5	1.358		00.8157		00.4030	
Reducing	Total	14						
sugars	Treatments	4	0.1927	5.880	56.7668	378.741	731.293	189.8409
	Error	10	0.0328		0.1499		3.852	
Amino	Total	14						
acids	Treatments	4	0.2123	61.119	0.1768	36.324	15.9671	179.5064
	Error	10	0.0035		0.0487		00.8895	

*Reference to Table 10

Table 30. Analysis of variance for the starch, reducing sugars, and amino acid content of GS-14254 treated and untreated mung bean and wheat seeds during germination*

Parameter	Source	df	24 h MS	24 h F	48 h MS	48 h F	72 h MS	72 h F
					Mung beans			
Starch	Total	9						
	Treatments	4	2.1730	86.047	3.801	1.472	3.0928	8.486
	Error	5	0.0253		2.581		0.3644	
Reducing	Total	14						
sugars	Treatments	4	0.0218	40.071	0.1559	1.868	134.783	583.89
	Error	10	0.0006		0.0835		0.2308	
Amino	Total	14						
acids	Treatments	4	1.172	1.083	0.1567	0.121	76.632	42.427
	Error	10	1.0827		1.294		3.137	
					Wheat			
Starch	Total	9						
	Treatments	4	11.634	3.176	52.665	9.213	116.959	130.295
	Error	5	3.663		5.716		0.8976	
Reducing	Total	14						
sugars	Treatments	4	0.5086	4.923	9.323	69.135	90.4526	114.826
	Error	10	0.1033		0.1348		0.7877	
Amino	Total	14						
acids	Treatments	4	0.1627	2.591	2.898	28.152	24.7732	190.789
	Error	10	0.0627		0.1029		0.1298	

*Reference to Table 11.

Table 31. Analysis of variance for the starch, reducing sugars, and amino acid content of alantolactone treated and untreated mung bean and wheat seeds during germination*

Parameter	Source	df	24 h MS	24 h F	48 h MS	48 h F	72 h MS	72 h F
				Mung beans				
Starch	Total	14						
	Treatments	4	8.444	7.311	16.220	13.380	72.612	17.887
	Error	10	0.9432		1.2120.		4.0600	
Reducing sugars	Total	14						
	Treatments	4	0.1410	2.325	9.738	82.927	16.1036	238.5411
	Error	10	0.0606		0.1175		0.06751	
Amino acids	Total	14						
	Treatments	4	3.380	8.676	0.3479	0.5497	322.364	30.6270
	Error	10	0.3896		0.6329		10.525	
				Wheat				
Starch	Total	14						
	Treatments	4	4.263	6.221	19.533	44.780	46.019	95.829
	Error	10	0.6851		0.4362		0.4802	
Reducing sugars	Total	14						
	Treatments	4	1.6005	2.281	39.759	37.500	1204.420	297.491
	Error	10	0.7015		1.060		4.0478	
Amino acids	Total	14						
	Treatments	4	0.2072	6.959	0.3682	0.5565	30.342	57.160
	Error	10	0.0298		0.6617		0.5308	

*Reference to Table 12.

Table 32. Analysis of variance for the starch, reducing sugars, and amino acid content of usnic acid treated and untreated mung bean and wheat seeds during germination*

Parameter	Source	df	24 h MS	24 h F	48 h MS	48 h F	72 h MS	72 h F
					Mung beans			
Starch	Total	14						
	Treatments	4	29.4200	77.790	12.2177	5.1313	2.5386	71.675
	Error	10	0.3781		2.3810		0.03541	
Reducing sugars	Total	14						
	Treatments	4	0.02569	2.944	1.0365	41.861	314.8400	144.920
	Error	10	0.00873		0.0247		2.172	
Amino acids	Total	14						
	Treatments	4	1.268	2.4908	12.755	0.7339	78.7400	14.003
	Error	10	0.5093		17.377		5.6230	
					Wheat			
Starch	Total	14						
	Treatments	4	36.631	29.094	25.693	30.9200	7.9689	141.440
	Error	10	1.258		0.8308		0.0563	
Reducing sugars	Total	14						
	Treatments	4	0.4780	4.640	207.387	130.470	146.7232	128.770
	Error	10	0.1030		1.589		1.1394	
Amino acids	Total	14						
	Treatments	4	0.0747	4.362	10.7016	14.767	60.249	194.414
	Error	10	0.01713		0.7246		0.3099	

*Reference to Table 13.

Table 33. Analysis of variance for the uptake and incorporation of ^{14}C-L-leucine leucine into protein of control and treated white potato tissue and germinating mung beans*

		Radioactivity			
		Alcohol soluble		Alcohol insoluble	
Source	df	MS	F	MS	F
		Potato discs			
Total	11				
Treatments	5	167741.33	424.88	1748821.11	98.54
Error	6	3948.68		17746.67	
		Mung beans			
Total	11				
Treatments	5	159984961.30	13.86	226395341.00	71.38
Error	6	11540140.83		3171804.92	

*Reference to Table 20.

VITA

R. R. Dalvi

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Biochemical and Physiological Studies on Phytotoxicity of Selected Pesticides and Allergens during Seed Germination of some Food Crops.

Major Field: Toxicology

Biographical Information:

Personal Data: Born at Talgaon, Ratnagiri, Maharashtra State, India, on November 8, 1938; son of Rajaram and Sundarabai Dalvi; married to Rekha B. Jadhav on January 22, 1969.

Education: Attended elementary school at Talgaon and passed S.S.C. examination from Kudal High School, Kudal, Maharashtra. Joined Rajaram College, Wilson College and received B.Sc. (Hons.) in Chemistry in 1962 from the University of Bombay. Admitted to Bombay University Department of Chemical Technology and received B.Sc. (Tech.) and M.Sc. (Tech.) in Food Science and Technology in 1964 and 1967, respectively. Completed requirements for the Ph.D. degree from Utah State University, Logan, Utah, U.S.A. in 1972.

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