EFFECT OF TEMPERATURE AND VARIOUS AGRICULTURAL CHEMICALS ON PHOSPHOLIPID FATTY ACID COMPOSITION OF SOYBEAN (GLYCINE MAX (L.) MERR.) ROOTS

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ABSTRACT

EFFECT OF TEMPERATURE AND VARIOUS AGRICULTURAL CHEMICALS ON PHOSPHOLIPID FATTY ACID COMPOSITION OF SOYBEAN (GLYCINE MAX (L.) MERR.) ROOTS

By

Carlos Manuel Rivera

Mitochondrial and plasmalemma membrane fractions isolated from soybean roots exhibited a rapid response in phospholipid fatty acid composition to shifts in temperature between 15C and 30C. As temperature was increased, the proportion of saturated fatty acids also increased. Changes were evident within 48 hr, often within 24 hr after a shift in temperature. Both membrane fractions responded similarly, however the mitochondria phospholipids were generally more unsaturated. As temperature was decreased the membranes shifted toward greater unsaturation though the mitochondrial fatty acid composition responded more quickly than the plasmalemma. An unsaturation ratio (UR) was calculated as percent weight contributed by linoleic acid plus linolenic acid divided by the percent weight contributed by palmitic acid. UR gave a measure of overall shifts toward greater saturation or unsaturation in the membranes.

Increased resistance to chilling injury was observed when soybeans were treated with pre-emergence applications of 2.24 kg/ha alachlor [2-chloro-2',6'-diethyl-*N*-(methoxymethyl)acetanilide), 0.56 kg/ha trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine), 2.24 kg/ha H-22234 [*N*-chloroacetyl-*N*-(2,6-diethylphenyl)-glycine], or 3.36 kg/ha vernolate (*S*-propyl dipropylthiocarbamate). Similar results were obtained when 0.14 kg/ha R-29148 [2,2-dimethyl-S-methyl (dichloroacetyl)oxazolidine], a potential thiocarbamate antidote, was combined with H-22234, alachlor or vernolate. Fatty acid analysis of the membrane phospholipid fraction demonstrated an increased degree of unsaturation. However both the mitochondria and plasmalemma did not always respond similarly.

Low calcium concentration of 0.4 mM (pH 6.5) stimulated linuron [3-(3,-dichlorophenyl)-1-methoxy-1-methylurea) uptake from nutrient solution and also increased the degree of saturation in the plasmalemma. A shift from linolenic to palmitic acid was seen. Increasing nitrogen, however, increased linuron uptake. The increase was not due to transpiration. Nitrogen also increased the degree of saturation in the plasmalemma but a shift from linoleic to stearic acid was seen.

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By

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INTRODUCTION

In the field some herbicides have been shown to be more phytotoxic in early spring, others during periods of high temperature. Though increased herbicidal activity may be due to the effects of temperature on various metabolic processes (e.g. respiration, photosynthesis, enzymic degradation of herbicides), such processes may not always explain increased phytotoxicity. On the other hand, the altered processes may actually be secondary effects due to a temperature-induced perturbation of a membrane. One such membrane, the plasmalemma, controls the permeability of the cell; any disruption or modification of the membrane will alter membrane permeability, perhaps herbicide uptake, and in turn affect cellular metabolism directly or indirectly, or both. There are numerous reports that support this.

However, a herbicide-induced alteration of the membrane has not been clearly documented. Generally, herbicide mode of action studies are concerned with isolated systems. Effects are measured and observed changes are reported. But isolated systems tend to loose the perspective of the involvement of the whole plant. Furthermore, in order for a herbicide to be active at a particular site of action, it must have traversed at least one membrane, the plasmalemma, and quite often a second, the mitochondria or chloroplast. Herbicide-membrane interactions though very real, have rarely been looked at as a primary event in the effect of herbicides on living systems. It is particularly surprising since a simple membrane interaction by several herbicide molecules could have far reaching effects on cellular metabolism and plant response to environmental stresses.

The objectives of this study were to (1) characterize the response of the plasmalemma and mitochondria lipid composition to altered temperature, (2) evaluate herbicide-temperature interactions on membrane lipid composition as related to chilling response, and (3) determine whether ion concentration influences membrane lipid composition which in turn could affect herbicide uptake.

CHAPTER 1

EFFECT OF HERBICIDES ON PLANT CELL MEMBRANE LIPIDS

Abstract

Lipids are ubiquitous components of plant cells and are involved in numerous processes within the cell. One of the processes concerns cellular uptake of herbicides and involves active transport or diffusion of herbicides across the plasma membrane. With respect to the latter, the diffusivity of herbicides across the membrane is influenced by the lipid composition of the membrane and the lipid solubility of the herbicide. But the permeability of the membrane to the herbicide may also be affected by a physical interaction of the herbicide with the membrane (e.g., binding), or by a herbicide disruption of the processes responsible for maintaining membrane integrity. Such alterations in permeability can affect the transport of other solutes into or out of the cell or may affect the uptake of subsequent molecules of the same herbicide. In either event an imbalance or disruption of cellular metabolism ensues, and eventual death of the plant may occur.

Various herbicides have been reported to cause ultrastructural changes in membranes which have been generally manifested as ruptured membranes, swelling of chloroplasts and mitochondria, inhibition of grana formation, and loss of membrane integrity. Such modifications may be due to herbicidal effects on the synthesis of lipids and lipid

turnover rates within the membrane. Several studies have reported shifts in membrane lipid composition due to herbicide treatment. Generally the shifts were similar to changes that occur in nature due to temperature fluctuations or aging. Shifts in lipid composition will also exert an influence on the fluidity of the membrane, thus causing a shift in the phase transition point of the membrane. Increased susceptibility to freezing injury may be a direct result of a herbicidal alteration of membrane lipid composition. The physical state of membrane lipids is important in regulating the biological activity of membrane-related functions such as respiration, phosphorylation, chlorophyll fluorescence, and electron transport. Herbicide alteration of membrane fluidity may also influence membrane maintenance, inhibiting the insertion of new membrane fragments.

Reported changes in the phytotoxicity of herbicides due to temperature may be due to a direct herbicide influence on the membrane rather than changes in transpiration, uptake, or reduction in a detoxification mechanism. However, the latter changes could conceivably result from a herbicide-induced perturbation of the membrane. Herbicide-induced membrane alterations may also increase the susceptibility of a plant to environmental stresses. Alternatively temperature-induced changes in membrane lipid composition may increase the diffusivity of the herbicide across the membrane, thus enhancing the level of herbicide within the cell to the critical point necessary for phytotoxicity.

Application of herbicide combinations often results in increased or decreased activity of one or the other herbicide. Interaction with membrane structure and function by one herbicide, thus altering membrane permeability to the second, may be an explanation. The area of herbicide influence on membrane lipids and function, however, is still in its infancy, and more work is needed to clarify the specific roles of each.

INTRODUCTION

For herbicides to be phytotoxic, they must at one time or another move through a membrane, whether the process be in the roots or the shoots. Plant membranes are involved as structural components of the cell and also function in cell permeability, ion transport, electron transport, and enzyme activity. Since membrane lipids are intimately associated with many of the membrane functions, any alteration of the lipid components could have a profound effect on membrane function or structure (3).

ROLE OF LIPIDS IN MEMBRANE STRUCTURE AND FUNCTION

Central to any discussion on the effects of herbicides on plant cell membranes is an understanding of the structure and function of biological membranes. The latter has been the subject of numerous review articles (12, 23, 54, 122, 124, 172, 173).

Membranes consist primarily of lipids and proteins; other constituents include cholesterol, metal ions (in some but not all membranes), and water. Phospholipids represent the principal lipid class in the plasmalemma; however, membranes may contain various proportions of neutral glycerides, sphingolipids, glycolipids, and sterols, depending on the species (122). In general, the major phospholipids found in most plant tissues are phosphatidylcholine, phosphatidylinositol, phosphatidylglycerol, and phosphatidylethanolamine. Of the glycolipids, monogalactosyl diglyceride and digalactosyl diglyceride predominate and are the major lipid fractions found in leaves. Non-photosynthetic tissues have similar lipids but generally less sulfolipid and phosphatidylglycerol and more sterols and cerebrosides (95). Each of the lipid classes has a variety of fatty acids associated with it, the distribution of which includes the following major fatty acids[1]: Myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic acid (18:3). In regard to cellular membranes such as plasmalemma, mitochondria, nuclear and chloroplast membranes, longer or shorter chain fatty acids are generally present in lesser amounts depending on the species and type of membrane concerned. It is of interest to note that the phospholipid and fatty acid composition of inner and outer mitochondria membranes differ (120). Inner membranes are characterized by predominantly unsaturated fatty acids and diphosphatidylglycerol, whereas outer membranes are composed of a high content of phosphatidylinositol and fatty acids which are mostly saturated. These differences may explain their dissimilar biochemical and osmotic properties.

At present there is no one accepted model for membrane structure, but it is generally acknowledged that most of the lipids are in a bilayer configuration with some globular membrane proteins located within the membrane while other proteins are located exterior to or partially submerged in the lipids. The precise organization of proteins and lipids within any membrane, however, remains undefined, yet interactions between proteins and lipids play an important role in membrane stability (19, 21). Although lipids are involved physically and chemically in the maintenance of biological membranes,

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[1] Numbers in parentheses refer to carbon chain length and number of double bonds, respectively.

they cannot be considered as structurally inert (20, 83, 172). Lipids and proteins are involved in membrane dynamics, continually associating and dissociating from the membrane (56, 93, 147, 148). Investigation into the turnover rate of membrane constituents provides considerable insight into the dynamic state of the membrane (30, 54, 107, 113, 147). For instance, the rate of phospholipid degradation differs depending on whether individual classes or different parts of the molecule are being degraded (172). Omura et al. (130) demonstrated a longer half-life for the fatty acid portion of phospholipids and a shorter half-life for the glycerol backbone, however, transfer of a complete fatty acid to a new phosphatide was not ruled out. In a recent review, Simon (155) indicated that modification of either the phospholipid or protein fraction might affect permeability since both fractions contribute to membrane structure. Hence, a solute could be prevented from diffusing through the membrane by phospholipids in one region or by protein molecules in another.

The significance of membrane turnover rate and variation in membrane fatty acid composition becomes apparent when one considers the transition temperature of a membrane; that is, the temperature at which a change of state from solid crystal to liquid or vice versa occurs. When warmed from the solid state, lipids do not undergo a direct transition from crystalline to liquid state but rather pass through an intermediate mesomorphic or liquid-crystal state. The temperature at which transition occurs is dependent on fatty acid chain length and degree of saturation (21, 37, 109, 143, 153, 154). For example, increasing the percentage of 1,2-dipalmitoyl lecithin from 0% to 100% in a lipid mixture with 1,2-dimyristoyl lecithin and water increases the phase transition temperature from about 27 C to 47 C (21). Molecular motion studies (ESR) have shown that membrane fluidity is related to temperature and lipid composition and that this fluidity continues along a gradient from the polar head groups of the phospholipid bilayers to the terminal methyl group of the phospholipid fatty acids where fluidity is the highest (83, 151).

Experiments with both artificial and natural membranes have shown that sterols may have an important functional role in biological membranes (61). Sterols appear to function in membrane structure (128) as membrane stabilizers (57, 59, 60) and in modulating the tansition temperature (20). In the latter case, the presence of cholesterol appears to abolish or lower the transition temperature depending on cholesterol concentration such that the lipids retain a loose, liquid-crystal configuration even when cooled below the physiologic range of temperatures (102). The amount of sterol influences membrane compactness, since increasing sterol content decreases the surface area for absorption (171). The primary role of sterols appears to be non-metabolic in nature, one of involvement as an architectural component of the membrane, though other molecules may substitute for sterols. Grunwald (60) has shown a structural requirement for sterol activity in membrane permeability. It was determined that to be active the sterols must conform to a flat molecular configuration similar to cholesterol. Sterol-carbohydrate interactions in membrane structure have also been implied. Jollow et al. (91) demonstrated well defined mitochondrial profiles in anaerobic cells of Saccharomyces cerevisiae cultured in excess ergosterol and galactose. Those cells grown in ergosterol and glucose, however, had poorly defined profiles. Nevertheless, the role of sterols in plant membrane remains unclear and more work is needed to elucidate its involvement in membrane structure and function.

Changes in membrane lipid composition during growth have been studied to elucidate the relationship between lipid composition and function during development. The fatty acid composition of membrane phospholipids can be altered by supplementing the growth medium of a variety of micro-organisms with various substances (28, 50, 114, 129). This method has proved valuable for studying the role of lipids in membrane structure and function. It is possible that a shift in composition might be related to a particular stage of development in higher plants, and any interference with that shift may result in detrimental or disruptive effects to the plant.

In the course of early plant development, some shifts in lipid composition have been noted. During germination, the rate of phospholipid synthesis in soybeans (*Glycine max* (L.) Merr.) was high, but since only small changes in total phospholipid content was noted, the rate of degradation or turnover must also be high (192, 193). Total sterols also increased during the germination of several species (15, 25, 48, 84, 85), but the increase shown in tobacco (*Nicotiana tabacum* L.) was due to free and esterified sterols, not steryl glycosides (15). However, in mung bean (*Phaseolus aureus* Roxb.) tissue, the sterol content decreased with age (48). It is interesting to note that seedlings of *Digitalis purpurea* L. sown throughout the year in a controlled environment study demonstrated annual variations in sterol level and had periods of poor germination coincident with periods of high sterol content (86). Since sterols have been implicated in regulating membrane permeability, high levels of sterols might have reduced the degree of permeability constituents such as terpenoids, which are volatile oils found in some plant species, have also been reported (1, 141). A marked increase in lipid content due to light was noted during chloroplast development (75, 106, 157, 169), but the rate of increase differed for individual lipids. Galactolipids, which are rich in linolenic acid, increased the most. The effect of light in stimulating the production of galactolipids appeared to be either directly on the biosynthetic chain or indirectly through the formation of a galactose pool since etiolated pea (*Pisum sativum* L.) seedlings already contained galactolipids (169).

As plant tissues approach maturity, other changes in lipid composition occur. Galliard (44) suggested a selective degradation of plastid membranes during ripening when no difference in concentration of total lipids between pre- and post-climacteric apples were observed. However, a decrease in linolenic acid as a result of a decrease in galactosyl diglycerides was noted. Grosbois (55) working with ivy (*Hedera helix* L.) fruit determined that lipids (particularly phospholipids and neutral lipids) accumulated during the process of maturation. The accumulation was characterized by a large amount of oleic acid in the neutral lipid fraction. Davis and Poneleit (29) noted an accumulation of sterols in developing maize (*Zea mays* L.) kernels from 10 days after pollination to maturity. On the other hand, the lipid composition of wheat (*Triticum vulgare* Vill.) roots has been shown to be affected by the level of mineral nutrition (101). High salt levels increased total phospholipids and sulfolipids but decreased glycolipid and sterol content. Therefore, it is evident that during normal plant growth and development, lipid composition is undergoing constant changes or shifts due to intrinstic plant factors or environmental influences.

HERBICIDE EFFECTS ON MEMBRANE FUNCTIONS

Permeability

The differential ability of solutes to pass through a membrane is an expression of the permeability of a membrane, but is also characteristic of that membrane since all membranes are not alike. Permeability can also change with time or in respone to external factors (e.g., dehydration). It is generally agreed that passive entry or transport of a molecule into a cell is a reflection of its lipid solubility (89, 124, 155, 171). This solubility serves to create the required gradient. However, the concept of lipid solubility implies that if permeation were solely governed by solubility, then the rate of diffusion through a membrane would be approximately the same as diffusion through a bulk lipid phase. The fact that membranes discriminate can be attributed to the rigid orientation of the lipids as opposed to a bulk lipid phase. Large molecules diffusing through a membrane would tend to have a more disruptive effect with greater steric hinderances involved (89).

A number of solutes can penetrate a membrane more rapidly than one would expect by simple diffusion which has led several investigators to theorize the existence of pores in the membrane (89, 171). Pores are difficult to visualize in a membrane that is liquid-crystalline in nature, although the area occupied by the lipids is determined by the nature of the fatty acids and by lipid-lipid and lipid-protein interactions (19). Membranes which contain fatty acids having a high degree of saturation have less fluidity and hence more compactness (171). The presence of cholesterol, furthermore, prevents the lipid bilayer from assuming an orderly crystalline-gel configuration. However, though the lipids retain a loose, liquid-crystal organization, the surface area is decreased by the condensing effect of the sterol (19, 171). Lipid-protein interactions, on the other hand, involve configurational changes in either the lipid or protein fraction (19). These configurational changes may be related to the diffusional characteristics of a molecule passing through the membrane, but some proteins in the membrane have a type of subunit structure which has often been associated with narrow water-filled channels which could provide pores for the passage of water and small molecules (155).

The rate of diffusion of a solute through a membrane can also depend on the properties of lipids. Smith (158) showed that some lipids increase the transport of 2,4-D (2,4-dichlorophenoxyacetic acid) across a model lipid membrane, particularly the more polar lipids, lecithin and monogalactosyl dilinolenate. Increasing the acidity of the herbicide solution, however, decreased the rate of exchange. Smith (158) concluded that there was a higher partitioning coefficient for the dissociated anionic species in polar lipids. When potato (Solanum tuberosum L.) tuber tissue was similarly treated with 2,4-D in the presence of lecithin, rate of uptake was also increased. However, the author noted that total 2,4-D uptake in tissue favored the undissociated form of the herbicide rather than the dissociated form. From the data presented by Smith (158) it appears that transport (or absorption) was not measured but rather adsorption. Increasing pH promotes dissociation of the herbicide into the anionic species which favors an adsorption-desorption exchange mechanism associated with polar groups of the lipids (175). Decreasing pH of the herbicide solution, on the other hand, results in measurements that indicate the diffusivity of the undissociated molecule into and out of the lipid fraction. A more rapid penetration by the undissociated 2,4-D molecule into lipids was discussed by Simon and Beevers (156) and by Blackman and Robertson-Cunninghame (9). In the model membrane used by Smith, the lower rate of exchange at lower pH levels actually represents the differences between the rate of adsorption at higher pH and absorption or diffusion at lower pH. With respect to the tissue study, the same principles are involved. Low pH would favor diffusion of 2,4-D into the membranes of the tissue as well as into the externally applied lecithin, whereas higher pH levels would favor adsorption of 2,4-D onto the lipids. The apparent enhancement of "uptake" by lecithin at each pH level reported by Smith probably represents herbicide interaction with additional lipid volume. Support for this view can be found in studies by Hilton and Christiansen (79) and Hilton et al. (80) who determined that externally applied lipids decrease the uptake of trifluralin (α,α,α-trifluoro-2,6-dinitro-_N,N_-dipropyl-_p_-toluidine) and metflurazone (4-chloro-5-(dimethylamino)-2-(α,α,α-trifluro-_m_-tolyl)-3(2_H_)-pyridazinone) in several species resulting in a preferential partitioning of the herbicides into the external lipids. Endogenous lipid levels in the seeds were found to correlate significantly with sensitivity of the species to the herbicide, indicating a possible

Table 1. Octanol-water partition coefficient of the benzoic acids. Taken from Glass (51).

Acid	Inhibition	Partition Coefficient
	%	
3,4,5-Trihydroxybenzoic acid	18	0.83
3,4-Dihydroxybenzoic acid	28	1.2
4-Hydroxy-3,4-dimethoxybenzoic acid	38	1.28
p-Hydroxybenzoic acid	41	1.58
Benzoic acid	54	1.87
4-Hydroxy-3-methoxybenzoic acid	71	1.72
o-Hydroxybenzoic acid	85	2.21

role for endogenous lipids (79).

The penetration of lipophilic solutes through a membrane involves the concept of lipid solubility in the lipoidal component of the membrane. Such a relationship appears to be involved in the inhibition of inorganic phosphate uptake by a series of substituted benzoic acids (51). Glass (51) postulated that benzoic acids alter barley (Hordeum vulgare L.) root membrane permeability to phosphate since it had been previously shown that intracellular concentrations of the phenolic compounds approached zero due to high glucosylation rates (52). Interestingly, increasing inhibition by the benzoics was strongly correlated with increasing lipid solubility (Table 1). Differential sensitivity to the phenolic compounds may be explained on the basis of differential rates of detoxification by conjugation which alters the lipid solubility of the phenolics. Work with labeled herbicides have shown that there are two routes of herbicide movement into plants, an aqueous route for water-soluble compounds and a lipoidal route for lipid soluble compounds (26, 27). Crafts (26, 27) suggested that the cuticle represents a matrix of wax which forms a lipoidal phase and pectin strands containing micropores form an aqueous route. Since many herbicides are fairly lipophilic, they probably would have little difficulty penetrating plant cells. Enhancement of herbicide phytotoxicity through altered membrane permeability characteristics in surfactant-treated plants may also be due to lipid interactions (118). The effect of herbicides on membrane permeability can be monitored by examining the flux of endogenous solutes. Wilkinson and Smith (189) measured betacyanin efflux from "ages" red beet (Beta vulgaris L.) root and found that as temperature and EPTC (S-ethyl dipropylthiocarbamate) concentration increased, so did betacyanin efflux. Dichlobenil (2,6-dichlorobenzonitrile) at 10^{-4} M has also been found to produce a significant increase in betacyanin efflux but only after 8 to 12 hours of treatment[2]. However, Magalhaes and Ashton (110) studied the effect of dicamba (2,6-dichloro-o-anisic acid) on purple nutsedge (Cyperus rotundus L.) membranes and found the herbicide to reduce the permeability of the membranes as measured by a decrease in efflux of endogenous solutes into an external solution. Herbicide enhancement of efflux from seeds or roots of plants may play a role in increased incidence of disease occasionally seen in the field (73, 145), particularly since fungal spore germination is often markedly influenced by exudation from plants (24, 149).

Herbicides have also been shown to affect the influx of solutes. Cells of Chorella pyrenoidosa were treated with several concentrations of 2,4-D and exhibited alterations in permeability to various solutes (178). With increasing concentrations of 2,4-D ranging from 10^{-7} to 10^{-2} M, Chlorella cells exhibited decreasing permeability to sucrose and mannitol but no inhibition of PO_4 uptake and no effect on SO_4 or NaCl uptake. It was suggested that the decrease in permeability might be due to an auxin effect (e.g., 2,4-D) on the membrane decreasing the area receptive to solute diffusion. This suggestion was substantiated by the work of Morre and Bracker (123) and Helgerson et al. (78) which

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[2] Price, H.C. 1969. The toxicity, distribution and mode of action of dichlobenil (2,6-dichlorobenzonitrile) in plants. Ph.D. dissertation. Michigan State University, East Lansing.

demonstrated a condensing effect in the plasma membrane from auxin treatment resulting in increased microviscosity. Wyse et al. (198) noted an increase in uptake of ^{14}C-atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine) in plants pretreated with 10^{-5} or 10^{-6} M EPTC; however, the increased uptake appeared to be associated with an increase in transpiration, but permeability to atrazine may have also been affected by EPTC-induced changes in the membrane. Likewise, Hamill and Penner (64, 65, 66) demonstrated a synergistic reduction of barley growth and increased herbicide uptake when treated with a combination of carbofuran (2,2-dimethyl-2,3-dihydrobenzofuranyl-7-N-methylcarbamate) and either alachlor (2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide), chlorobromuron (3-(4-bromo-3-chlorophenyl)-1-methoxy-1-methylurea), or butylate (S-ethyl diisobutylthiocarbamate). It was thought that carbofuran seed treatment modified barley permeability to the herbicides. In a similar study, Schulz et al. (150) found that EPTC enhanced the uptake of the insecticide, phorate, in corn. Addition of the antidote, R-25788 (N,N-diallyl-2,2-dichloroacetamide), had no effect on the EPTC-enhanced uptake of phorate but did counteract the phytotoxic effect of EPTC on corn. These studies and others (17, 34, 103, 104, 134, 179) suggest that membrane permeability and, hence, solute uptake is affected by chemical interaction with membrane lipids.

There is evidence that the action of several herbicides results in peroxidation of membrane lipids (in particular, unsaturated lipids) resulting in loss of membrane integrity and inactivation of membrane-bound enzymes, such as ATPase. The most notable of these are the bipyridyliums, namely paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) and diquat (6,7-dihydrodipyrido(1,2-α:2',1'-*c*)pyrazinediium ion). These herbicides accept electrons from the light reaction in photosynthesis in a reductive step. Subsequently, autoxidation yields the original herbicide ion and generates the potentially damaging free radical, superoxide anion, singlet oxygen, or hydrogen peroxide (14, 38). In the light, diquat-induced superoxide anion involvement in the peroxidation of lipids in *Scenedesmus* cells has been studied by Van Rensen (174), and shown to cause a reduction in chlorophyll content, primarily chlorophyll *a*. Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) reduces lipid peroxidation caused by diquat, indicating the diquat reduction occurred at a point in the photosynthetic electron transport system beyond the inhibition of the Hill reaction. Likewise, cysteine protected lipids from peroxidation. On the other hand, the antioxidant, butylated hydroxytoluene, did not counteract diquat-induced lipid peroxidation in whole cells of *Scenedesmus*, but did in isolated chloroplasts (77). Lipid peroxidation in animals appears to involve the production of fatty acid hydroperoxides from the reaction between singlet oxygen (formed from superoxide radical) and lipids (14). A similar situation may exist in plants. Disruption of membrane integrity by lipid peroxidation caused increased efflux of potassium ions from flax (*Linum* *usitatissium* L.) cotyledon leaves treated with paraquat (70).

In addition to lipids, sterols have been implicated in controlling membrane permeability (57, 59, 60, 61). Sterols are involved as structural components of membranes (58, 97) and occur in higher plants as free sterols, sterol esters and sterol glycosides (58). However, only free sterols are thought to be involved in changing membrane permeability (59, 60). Sterol metabolism in plants has been studied extensively, but there are no known reports on the effect of herbicides on sterol production; only the effect of other chemicals on sterols have been reported. Geuns and Vendrig (49) studied the effect of the hormone, naphthalene acetic acid (NAA), on sterol biosynthesis and found that the sterol content of mung bean hypocotyl sections increases primarily in the zone of elongation. The increased sterol synthesis could be explained on the basis of an enhanced conversion of cycloatenol into sterol. The NAA treatment also increased the stigmasterol/sitosterol ratio. Gibberellic acid, on the other hand, had been shown to effect little change in the sterol content of hazel (Corylus avellana L.) cotyledons but exhibited increased 2-^{14}C-mevalonic acid incorporation into free and esterified sterols (152). Gibberellic acid has also been reported to interact with lecithin, suggesting a possible hormonal mechanism for the control of permeability (195, 196). Ozone has been shown to decrease the total amount of sterols in Phaseolus vulgaris leaves but increase the steryl glycosides and acylated steryl glycosides (166). However, the effect could be counteracted with antisenescents such as benzimidazole, kinetin, or N-6-benzyladenine(167). Since sterols appear to be involved in membrane permeability, it is interesting to note the effect of antisenescents on sterol production. Grunwald (61) postulated that ozone may act to increase membrane permeability to converting free sterols to steryl glycosides and acylated steryl glycosides, but the antisenescents inhibit the conversion and, hence, the increased permeability. Kuiper et al. (101) determined that high salt solutions caused wheat and oat (Avena sativa L.) roots to be low in glycolipid and sterol content; however, the proportion of sterols in the membrane fractions was enhanced. Another aspect of sterol function in membranes concerns the property of polyene antibiotics that lyse various fungi (e.g., *Neurospora crassa*) but not bacteria (32). Of importance in the lipid composition is the lack of sterols in bacteria which are present in the fungi.

Increase the cell permeability often occurs at sites of infection by facultative pathogens (53), but increases or decreases in cell permeability some distance from the lesion have also been reported (68, 105). Hancock (68) noted that permeability of cells in sunflower (*Helianthus annus* L.) hypocotyl sections above lesions caused by *Sclerotinia sclerotiorum* were less than for control plants. Lai et al. (105) on the other hand, reported increases in permeability in *Phaseolus aureus* in advance of pathogenesis by *Rhizoctonia solani*. Frequently the use of herbicides has been shown to affect the incidence of disease. Some of the herbicides reported to increase disease occurrences are 2,4-D and dalapon (2,2-dichloropropionic acid) in tomato (*Lycopersicon esculentum* Mill.) (145) and trifluralin in cotton (*Gossypium hirsutum* L.) (16). Increases in incidence of disease due to herbicide treatment is often attributed to enhanced exudation from the roots which implies increased membrane permeability (24, 73, 149). Those herbicides reported to decrease the incidence of disease are DMPA (*o*-(2,4-dichlorophenyl)-*o*-methyl-isopropylphosphoramidothioate) (39) and trifluralin (73) in peas and propham (isopropyl carbanilate) and dinoseb (2-*sec*-butyl-4,6-dinitrophenol) in tomatoes (145). The reduction in disease may be due to an inhibition of exudation or a fungistatic effect of the herbicide.

Modification of cellular membrane permeability appears to be affected by a number of factors. Increases or decreases in membrane permeability may result from fluctuations in temperature or by herbicide treatment. In the latter case, pretreatment with one herbicide may affect uptake of another herbicide or systemic insecticide. Additionally, herbicide-induced increases in exudation may enhance the incidence of disease. Membrane integrity is maintained in plants through the expenditure of energy (ATP) which is used in the synthesis of membrane components and in the active transport of materials across the membrane. Herbicide interference with the latter through an inhibition of ATP production would be expected to manifest itself as a disruption of membrane integrity and increased membrane permeability. Inhibition of ATP synthesis has been postulated to be partially responsible for the accumulation of lipid globules and poor thylakoid development in pyrazon-treated (5-amino-4-chloro-2-phenyl-3(2H)-pyradazinone) bean (Phaseolus vulgaris L.) leaves (2). St. John and Hilton (164) demonstrated a rapid increase (within one hour) in membrane permeability in roots of 4-day-old wheat seedlings treated with 10^{-4} M dinoseb, though membrane integrity was not ultrastructurally examined. But dinoseb was also observed to decrease the polar lipid content of the seedlings suggesting an alteration of membrane function through lipid synthesis inhibition. These studies suggest that herbicides interfere with membrane permeability probably through interaction with the lipoidal component of the membrane. This interference can influence the transport of solutes across the membrane whether the solutes are endogenous compounds or herbicides.

Metabolism

Cellular membranes are involved in multienzyme system activities in addition to functioning as a permeability barrier and in the transport of solutes. Most membranes have enzymes associated with their surfaces, and enzymes, such as cytochrome oxidase (170), require a membrane surface or exhibit a lipid requirement to work efficiently (23, 122). Many of the studies on effect of herbicides have dealt with the processes of oxidative phosphorylation, electron transport, and ATP production. Individual enzymes, like cytochrome oxidase, of the electron transport chain of mitochondria were not affected by 2,4-D or 2,4,5-T (2,4,5-trichloroacetic acid) but rather the entire enzyme complex was affected (165). Since these enzymes are membrane-bound in the mitochondria (36, 40), it is conceivable that auxin-like herbicides (e.g.,2,4-D) might act to interfere with membrane structure, forming complexes with metallic ions such as copper, affecting membrane structure and proton equilibrium, and thereby affecting oxidative phosphorylation (4). Moreland et al., (121) studied the effect of diphenylether herbicides on mitochondria and chloroplasts of spinach (Spinacia oleracea L.) and concluded that phytotoxicity might be due to intereference with ATP generation. Likewise, picloram (4-amino-3,5,6-trichloropicolinic acid) inhibits ATP generation in oxidative phosphorylation (42). Picloram also caused a swelling in mitochondria that had previously been contracted by ATP; however, as opposed to known mitochondrial swelling agents, it could not overcome a succinate-induced contraction or enhance 0.3 M KCl swelling (18). Pyriclor (2,3,5-trichloro-4-pyridinol) or a metabolite of pyriclor also inhibited oxidative phosphorylation, but it additionally increased the number of mitochondria in tobacco (*Nicotiana tobacum* L.) chloroplast (47). Chlorpropham (isopropyl-*m*-chlorocarbanilate) inhibited both the photochemical and oxidative production of ATP, whereas diuron only inhibited the photochemical production (161).

Inhibition of oxidative phosphorylation and hence ATP production may be related to a phtotoxin-induced increase in mitochondrial membrane fragility. Peterson et al. (138) demonstrated a genetically controlled effect of a pathotoxin from *Helminthosporium maydis*, race T., on mitochondria isolated from etiolated maize seedlings. The pathotoxin (not identified) could inhibit oxidative phosphorylation and electron transport and overcome malate and succinate inhibition of ATPase in sensitive mitochondria. These mitochondria were obtained from plants with the Texas cytoplasm which confers male sterility and field susceptibility to *H. maydis* race T. It was hypothesized that the pathotoxin binds to the inner membrane of sensitive, but not resistant mitochondria and the that binding site is controlled by cytoplasmic DNA. This suggests that possibility of genetically controlled sites of action for other toxins such as herbicides. In contrast, Haslam et al. (74) suggests that the loss of energy-linked reactions in mitochondria may be due to increased proton permeability caused by a change in the physical properties of the lipid phase of the inner mitochondrial membrane. However, it is conceivable that any herbicide action that results in the loss of energy-linked reactions in mitochondria might be due to a binding of the herbicide to the membrane such that permeability is affected; hence the observed increase in flux of protons. Herbicide binding to chloroplast thylakoid membranes of *Bumilleriopsis filiformis* Vischer has been suggested by Roger et al.

(10) as a mode of action for bentazon (3-isopropyl-2,1,3-benzothiadiazin-4-one-2,2-dioxide). The length of time chloroplasts were incubated with bentazon influenced the recovery potential for electron transport inhibition. Since whole cells exhibited slow but partial recovery from inhibition of photosynthesis (photosytem II), it appeared that bentazon did not destroy the redox functions of the thylakoids, although binding seemed to be quite strong. In isolated chloroplasts, however, the binding was not as strong and recovery was quick, suggesting that a metabolite may be the active agent in whole cells. If the binding site were genetically controlled as Peterson et al. (138) suggests, it may provide an explanation for the mode of action of some herbicides. But concerning herbicides, there is a paucity of published work regarding effect on energy driven transport processes in the membrane; however it is due in part to the lack of knowledge regarding membrane structure and function.

EFFECT ON MEMBRANE STRUCTURE

Ultrastructure

Several studies have shown that herbicides can produce observable modifications of cellular membranes (3, 124). These changes were primarily manifested as separations in the thylakoids of chloroplasts and ruptured membrane envelopes (2, 5, 6, 47). Other common effects include loss of starch, inhibition of grana formation, swelling of chloroplasts and mitochondria and loss of membrane integrity by herbicides such as pyriclor (47), pyrazon (2), atrazine (5, 6), and bromacil (5-bromo-sec-butyl-6-methyluracil) (3). Interestingly, pyrazon also caused an increase in the size and quantity of lipid globules, lipids that probably would have been used in thylakoid development. On the other hand, pyriclor caused an increase in the number of mitochondria in tobacco chloroplasts but did not affect their size or shape (47). White and Hemphill (181) found that the response of tobacco leaf mesophyll cells to 2,4-D was related to the age of the leaf at the time of treatment. Whereas young leaves from the upper portion of the plant exhibited high tolerance to 2,4-D, older, more mature leaves were highly sensitive to the herbicide. Response to 2,4-D involved rupturing and disintegration of the tonoplast, plasmalemma and membranes of chloroplasts and mitchondria, suggesting an involvement of membrane lipid composition. But it is also possible that since membrane lipid turnover rate and, hence metabolism, is slower in older tissues, 2,4-D activity directly on the membrane might be less effective in younger tissues with higher metabolism and lipid turnover rates. Of course, the activity of 2,4-D would be concentration dependent. Baur et al. (7) studied the effect of paraquat on mesquite (Panicum obtusum H.B.K.) and reported that membrane disintegration occurred within 5 minutes after treatment followed by disruption of chloroplast membrane. Interference with membrane formation was also reported by St. John and Hilton (164). The authors noted that membrane structure and function were altered by dinoseb and MBR 8251 (1,1,1-trifluoro-4'-(phenylsulfonyl)-methanesulfono-o-toluidide) activity which decreased polar lipids that were required for membrane formation.

Herbicide action on membrane ultrastructure appears to commonly affect membrane integrity as evidenced by ruptures and disintegration of membrane envelopes. If membrane integrity was affected quickly, herbicides may have acted directly on the membrane by binding to membrane lipids and modifying permeability. If membranes were affected over a longer period of time, herbicides may have indirectly influenced membrane structure by affecting lipid biosynthesis.

Lipid biosynthesis

The effect of herbicides on lipid synthesis and degradation has only recently been examined. Early studies were concerned with the effects of herbicides on cuticular waxes. As early as 1957, Juniper (92) observed that TCA (trichloroacetic acid) altered the surface of pea leaves and correlated this with increased wettability and susceptibility of subsequent herbicidal sprays. Kolattukudy (98) showed that TCA inhibited wax synthesis from ^{14}C-acetate by cabbage and broccoli (Brassica oleracea L.) leaves. The synthesis of fatty acids in the leaves was not inhibited at TCA concentrations that inhibited surface wax deposition or synthesis, indicating that the two processes are separate. The effect of TCA on epicuticular waxes has been reported by a number of investigators (33, 41). The thiocarbamates, EPTC and diallate (S-(2,3-dichloroallyl) diisopropylthiocarbamate have also been shown to be active in reducing wax deposition on cabbage (45) and pea leaves (160). Gentner (45) further reported that the degree of wax inhibition and the rate of transpiration in cabbage was dependent on the rate of EPTC applied. Wilkinson (182) examined the activity of diallate in relation to photoperiod on sicklepod (Cassia obtusifolia L.) leaflets and similarly determined that as diallate concentration was increased, epicuticular fatty acid content decreased. Fatty alcohols, however, were increased 200% over control levels with only 0.28 kg/ha diallate. Treatment with increasing photoperiod increased fatty acid, fatty alcohol and hydrocarbon content. Kolattukudy and Brown (99) also reported a reduction in cuticular lipid biosynthesis in young pea leaves by several thiocarbamates. The authors furhter noted an inhibition of ^{14}C-acetate incorporation into long-chain cuticular lipids, resulting in an accumulation of label in shorter-chain lipids. The effect of thiocarbamates in reducing cuticular lipid formation appears to be through an inhibition of various chain-enlongating enzyme systems. The reduction of wax deposition in plants may play a role in split applications of herbicide combinations that result in greater injury to the crop by the post-emergence application (33, 139).

It is known that herbicides inhibit many of the processes that involve lipids, but information regarding the interaction between lipids and herbicides has only recently been studied. Mann and Pu (111) demonstrated an inhibition of lipogenesis by several herbicides as measured by the reduction of label incorporation from malonic acid-2-^{14}C into lipids by excised hypocotyls of hemp sesbania (Sesbania exaltata (Raf.) Cory). Similarly Wilkinson and Smith (188) noted an inhibition of incorporation of ^{14}C-malonic acid as well as ^{14}C-acetate into lipids of isolated spinach chloroplasts treated with 33 μM EPTC or 90 μM diallate. However, when excess acetate or malonate was added, the inhibition was not seen. Treatment of the chloroplasts with 1,8-naphthalic anhydride or R-25788 or 10^{-7} M reversed the inhibition by 10^{-5} M EPTC (187). Similar data was reported for red beet root tissue (189). Hardcastle et al. (69) found that metribuzin (4-amino-6-tert-butyl-3-(methylthio)-as-triazin-5(4H)-one) increased the total fatty acid content of soybean oil in several varieties over the levels found in untreated plants. In 'Hampton' cultivar several saturated fatty acids (namely, myristic, palmitic and stearic acids) decreased, whereas two unsaturated fatty acids (linoleic and linolenic acids) increased. Other studies with soybeans showed no effect on oil content with various herbicides (90, 136, 177), but some shifts in fatty acid composition were observed (136). Increases in linoleic acid content appeared to correlate with decreases in stearic acid, indicating a possible stimulation in the conversion of stearic acid to oleic acid to linoleic acid. Investigations on the effect of various herbicides on oil content of seeds from flax (126), corn (137), and cotton (185, 186) also proved negative. Wilkinson and Hardcastle (183, 184) studied the effect of EPTC on sicklepod and reported no alteration in the fatty acid content of sicklepod petioles by EPTC; however, cuticle thickness of the petiole decreased as much as 35% as herbicide concentration was increased from 0.14 to 4.48 kg/ha. But when sicklepod leaflets were examined, the authors noted that EPTC had affected the fatty acid content quantitatively but not qualitatively. Nonphytotoxic levels of EPTC increased total fatty acid content even though there was a corresponding inhibition of cuticle formation. The authors suggested that EPTC acts to block the conversion of fatty acids into hydrocarbons (a main constituent of cuticular wax). Their suggestion was substantiated by an observed decrease in long chain hydrocarbon content with increasing EPTC rates (184). EPTC has also been shown to affect the fatty acid composition of wheat root phospholipids (94). Total fatty acid content was reduced 39% by as little as 1 mg EPTC per kg sand. In fact, all major fatty acids were decreased 50% to 70% of the control by nonphytotoxic levels of EPTC. Hilton and Christiansen (79) found that lipids applied to filter paper

Figure 1. Relative effectiveness of various lipids in circumventing metflurazone inhibition of chlorophyll formation in mustard cotyledons. Taken from Hilton et al. (80).

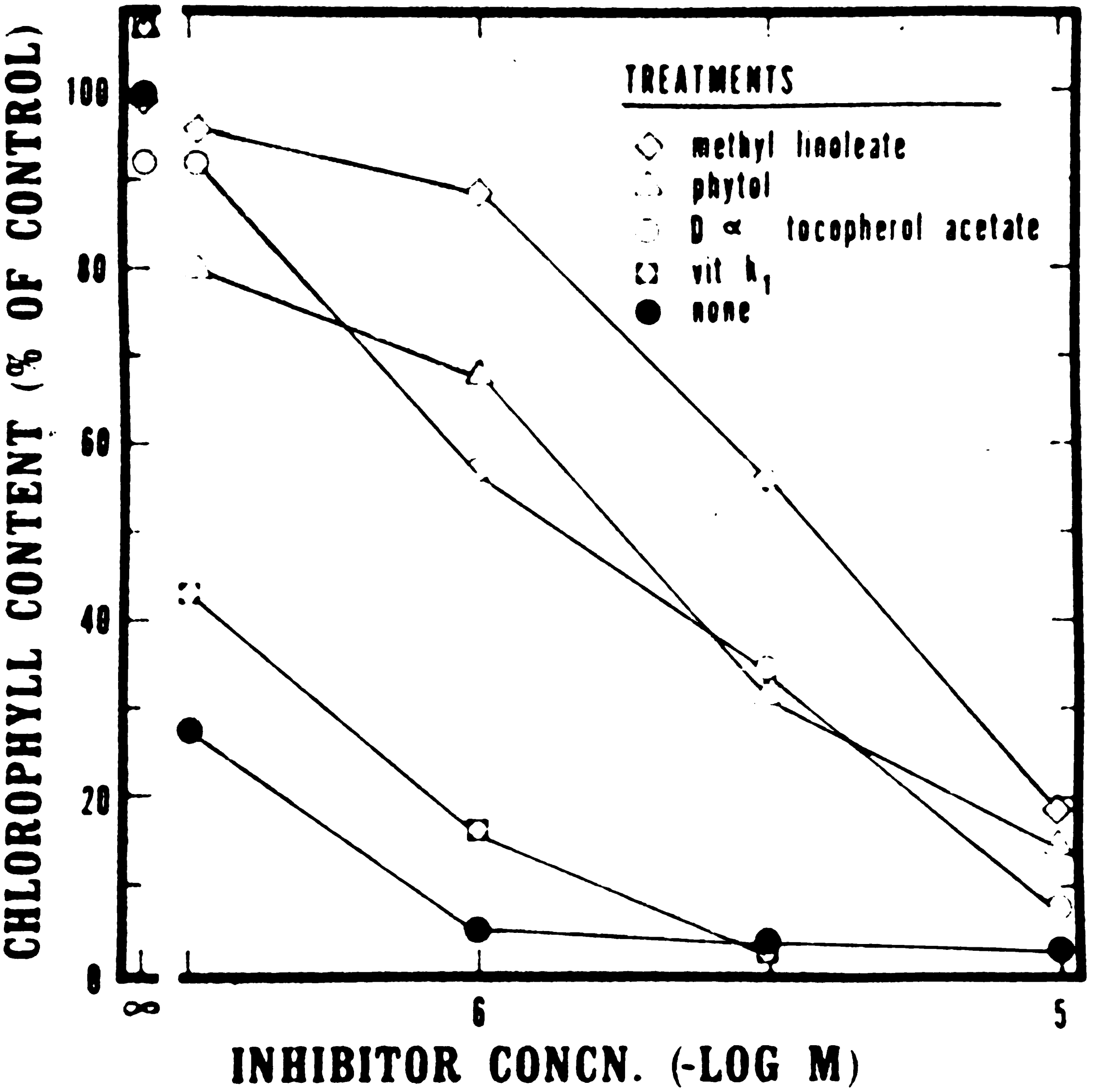
CHLOROPHYLL CONTENT (% OF CONTROL)
100
80
60
40
20
0
∞
6
5
INHIBITOR CONCN. (-LOG M)
TREATMENTS
◇ methyl linoleate
△ phytol
○ D α tocopherol acetate
☐ vit K1
● none

Figure 1

or to soil decreased the phytotoxic action of trifluralin to numerous plant species. A significant correlation between endogenous lipid level and sensitivity of the species to trifluralin indicated a possible role for endogenous lipids in imparting tolerance.

Hilton et al. (80) also investigated the role of certain lipids in chloroplast development as affected by a pyridazinone compound known to inhibit chlorophyll pigment formation. Metflurazone completely inhibited the formation of chloroplast pigments in mustard (Brassica juncea (L.) Coss.) cotyledons and in barley first leaves, but the inhibition was prevented by the presence of D-αtocopherol acetate, phytol, farnesol, squalene, or by certain unsaturated fatty acids such as palmitoleate, oleate, linoleate, and linolelaidate and their methyl esters (Fig. 1). Interestingly, more protectant was needed at a lower temperature (27 C) than at a higher temperature (34 C). Metflurazone also reduced the amount of fatty acids in polar galactolipids of barley chloroplasts and increased the fatty acids in the nonpolar lipid fraction. The protective action afforded by methyl linoleate and other compounds (e.g., tocopherol) was thought to be due to a preferential partitioning of metflurazone into the externally applied lipids. Although the inhibitor was ineffective, it was present at an inhibitory level in mustard cotyledons. Since metflurazone was not prevented from reaching the chloroplast nor from influencing polar lipid formation (specifically, the conversion of linoleic to linolenic acid), the authors felt that the protective action of methyl linoleate might be explained on the basis of a substrate-product effect. On the other hand, the action of tocopherol appeared to be related to the protection of existing unsaturated fatty acids from peroxidation.

It appeared that the mode of action of metflurazone may be to inhibit chlorophyll formation and the utilization of other lipids for lamellar membrane formation. In contrast to metflurazone action (80), Weinberg and Castelfranco (180) noted that EPTC enhanced chlorophyll accumulation in cotyledons of etiolated cucumber (*Cucumis sativus* L.) seedlings. EPTC-treated plants had 35-45% more protochlorophyll and 30-40% more glycolipids than control plants. Other herbicides tested, namely CDAA (*N*,*N*-diallyl-2-chloroacetamide), propachlor (2-chloro-*N*-isopropylacetanilide), chloropropham and propham, did not produce similar effects.

Studies with other compounds indicate that effects on chloroplasts lipids may be common. When isolated spinach chloroplasts were treated with either simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine) or atrazine at several concentrations, total free fatty acid content increased (159). The level of free fatty acid was approximately the same regardless of herbicide concentration; however, qualitatively the herbicide treatments increased the amount of unsaturated rather than saturated fatty acids. St. John (162) demonstrated an ability to manipulate the fatty acid composition of wheat chloroplast galactolipids with various substituted pyridazinones such that there was either a shift in the linoleic to linolenic ratio, or a greater proportion of saturated to unsaturated fatty acids, or a preferential biosynthesis of digalactosyl diglycerides over monogalactosyl diglycerides. Manipulation of lipid composition by chemical treatment may be a valuable tool in determining the role of lipids in the structure and function of membranes.

The effect of herbicides on lipid metabolism and possible physiological significance was studied by St. John and Hilton (164). Dinoseb and MBR 8251 inhibited the enzymic synthesis of glycerides *in vitro*. However, *in vivo*, there was an accumulation of free fatty acids and decreases in neutral and polar lipids in wheat (*Triticum aestivum* L.),'Mediterranean' (C.I. 5303) seedlings. The authors suggested that the herbicides act through an interference with membrane formation by limiting the synthesis of lipids required for membrane structure. Since membrane structure was altered, it appeared that membrane function might also be affected. Membrane permeability studies by the authors with intact wheat roots indicated that dinoseb was more effective in increasing membrane permeability than was MBR 8251, although MBR 8251 was taken up more slowly. Therefore, it was thought that the mode of action of both herbicides was through an alteration of membrane stucture and function by inhibiting polar lipid biosynthesis.

Herbicide alterations of membrane lipid components have not been studied in terms of whether the alterations were permanent or temporary (94, 162, 163, 164). One would suspect that as long as the herbicide were present in an effective concentration, membrane lipid composition would continue to be affected. Information of this nature may provide insight into a possible mode of action.

Phase transitions

Lateral diffusion and phase separations in intact and functional membranes are regulated by phospholipid hydrocarbon chain dynamics (116, 119, 125, 129, 131, 132, 153). Hydrocarbon chain dynamics are in turn influenced by temperature (143), external ion concentration (87), lipid-protein interactions (21), and lipid-lipid interactions

(54). From studies with model membranes the temperature at which a phase transition between liquid crystalline and solid state occurs is dependent on the lipid species as well as the fatty acid composition (20, 21, 82, 83, 151); increasing the chain length or the degree of saturation will raise the transition temperature. On the other hand, the presence of cholesterol lowers the transition temperature (102). This transition temperature appears in a fairly narrow temperature range in simple model membrane, but in biological membranes with a mixture of lipid species and a heterogenous fatty acid composition, the transition occurs over a wider range of temperatures (21, 125). Although some herbicides have been shown to alter lipid composition (94, 162, 164), no reports could be found relating the effects of herbicides on plant membranes to phase transitions.

Studies on the physical state of membrane lipids have demonstrated its importance in determining the biological activity of membrane-related functions (87, 100, 119, 125, 142, 143, 153). Recently, electron spin resonance (ESR) methods have enabled researchers to probe the membrane matrix with spin-labeled compounds specific for membrane lipids to study the structural and kinetic features of biological membranes. It is apparent from such studies that a change in the physical state of membrane lipids due to various factors (e.g., temperature, ions, pH, etc.) causes a conformational change in membrane proteins or enzymes which appears as a discontinuity in Arrhenius plots of membrane-related functions. Using ESR techniques, Miller et al. (119) noted adverse changes in the rates of wheat mitochondrial respiration and phosphorylation efficiency in the temperature regions at which transitions occur. The authors reasoned that since those functions involve lipoproteins, lipid-protein interactions may be responsible for the structural changes seen. Transition of the physical phase of thylakoid membrane lipids of Anacystis nidulans influenced its photosynthetic characteristics (125). The transition point of the membrane lipids coincided with maximum yield of chlorophyll a fluorescence, a change in the activation energy of electron transport reactions, and a configurational change in the thylakoid membrane. Temperature-dependent phase transitions in the mitochondrial membranes have also been shown to coincide with the temperature below which chilling injury was observed (143). Mitochondria obtained from sweet potato (Ipomoea batatas L.), a chilling-sensitive plant and rat liver (Sprague-Dawley Albino), a homeothermic animal, were observed to have a phase change at 12 and 23 C, respectively. Chilling injury occurred in sweet potato below 12 C. In contrast, mitochondria from potato tuber, a chilling-resistant plant, and rainbow trout (Ictalurua punctatus) liver, a poikilothermic animal, did not show a phase change. It was noted that although potato and trout mitochondria had greater amounts of unsaturated fatty acids, there was a temperature-dependent disruption in the uptake of oxygen by sweet potato and rat mitochondria. This led the authors to suggest that the physical state of the membrane lipids had a considerable influence on the function of some membrane-bound enzymes since oxygen uptake is enzyme dependent. Support for their suggestion concerning membrane lipid influence came from an additional study by Raison et al. (144) that reported no temperature-induced changes in succinate oxidase, succinate dehydrogenase or cytochrome c oxidase activity in sweet potato or rat liver mitochondria after treatment with detergent to remove lipid material.

Although lipid phase transition affect membrane-related functions, it is possible phase transitions might also play a functional role in cell membranes. Membranes in a crystalline-gel or semi-crystalline state contain lipids expressing a high degree of "lateral compressibility and extensibility" (153). This would permit the addition of new protein or lipid molecules or new membrane fragments to the membrane without increasing its surface area. It would also impart the necessary fluidity for the translocation of solutes by protein carriers across the membrane.

Herbicide-induced changes in membrane lipid composition will affect membrane-related functions and could cause a shift in the temperature at which phase transitions occur. Thus, reports of herbicide inhibition of various mitochondrial enzymes systems (42, 108, and others) may be measurements of the indirect effects of a herbicide-induced perturbation of the mitochondrial membrane. Likewise, enhancement of fatty acid saturation by herbicides would be expected to shift the phase transition point to a higher temperature and possibly increase a plant's susceptibility to chilling injury or alter the temperature-herbicide absorption relationship. The relationship between fatty acid saturation and chilling injury has been shown for vernolate (S-propyl dipropylthiocarbamate) in soybeans (Rivera and Penner, unpublished) and pyridazinones in cotton seedlings (163). Unfortunately, no phase transition temperatures were measured, although one would susepect that since changes in fatty acid composition have already been shown to shift phase transition temperature, any herbicide-induced changes would do the same. Alterations in membrane lipid composition, if in effect for a sufficient period of time, could disrupt cellular functions, inhibit membrane maintenance, enhance plant sensitivity to environmental stresses, and lead to the eventual death of the plant.

INTERACTION OF HERBICIDES AND TEMPERATURE ON MEMBRANE LIPIDS

Changes in environmental temperature can alter herbicide activity. Johnsongrass (*Sorghum halepense* (L.) Pers.) control with dalapon at 1.12 kg/ha was greater when plants were grown at 16 C than at 27 or 38 C (115). Penner and Graves (135) demonstrated greater injury to some but not all navy bean varieties that were treated with alchlor at 20 or 25 C, but no injury was observed at 30 C. Likewise, trifluralin showed greater activity on peanuts (*Arachis hypogaea* L.) (76) and on tomatoes transplanted early in the spring (194) when temperatures were cooler. Increased phytotoxicity at low temperatures has also been shown for MSMA (monosodium methanearsonate) on cotton (96), 2,4-D on spring wheat (11), and bentazon on redroot pigweed (*Amaranthus retroflexus* L.) (127). In contrast to increased activity at low temperatures, EPTC injury to several corn cultivars was greater at 30 C than at 20 C (13). Dudek et al. (35) similarly noted that terbutryn [2-(*tert*-butylamino)-4-(ethylamino)-6-(methylthio)-*s*-triazine] and propazine [2-chloro-4,6-bis(isopropyl-amino)-*s*-triazine] had greater phytotoxicity to wheat and sorghum (*Sorghum bicolor* (L.) Moench) at 32 C than at 16 C. Likewise, desmediphan (ethyl-*m*-hydroxycarbanilate carbanilate (ester) activity on sugarbeet (*Beta vulgaris* L.) was most severe at 25 to 30 C but decreased with higher or lower temperatures (8). But the greater activity of various herbicides at higher or lower temperatures may be due to increased transpiration (133), greater uptake (63, 133, 176), or a reduced enzymic capacity to detoxify (168). However, a temperature effect at the site of herbicide action could also explain the increased activity since plants grown at different temperatures appear to vary metabolically as well as morphologically (67).

In a variety of biological systems the response of lipid composition to temperature has been shown to increase in unsaturated fatty acid content with a decrease in environmental temperature (27, 72, 81, 117, 129, 146, 190). Such a response has been shown for Escherichia coli (112), Bacillus licheniformis (43), alfalfa (Medicago sativa L.) (46), and flax, sunflower and castor (Ricinus communis) (71). All species exhibited increases in unsaturated fatty acid content, primarily linoleic and linolenic acids, with decrease in temperature. But total fatty acid measurements may occasionally mask shift in fatty acid composition due to a single lipid species. Thus Wilson and Crawford (191) could find no difference in leaf fatty acid content between four chill-resistant and chill-sensitive plant species. Likewise, de la Roche et al. (31) analyzed for total lipid and total fatty acid content in winter wheat seedlings grown at 2 C or 24 C and could find no differences. But separate analysis of the phospholipid fraction determined its content to be higher at the lower temperature and its fatty acid composition to contain a greater amount of linolenic acid. Similar observations were noted in Musca domestica larvae by Robb et al. (146), but the authors additionally examined individual phospholipids. They cited temperature-dependent changes in the fatty acid composition as being greatest for phosphatidylcholine when compared to phosphatidylethanolamine or phosphatidylserine. Of the fatty acids, palmitoleic acid in phosphatidylcholine had the greatest changes. Low temperature (5 C) also changes the level of each of the major phospholipids in leaves of cucumber and cotton, both cold-sensitive species, but not in broccoli, a cold-resistant species (197). The amount of each of the phospholipids decreased during the chilling treatment; however, there was a humidity interaction. After 3 days at 5 C and 85% relative humidity, the levels of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol declined, but at 100% relative humidity there were no changes. The role of relative humidity in phospholipid metabolism is unclear but may be related to hydration of the membrane since about 40% of the leaf fresh weight had been lost after 12 hours at 85% relative humidity. Guinn (62) could not demonstrate a relationship between chilling resistance in cotton and leaf lipid-soluble phosphate because total lipid analysis was masking subtle fatty acid changes in the phospholipids. St. John and Christiansen (163), however, analyzed the polar lipid fatty acid composition of cotton hypocotyl sections and determined a relationship between phospholipid linolenic acid content and chilling resistance in cotton. Using a pyridazinone compound, Sandoz 9785 [4-chloro-5(dimethylamino)-2-phenyl-3(2*H*)-pyridazinone] and germinating seeds at various temperatures from 15 to 30 C, Sandoz-treated plants had less linolenic acid in the polar lipid fraction regardless of temperature than the control plants. Since the degree of unsaturation in fatty acids has often been implicated in chilling resistance, the authors exposed plants to 8 C for 3 days and found greater chilling injury

Table 2. Linoleic/linolenic fatty acid ratios of cotton seedling root tip polar lipids. Taken from St. John and Christiansen (163).

Growth Temperature	Treatment	
	Control	Sandoz 9785 (10 μM)
degree C		
30	2.67	4.04
25	1.46	3.08
20	1.18	3.07
15	1.16	2.56

in the Sandoz-treated plants which had less linolenic acid than the control plants (Table 2). Hardening the plants prior to chilling reduced the effect of the Sandoz 9785 treatment only slightly. In contrast, cucumber plants, a cold-sensitive species, exhibited increased resistance to freezing when treated with N-dimethyl amino succinamic acid (B-Nine) compared to control plants grown at the same temperature (16 to 21 C) (88). But the chemical also caused dwarfing which suggests an interrelationship between cold-hardiness and hormone activity. Unfortunately, lipid analysis were not performed.

As a possible consequence of the shifts that occur in the level of various phospholipids and in their fatty acid composition at different temperatures, membrane permeability may also be affected. The effect of temperature on membrane permeability has often been investigated in relation to chilling injury and usual measurements include uptake or exudation of solutes from leaves or roots. Enhanced leakage of solutes from cotton radicles exposed to 5 C has been reported (22). However, when the seedlings were transferred to a warmer temperature (31 C), the loss of solutes decreased, indicating a reversal of the previously increased membrane permeability. Christiansen et al. (22) also demonstrated a reversal and even prevention of the temperature-induced exudation with calcium or magnesium ions but noted a pH effect. At low pH (ca. pH 3) the cations did not work. However, at low pH the cations may be hydrated or exist as undissociated salts and thus be less available to the membrane. Leakage of solutes from leaf disk cells were also enhanced by chilling temperatures (140). An interesting ramification of these temperature-induced increases in permeability is that cell metabolism may be affected such that herbicide uptake and phytotoxicity is enhanced.

The effect of temperature or herbicides on plant membrane lipid composition and metabolism has been studied by several investigators, but virtually no one has examined temperature-herbicide interactions as they may relate to lipid biosynthesis and metabolism, membrane permeability, and herbicide uptake and phytotoxicity. It has been shown that temperature affects herbicide activity, but temperature also affects lipid composition, membrane permeability and many other factors. The increase in herbicide activity with increased or decreased temperature may be a result of temperature-induced changes in the lipoidal complex of the membrane such that membrane permeability is affected and herbicide solubility in the membrane lipid fraction is enhanced.

CONCLUSIONS

When one considers that in order to be phytotoxic, a chemical must traverse the plasmalemma, it is striking that very few studies have been concerned with the chemical effects of herbicides on the membrane. Rather, the plasmalemma is generally thought of as a semi-permeable barrier, possibly even inert to the substance which must pass through. And yet, it is entirely conceivable that the primary site of action of some herbicides may reside at the membrane, and the effects subsequently observed are simply manifestations of a metabolic imbalance created by disruptions at the cellular membrane. Mode of action studies involving in vitro systems that look at specific biochemical effects ignore possible membrane interactions but are invaluable in determining a basis for selectivity. Consequently, if one considers herbicide-membrane interactions, it becomes apparent that herbicide interaction with the membrane causes increased or decreased permeability such that (a) disruption of cellular metabolism occurs, or (b) uptake of the same herbicide or that of another is allowed to increase to a critical level necessary to inhibit a biochemical process. Alternatively, the herbicide diffuses through the membrane without interaction and a herbicidal effect ensues on some biochemical process. In the latter case herbicide diffusion through a membrane will be a function of its lipid solubility, the lipid composition of the membrane, and temperature. Membranes differing in composition may transport the same compound at different rates, thus imparting a physical basis of selectivity. Temperature, on the other hand, influences the fluidity of membranes as well as the lipid species present, thereby also affecting diffusion.

Recent improved methods for isolating the plasmalemma have aided in our understanding of the effects of herbicides on membrane composition and function. But further advancements are needed to elucidate membrane structure, to pinpoint areas of membrane-herbicide interaction, and to clarify mechanisms of active and passive transport. The effect of herbicides on membrane composition and therefore membrane structure and function may explain some of the anomalies seen in the field. Increased herbicide activity on crop species early in the spring or during period of intense heat may be due solely to transpiration or metabolism changes. Instead, it has been shown that temperature affects membrane lipid composition as well as cell permeability, and these two factors taken together may account for the enhanced herbicide activity via altered herbicide uptake. In addition, the increased practice of applying pesticides in combination has focused attention on numerous pesticide interactions involving synergism or antagonism. Some of these may be explained on the basis of interaction with membrane structure and function by one pesticide, thus altering membrane permeability to another and increasing its effectiveness. However, it is clearly evident that much more experimental work is needed to clarify the mechanisms underlying the effect of herbicides on membrane structure and function.

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CHAPTER 2

RAPID CHANGES IN ROOT MEMBRANE LIPID COMPOSITION WITH ALTERED TEMPERATURE

Abstract

Mitochondrial and plasmalemma fractions were isolated from soybean [Glycine max (L.) Merr.] roots and the influence of temperature on their phospholipid fatty acid composition determined. Seeds were germinated at 15, 22, or 30 C. At the second trifoliolate state of growth, the seedlings were subjected to a temperature shift from the germination temperature to the next highest or lowest temperature regime. The plants were harvested 0, 24, and 48 hr after treatment. As temperature was increased between 15 and 30 C, both membrane fractions exhibited compositional increases in saturated fatty acids (palmitic and stearic acids) and decreases in unsaturated fatty acids (oleic, linoleic, and linolenic acids). As temperature was decreased, the reverse trend occurred. Both the mitochondria and plasmalemma exhibited a rapid response to changes in temperature; a significant shift in fatty acid composition occurred in 48 hr, often in 24 hr. Mitochondria responded more quickly than the plasmalemma, particularly at the lower temperature. Unsaturation ratio (taken as the percent weight contributed by linoleic plus linolenic acids divided by the percent weight contributed by palmitic acid) indicated a higher degree of unsaturation in the mitochondria at each temperature regime

than in the plasmalemma. The results are consistent with previous reports and stress the importance of examining lipids of individual membranes rather than whole tissues and the rapidity of the membrane response to changes in temperature.

INTRODUCTION

The literature contains numerous citations where membrane fractions from mammalian, fungal, and bacterial cells have been purified and characterized (8, 19, 27). In contrast, there is a paucity of information regarding the lipid composition of plant plasma membranes (plasmalemma). Much of the effort in this area has been directed at the characterization of lipid extracts of whole leaves, roots, or seeds (9, 24, 25) or is drawn from studies of mitochondria or chloroplasts (2, 21, 26, 35, 40). However, improved methods for isolating membrane fractions have permitted separate analyses of membrane lipid composition (1, 15, 32, 38).

Lipids have high turnover rates (5, 11, 23, 28), continually associating and dissociating from membranes (18, 37, 39). Lipid composition varies during plant growth. It has been shown, for instance, that phospholipids and neutral lipids, and one fatty acid, linoleic acid, accumulate during the process of maturation of ivy fruit (*Hedera helix* L.) (12). Similarly, Wilson and Rinne (49) reported decreases in phosphatidic acid and n-acylphosphatidylethanolamine, and increases in phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine in soybean [*Glycine max* (L.) Merr.] from 60 days after flowering to maturity. Likewise, maize (*Zea mays* L.) kernels have been reported to accumulate sitosterol from the time of pollination to maturity (4). Therefore, it is apparent that lipid composition in plant tissue is in a constant state of flux, responding to external stimuli such as temperature, but also internal processes such as maturation. But previous studies have generally ignored the possibility that differences in lipid composition may be due to a single membrane species such as the plasmalemma or mitochondria. Keenan *et al.* (20) compared the lipid composition of mitochondria and plasmalemma from oat (*Avena sativa* L.) roots and determined the composition to be distinctly different. Mitochondria were higher in total phospholipid and free fatty acids but lower in triglycerides. There were also differences in individual phospholipids (18.2% phosphatidylcholine in plasmalemma versus 9.9% in mitochondria) and in the percentage of unsaturated fatty acids (mitochondria had twice as much). However, reported high levels of phosphatidic acid, 41.3% and 13.9% in the mitochondria and plasmalemma, respectively, are suggestive of phospholipid degradation probably occurring during the membrane separation process. Schwertner and Biale (40) similarly analyzed the lipid composition of mitochondria and chloroplasts from avocado (*Persea americana* Mill.) and cauliflower (*Brassica oleracea* L.). However, differences in fatty acid composition or individual phospholipids between organelles of the same plant species were not as great as the differences between plasmalemma and mitochondria from oat roots (20). The study by Schwertner and Biale (40) included organelles from different tissues of the same species and also attempted comparison of fatty acids from mitochondria isolated a year after lipid determinations were performed on other mitochondria. Comparing mitochondria from one tissue with chloroplasts of another may confuse attempts to reconcile functional roles for lipids. Also, the validity of comparing fatty acids isolated from tissue one year after previous lipid analysis is questionable.

In a variety of biological systems, lipid composition has been studied in relation to growth at a single temperature (3, 13, 16, 30, 33, 36, 47). Thus, tissues have been observed to contain a higher degree of saturated fatty acids at higher temperatures and a higher degree of unsaturated fatty acids at lower temperatures. Prominent shifts in two of the principal fatty acids, namely linoleic and linolenic acids, were generally involved. But no reports could be found dealing with continuous shifts in lipid composition as a function of shifts in temperature. In addition, total lipid measurements run the risk of masking subtle shifts in the fatty acid composition of individual lipid components. Such may have been the case when Wilson and Crawford (48) could not demonstrate a difference in leaf fatty acid content between four chill-resistant and chill-sensitive plant species. Similarly, de la Roche _et al_. (6) could not show a difference in total lipid and total fatty acid content in winter wheat (_Triticum aestivum_ L.) seedlings grown at 2 C or 24 C. However, in the phospholipid fraction, the authors observed a higher total phospholipid and linolenic acid content of seedlings grown at 2 C. Likewise, Thomas and Zalik (44) reported decreases in phosphatidylcholine and linoleic acid and increases in phosphatidic and linolenic acids in two rye varieties (_Secale cereale_ var. Sangaste and var. Prolific) during vernalization treatment. In addition, it was noted that the glycolipids were more unsaturated than the phospholipids. But in these studies and others, constant temperatures were utilized and shifts in lipid composition from one temperature regime to another were either not performed or inferred from the data.

In addition to separate analysis of membrane fractions, the dynamics of lipid compositional changes have not been documented, particularly with respect to temperature-induced changes in the composition of the plasmalemma lipids. Therefore, the present investigation was undertaken to examine two specific membrane source, the plasmalemma and mitochondria, and to characterize the dynamic nature of the fatty acid composition while the membranes were being subjected to shifts in temperature.

MATERIALS AND METHODS

Germination Procedure and Temperature Treatment

Seeds of soybean [*Glycine max* (L.)Merr., var. 'Hark'] were planted in quartz sand in 946 ml wax-coated paper cups containing drainage holes. Seeds were germinated at constant 15, 22, or 30 C with a 16-hr daylength in controlled environment chambers. Illumination was provided by incandescent and fluorescent bulbs with the light intensity between chambers ranging from 15 klux to 20 klux. At each temperature regime seedlings were grown to the second trifoliolate stage at which time they were subjected to a shift in temperature. Treatments consisted of constant temperature (no shifts) or a shift to the next highest or lowest temperature; that is, a shift from 15C to 22 C, from 22 C to 15 or 30 C, or from 30 C to 22 C. The plants were then harvested 0, 24, and 48 hr after treatment. Plants were watered daily as required and were fertilized once with Hoagland's No. 1 solution (14) 2 to 3 weeks after planting. Data presented are the means of three experiments with three replications each.

Membrane separation

Plasmalemma and mitochondria membranes were obtained according to a modified procedure of Hodges and Leonard (15). Thirty grams of fresh root tissue were homogenized by grinding in a mortar and pestle for 90 seconds in 30 ml of an ice-cold medium of 0.25 M sucrose, 3mM EDTA, 50mM tricine (N-tris(hydroxymethyl)methyl glycine), and 1% (w/v) BSA (fatty acid free) (pH 7.8). The homogenate was strained through four layers of cheesecloth and centrifuged at 13,000 g for 15 min. All centrifugations were performed at 2 C. The 13,000 g pellet containing mitochondria was resuspended in homogenizing medium and centrifuged at 2500 g for 10 min to remove cell walls and other large cellular fragments. The resulting supernatant was then pelleted at 13,000 g for 15 min, the pellet rinsed and suspended in deionized water, and repelleted at 13,000 g for an additional 15 min. The resulting mitochondria were then held at -15 C for further analysis. The supernatant containing plasmalemma from the original 13,000 g centrifugation was further centrifuged at 80,000 g for 30 min. The pellet was then resuspended in 2 ml 20% (w/w) sucrose containing 1 mM MgSO and 1 mM tris-MES (2-N-morpholino ethane sulphonic acid), pH 7.8. The suspension was layered onto a discontinuous sucrose gradient consisting of 28 ml of 45% (w/w) sucrose and 8 ml of 34% (w/w) sucrose. The sucrose solutions each contained 1 mM $MgSO_4$ and 1 mM tris-MES, pH 7.8. The gradient tubes were centrifuged for 2 hr at 95,000 g in a swinging bucket rotor (Beckman SW27 rotor). The plasmalemma were then obtained from the 34% to 45% interface, diluted in deionized water and pelleted at 80,000 g for 10 min. The plasmalemma samples were held at -15 C for futher analysis.

Extraction and Analysis of Phospholipids

The frozen membrane samples were lypholized and the lipids extracted by a modified procedure of Folch *et al.* (7). All solvents used contained 0.1 mg/1 antioxidant BHT (2,6-ditert-butyl-*p*-cresol). Ten ml of 2:1 (v/v) chloroform/methanol (C/M) was added to the lypholized tissue and the samples shaken in a water bath at 33 C for 30 min. The extract was filtered (Whatman No. 42) into a second tube and the residue re-extracted twice with 5 ml C/M (2:1) by shaking in a water bath for 15 min. The filtered extracts were combined and washed with 0.2 volumes of 0.9% (w/v) NaCl (5) in a tube stirrer for 1 min. After the mixture was allowed to settle, the upper phase was discarded and the lower phase was washed two more time with 0.2 volumes of chloroform/methanol/water (3:48:47, v/v/v) containing 0.9% (w/v) NaCl. The lower phase containing the lipids was then taken to dryness under nitrogen at 33 C and the residue dissolved in 50 μl chloroform. The lipids were then applied to TLC plates (20 x 20 cm) pre-coated with 0.25 mm silica gel GF (Analtech, Inc.). The phospholipids were separated from the remaining lipids in acetone/acetic acid/water (100:2:1) using published Rf values (40) and verifying with known phospholipid standards. Glycolipid contamination as indicated by orcinol-sulfuric acid spray (42) was minimal. The phospholipid band was scraped from the plates, extracted with 2 ml C/M (2:1) followed by 1 ml methanol, and the resultant solution taken to dryness under nitrogen at 33 C. Fatty acid methyl esters were prepared according to a modified method of Metcalfe *et al.* (29). One ml of 0.5 N methanolic KOH was added to the dried sample followed by boiling for 5 min.

After the tubes had cooled, 1 ml of 14% BF_3-methanol (Applied Science Laboratories) was added and the samples boiled for an additional 2 min. One drop of saturated NaCl was then added and the methyl esters extracted three times with 1 ml hexane. The extracts were combined, dried under nitrogen, and the residue taken up in 50 μl hexane for analysis by gas chromatography (Beckman GC-5). Fatty acid composition was determined by flame ionization using a 1.83 m long by 2 mm i.d. glass column packed with 12% stabilized DEGS on anakrom ABS (90/100 mesh) (Analabs, Inc.) and operated at 170 C. N_2 was the carrier gas. Peak identification and quantification was performed by a computer-interfaced system using authentic fatty acid methyl ester standards (Analabs, Inc.). Data were analyzed as a completely randomized design.

RESULTS AND DISCUSSION

Purification of the plasmalemma from other cell contaminants was verified by electron microscopy using a phosphotungstic-chromic acid stain that is specific for plant plasmalemmas (22). Quantitative analysis of membrane sections revealed a plasmalemma purity in excess of 70% which was sufficient for analysis. Preliminary studies also indicated that five major fatty acids were present: Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. Trace amounts of palmitoleic (16:1), arachidic (20:0), and behenic (22:0) acids were occasionally observed.

Progressively raising or lowering the temperature between 15 and 30 C resulted in rapid measurable responses in the fatty acid composition of both the plasmalemma and mitochondria (Table 1 and II). The largest observed changes occurred in palmitic and linolenic acids.

Table I. Influence of temperature shift and time of harvest on the fatty acid composition of soybean root plasmalemma phospholipids.

Growth Temperature	Treatment Temperature	Sample Time	Fatty Acid Composition				
			C 16	C 18	C 18:1	C 18:2	C 18:3
degree C	degree C	hr	% of total weight				
15	22	0	26.5	17.5	10.0	26.2	19.8
		24	27.9	14.2	14.0	25.7	18.2
		48	36.7	17.0	11.4	17.5	17.5
22	30	0	30.1	16.1	8.5	24.5	20.7
		24	32.4	16.8	8.9	20.2	21.7
		48	33.7	18.2	8.4	20.5	19.1
30	22	0	37.2	14.3	12.7	23.0	12.5
		24	30.1	13.7	12.5	27.4	16.2
		48	24.8	11.8	8.9	29.0	25.6
22	15	0	30.1	16.0	8.5	24.5	20.7
		24	28.4	16.6	10.5	24.4	20.0
		48	26.5	14.3	10.2	24.7	24.3

Table II. Influence of temperature shift and time of harvest on the fatty acid composition of soybean root mitochondria phospholipids.

Growth Temperature	Treatment Temperature	Sample Time	Fatty Acid Composition				
			C 16	C 18	C 18:1	C 18:2	C 18:3
degree C	degree C	hr	% of total weight				
15	22	0	24.0	16.4	10.3	28.7	20.5
		24	25.7	15.2	14.1	29.2	15.7
		48	31.6	11.3	12.7	27.5	16.8
22	30	0	22.8	17.1	11.6	24.4	24.0
		24	31.4	17.6	10.8	20.1	19.9
		48	29.9	21.1	8.5	21.1	19.9
30	22	0	29.1	12.0	11.6	29.8	17.3
		24	29.2	12.0	9.6	27.0	22.2
		48	23.7	10.9	9.3	26.7	29.3
22	15	0	22.8	17.1	11.6	24.4	24.0
		24	21.6	12.7	4.6	30.8	30.1
		48	18.6	12.8	5.9	29.8	32.9

As treatment temperature increased, the percentage of saturated fatty acids, palmitic acid, and to a lesser extent, stearic acid also increased while the percentage of unsaturated fatty acids, oleic, linoleic, and linolenic acids decreased. The trend was reversed as treatment temperature decreased. The relationship between temperature and fatty acid composition supports previous investigations concerning other plant species (1, 6, 20, 40, 44), bacteria (3), fungi (38), and animals (19, 33, 36). However, in contrast to the steady state conditions used in previous studies, the present study demonstrates the rapidity by which membranes respond to fluctuations in temperature. Rapid lipid turnover rates have also been reported for dictyosomes and microsomes in stem explants of onion (Allium cepa L.) and were demonstrated to have half-times of 1.2 to 5 hr (31). In contrast, lipid turnover rates in the endoplasmic reticulum of rat hepatocytes were between 29 and 44 hr (34). Interestingly, the rate of lipid replacement in the endoplasmic reticulum differed depending on the portion of the phospholipid being considered; the hydrophilic moiety (glycerol backbone) had a much shorter half-life than the hydrophobic portion (fatty acids). However the turnover rate for the fatty acids could be complicated by possible transference of whole fatty acids to another lipid molecule. Although the response to temperature in the present investigation is shown to be measurable within 24 hr, younger plants would be expected to exhibit a quicker response due to a higher proportion of actively growing root initiates.

In contrast to the study by Keenan et al. (20) which reported distinct differences between mitochondria and plasmalemma of oats, no marked differences were seen in the fatty acid composition between the plasmalemma and mitochondria of soybeans (cf. Table I and II) even though separation techniques were similar. However, lipase activity may have been high enough even at pH 7.2 used by Keenan _et al_. (20) to cause the differences seen. The high levels of phosphatidic acid, a phospholipid degradation product, that were reported tend to support this conclusion. Angelo and Altschul (43) and Galliard (10) reported lipase activity maxima and free fatty acid-stimulated enzymic deacylation of lipids in the range of pH 6.2 to 7.2. Inclusion of bovine serum albumin and a high pH in the homogenizing medium minimizes the deacylation process.

An unsaturation ratio (UR) was calculated as the percent weight contributed by linoleic plus linolenic acids divided by the percent weight contributed by palmitic acid to obtain some measure of a temperature-induced shift in the balance between saturated and unsaturated fatty acids. It was felt that these particular fatty acids represent opposing ends of the portion of the biosynthetic pathway in consideration and that changes in their overall weights would reflect shifts toward saturation or unsaturation. Shifts in UR permit a membrane to retain its fluidity at a particular temperature (13, 45, 46). Plasmalemma phospholipids (Table III) exhibited a significant decrease in UR within 48 hr when subjected to a rise in temperature. When the temperature was increased from 15 to 22 C, a significant decrease in unsaturation was noted within 24 hr. However, when the temperature was increased from 22 to 30 C, 48 hr was required for a significant response. Decreasing the treatment temperature increased the degree of unsaturation. A significant response was exhibited within 48 hr when the temperature was shifted from 30 to 22 C.

Table III. Unsaturation ratios[1] of fatty acids from soybean root plasmalemma phospholipids.

Sample Time	Growth/Treatment Temperature (°C)[2]			
	15/22	22/30	30/22	22/15
hr				
0	2.11 c	15.0 b	0.94 a	1.50 a
24	1.76 b	1.31 ab	1.49 ab	1.57 a
48	1.41 a	1.18 a	2.24 b	1.56 a

[1] Unsaturation ratio is calculated as (18:2% + 18:3%)/(16:0%)

[2] Means followed by the same letter within a column do not differ significantly at the 5% level by Duncan's Multiple Range Test.

Table IV. Unsaturation ratios[1] of fatty acids from soybean root mitochondria phospholipids.

Sample Time	Growth/Treatment Temperature (°C)[2]			
	15/22	22/30	30/22	22/15
hr				
0	2.05 c	2.18 b	1.67 a	2.18 a
24	1.74 b	1.30 a	1.72 ab	2.85 ab
48	1.40 a	1.50 ab	2.37 b	3.40 b

[1] Unsaturation ratio = (18:2% + 18:3%)/(16:0%)

[2] Means followed by the same letter within a column do not differ significantly at the 5% level by Duncan's Multiple Range Test.

However, shifting the temperature from 22 to 15 C did not alter the degree of unsaturation, at least within the 48 hr observation period, although trends were evident in the fatty acid composition (Table I).

The mitochondrial phospholipids responded similarly (Table IV). Increasing the temperature significantly reduced the degree of unsaturation within 24 hr. Decreasing the temperature, significantly increased the degree of unsaturation; however, 48 hr was required. In contrast to the plasmalemma, when the temperature was lowered from 22 to 15 C, a marked increase in unsaturation was observed after 48 hr. One possible explanation may be that the mitochondria are metabolically more active at the lower temperature than the plasmalemma and, hence, would exhibit a more rapid response. The mitochondria were also observed to have a higher degree of unsaturation (cf. Table III and IV), an observation not readily apparent in Tables I and II. Higher UR values at lower temperatures allow for greater membrane lateral compressibility and extensibility (41), thus facilitating insertion of new membrane components with little increase in membrane surface area (17). It would also provide the necessary flexibility for protein carrier kinetics in the transport of solutes across the membrane. The quicker response by mitochondria might also be explained on the basis of the synthesis and transfer of membrane lipids to the various membranes. Kagawa _et_ _al_. (18) and Morre (31) demonstrated a time lapse in incorporation of ^{14}C-choline as a phospholipid precursor into lecithin of the endoplasmic reticulum first, followed in order by dictyosomes, mitochondria, and plasma membrane. Consideration of this labeling sequence and the low temperature utilized in the present investigation (which is near the minimum temperature at which soybeans can be successfully grown) might account for the lack of response seen in the plasmalemma within the 48 hr observation period.

The data presented emphasize the importance of examining individual cell membranes as well as examining the continuous response of those membranes to fluctuations in temperature. Such separate analyses of temperature-induced changes in the lipid composition of membranes from different organelles of the same tissue may provide insight into differences in the dynamic nature of each. This was demonstrated between the phospholipids of mitochondria and plasmalemma from soybean roots. Although there were no apparent differences in fatty acid composition, UR values indicated a higher degree of unsaturation in the fatty acids of the mitochondria than of the plasmalemma. Reducing the temperature between 12 and 15 C resulted in a more rapid response in the mitochondria. Secondly, previous studies have performed lipid analyses on plant species grown at a single temperature. However, with fluctuating temperatures in nature, membrane lipid composition must be in a constant state of flux. Soybean roots responded to temperature changes fairly rapidly. However, the mitochondria and plasmalemma utilized in this study were derived from whole root samples. Had actively growing root initiates only been used, a more rapid response (less than 24 hr) might have been seen.

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CHAPTER 3

EFFECT OF HERBICIDES AND ANTIDOTE ON CHILLING RESISTANCE AND MEMBRANE LIPID COMPOSITION IN SOYBEAN (GLYCINE MAX (L.) MERR.)

Abstract

Modification of chilling injury tolerance was evaluated in soybean (Glycine max (L.) Merr.) seedlings treated with 10^{-6} M solutions of either alachlor (2-chloro-2',6'-diethyl-N-(Methoxymethyl)acetanilide), trifluralin (a,a,a-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine), vernolate (S-propyl dipropylthiocarbamate), H-22234 (N-chloroacetyl-N-(2,6-diethylphenyl)-glycine), or a combination of the above and 10^{-7} M R-29148 (2,2-dimethyl-5-methyl(dichloroacetyl)oxazolidine), a potential thiocarbamate antidote. Seedlings were grown at 30 C and chilled at 8 C for four days. All treatments enhanced chilling tolerance compared to the control. Separate analysis of the phospholipid fraction of the plasmalemma and mitochondria membranes revealed that many of the herbicide treatments increased the degree of fatty acid unsaturation. The effect was generally coupled with a corresponding decrease in palmitic acid content, a saturated fatty acid. Of the treatment examined, H-22234 plus R-29148 and alachlor plus R-29148 significantly increased the degree of unsaturation in both membrane fractions at 30 C. At 15 C, H-22234 significantly increased the degree of unsaturation in the mitochondria, however, it had an opposite effect in the plasmalemma, thus the two membrane fractions did not always

respond similarly to the same herbicide-temperature treatment. Other herbicides tested did not significantly affect membrane lipid composition. Fatty acids of the mitochondria phospholipids were generally more unsaturated than fatty acids of the plasmalemma regardless of temperature.

INTRODUCTION

The lipid composition of a wide range of species has been shown to be higher in unsaturated fatty acids at low temperatures, and lower in unsaturated fatty acids at higher temperatures (10, 15, 18). The major unsaturated fatty acids that vary are linoleic and linolenic acids. In general, lipid composition studies have dealt either with whole tissues such as roots, leaves, or seeds (6, 8, 12), or with whole organelles such as chloroplasts or mitochondria (9, 24).

More recently interest has centered on individual membrane systems, in particular mitochondrial and plasmalemma membranes (1, 13, 23, 32). In one study, a differential lipid composition between inner and outer mitochondrial membranes of cauliflower (*Brassica oleracea* L.) buds has been shown (17). A similar difference in lipid composition between inner and outer mitochondrial membranes in maize (*Zea mays* L.) was suggested in the pathotoxic action of *Helminthosporium maydis* Race T toxin (22, 31). The toxin (not identified) was demonstrated to bind selectively to the inner mitochondrial membrane of sensitive varieties of maize but not to resistant varieties. When the outer mitochondrial membrane was removed, however, the toxin was able to bind non-selectively to the inner membrane of both varieties, thus demonstrating a physical selectivity between varieties based on differential permeability of the outer membrane. Since membrane permeability is a function of its lipid composition and the lipid solubility of the solute (30), differences in mitochondrial membrane lipid composition provide insight into possible modes of phytotoxic action of some herbicides. Recently, Boger et al. (2) suggested that bentazon (3-isopropyl-1H-2,1,3-benzothiadiazin-(4) 3H-one 2,2-dioxide) may act by binding to chloroplast thylakoid membranes of Bumilleriopsis filiformis Vischer, thus interfering with electron transport. Binding of bentazon to the thylakoids of whole cells appeared to be quite strong, whereas in isolated chloroplasts, reversal of inhibition was rapid. Reports of preferential binding of toxins to specific membranes demonstrate the importance of examining the lipid composition of individual membranes rather than whole tissues.

Not only will membrane lipid composition respond to temperature, thus affecting herbicide movement into the cell (16, 19, 35), but herbicides may also affect membrane lipid composition (12, 28) and limit ion uptake (5).

St. John and Christiansen (28) reported lipid composition alterations in cotton (Gossypium hirsutum L.) seedlings treated with Sandoz 9785 (4-chloro-5-(dimethyl-amino)-2-phenyl-3(2H)-pyridazinone), and noted an increase from 10.9% to 17.1% in the amount of linolenic acid in the polar lipids as temperature was lowered from 30 to 15 C. This is in contrast to control seedlings that exhibited an increase in linolenic acid from 13.6% to 27.5%. Interestingly, Sandoz-treated plants were more susceptible to chilling injury even with a hardening treatment which suggests a role for herbicides as potential agents in manipulating the chilling resistance of crops. Additional manipulations of lipid composition were demonstrated by St. John (27) in wheat (Triticum aestivum L.) chloroplasts.

No reports could be found in the literature dealing with the effect of herbicides on the lipid composition of specific membranes, in particular mitochondrial and plasmalemma. Early studies on the effect of herbicides on lipids concerned cuticular waxes (4, 26), crop quality (11, 20, 21), or lipid synthesis (14, 32). Interestingly, inhibition of lipid synthesis in spinach (Spinacia oleracea L.) chloroplasts by 10^{-5} M EPTC (S-ethyl dipropylthiocarbamate) was reversed by 10^{-7} M R-25788 (N,N-diallyl-2,2-dichloroacetamide), a commercial thiocarbamate antidote (33). A similar observation was reported for red beet (Beta vulgaris L.) root tissue (34). Most frequently, however, investigators have examined the effects of herbicides on chloroplast lipid (9, 25, 27, 32), or have analyzed the lipid composition of whole tissues (12, 28, 29) and inferred membrane alterations from any observed changes. However, individual membranes may not be affected in a similar manner.

Endogenous lipid levels appeared to play a role in imparting tolerance to herbicides (8, 9). The protection provided was thought to be due to a preferential partitioning of the herbicides into the lipid phase.

The objectives of the present investigation were to determine whether several herbicides affected soybean susceptibility to chilling injury and to determine whether the effects could be associated with differences in the lipid composition of mitochondria and plasmalemma giving rise to a temperature-herbicide-membrane interaction.

MATERIALS AND METHODS

Chilling injury study

Plants were chilled according to a modification of the procedure of St. John and Christiansen (28). Fifteen soybean seeds were planted per cup in quartz sand in 250-ml wax-coated paper cups containing a drainage hole. The cultures were moistened with water or a 10^{-6} M herbicide solution. Herbicide treatment solutions consisted of alachlor, alachlor plus 10^{-7} M R-29148, trifluralin, vernolate, vernolate plus R-29148, H-22234, and H-22234 plus 10^{-7} M R-29148. All treatments were watered daily. Plantings were germinated at 30 C and 16-hr day (15 klux) until the leaf primordia of the first trifoliolate was clearly visible. Two replications of each treatment were then chill-hardened according to the following schedule: 2 days at 20 C and 2 days at 15 C. Two additional replications of each treatment were not chill-hardened and remained at 30 C during the interim period. After the first group of seedlings were chill-hardened, all plants were subjected to chilling for 4 days at 8 C. After chilling, all seedlings were returned to 30 C for observation of recovery and injury symptoms. The experiment was repeated three times.

Lipid composition study

Seeds of soybean were planted in quartz sand in 846 ml wax-coated paper cups containing drainage holes. Various herbicide treatments were applied pre-emergence to the sand at 935 L/ha and 2.11 kg/cm^2 pressure. The herbicide treatments used were: Alachlor at 2.24 kg/ha; H-22234 at 2.24 kg/ha; trifluralin at 0.56 kg/ha; vernolate at 3.36 kg/ha; vernolate plus R-29148 at 3.36 plus 0.14 kg/ha; and either alachlor or H-22234 plus R-29148 at 2.24 plus 0.14 kg/ha. The cups were placed into larger diameter cups that served as water reservoir containers between irrigations. Seeds were germinated at 15 or 30 C with a 16 hr daylength in controlled environment chambers. Illumination was provided by incandescent and fluorescent bulbs with a light intensity of 20 klux. The seedlings were grown to the second trifoliolate stage at which time they were harvested. Plants were

Figure 1. Typical herbicide effect on chilling tolerance in nonhardened soybean seedlings. Soybean germinated at 30 C and chilled at 8 C for four days.

Figure 1

Table 1. Effect of herbicide treatment on mitochondrial phospholipid fatty acid composition of soybean plants grown at 15 C.

Treatment	Application rate (kg/ha)	Fatty acid composition (%)				
		C 16	C 18	C 18:1	C 18:2	C 18:3
Control	--	26.9	11.4	6.4	30.8	24.6
Trifluralin	0.56	23.8	5.3	8.5	37.9	24.5
Alachlor	2.24	28.9	7.9	8.7	32.5	22.2
H-22234	2.24	23.9	9.0	6.2	22.4	38.5
Vernolate	3.36	28.6	8.1	6.3	31.9	25.1
Vernolate + R-29148	3.36 + 0.14	27.6	13.8	11.2	28.8	18.7
H-22234 + R-29148	2.24 + 0.14	38.3	14.2	7.4	22.2	18.0
Alachlor + R-29148	2.24 + 0.14	24.1	5.7	5.8	36.5	28.0

watered daily and fertilized once with Hoagland's No. 1 nutrient soluiton (7) 2 to 3 weeks after planting. Data presented are the means of three experiments with three replications each.

Tissue analysis

Separation of membrane fractions, extraction and analysis of the phospholipids were performed as previously described (23).

RESULTS AND DISSCUSSION

None of the herbicide treatments caused increased susceptibility of soybean plants to chilling injury (Figure 1). The unhardened control plants, however, exhibited immediate wilting (within 24) when the temperature was lowered from 30 C to 8 C, but completely recovered by the fourth day of chilling. In contrast, none of the herbicide-treated plants showed signs of wilting. Hardening the plants prior to chilling prevented the initial wilting response by the control plants. Increased chilling tolerance exhibited by the herbicide-treated plants was further examined in terms of possible modification in fatty acid composition.

When soybean plants were treated and grown at 15 C, most of the herbicide treatments resulted in slight shifts in the fatty acid composition of the mitochondrial phospholipids (Table 1). Palmitic, linoleic, and linolenic acids were most affected by the treatments. At the rate used, H-22234 plus R-29148 caused the largest increase in palmitic acid, but also the greatest reduction in the amount of linoleic and linolenic acids. This suggests that if one considers the series of fatty acids from palmitic to linolenic acid as representing a sequence in the biosynthesis of fatty acids, it appears that the increase in palmitic acid due to treatment with H-22234 plus

Table 2. Effect of herbicide treatment on plasmalemma phospholipid fatty acid composition of soybean plants grown at 15 C.

Treatment	Application rate (kg/ha)	Fatty acid composition (%)				
		C 16	C 18	C 18:1	C 18:2	C 18:3
Control	--	31.0	14.0	4.3	27.2	23.5
Trifluralin	0.56	33.2	11.4	5.3	28.6	21.5
Alachlor	2.24	31.0	12.4	4.9	28.1	23.6
H-22234	2.24	36.1	13.1	4.5	26.9	19.5
Vernolate	3.36	38.5	14.8	4.1	21.4	21.2
Vernolate + R-29148	3.36 + 0.14	30.1	16.1	4.8	26.0	23.0
H-22234 + R-29148	2.24 + 0.14	32.3	14.2	4.7	25.4	23.4
Alachlor + R-29148	2.24 + 0.14	37.1	14.7	3.5	25.1	19.6

Table 3. Effect of herbicide treatment on mitochondria phospholipid fatty acid composition of soybean plants grown at 30 C.

Treatment	Application rate (kg/ha)	Fatty acid composition (%)				
		C 16	C 18	C 18:1	C 18:2	C 18:3
Control	--	31.4	17.9	8.5	24.6	17.6
Trifluralin	0.56	29.4	9.9	5.7	31.8	23.3
Alachlor	2.24	33.9	16.1	10.5	16.1	23.4
H-22234	2.24	29.3	10.7	6.2	31.8	22.0
Vernolate	3.36	26.2	10.3	7.3	26.8	29.4
Vernolate + R-29148	3.36 + 0.14	31.3	12.6	6.4	23.6	26.1
H-22234 + R-29148	2.24 + 0.14	26.9	9.5	5.7	26.3	31.6
Alachlor + R-29148	2.24 + 0.14	30.3	6.8	3.0	33.8	26.2

Table 4. Effect of herbicide treatment on plasmalemma phospholipid fatty acid composition of soybean plants grown at 30 C.

Treatment	Application rate (kg/ha)	Fatty acid composition (%)				
		C 16	C 18	C 18:1	C 18:2	C 18:3
Control	--	36.9	15.6	6.5	26.6	14.4
Trifluralin	0.56	34.3	15.2	6.9	28.9	14.7
Alachlor	2.24	35.2	15.5	7.8	23.3	18.2
H-22234	2.24	33.6	10.1	4.4	34.3	17.6
Vernolate	3.36	31.8	19.4	11.4	22.2	15.2
Vernolate + R-29148	3.36 + 0.14	31.3	14.7	7.1	30.5	16.4
H-22234 + R-29148	2.24 + 0.14	24.1	18.9	9.1	27.7	20.4
Alachlor + R-29148	2.24 + 0.14	32.4	10.4	5.7	31.4	20.2

R-29148 occurred at the expense of linoleic and linolenic acids. Vernolate plus R-29148 also inhibited linolenic acid production but tended to accumulate more stearic and oleic acids. Interestingly, treatment with H-22234 alone increased the amount of linolenic acid in contrast to H-22234 plus R-29148 which affected both linoleic and linolenic acids.

The effect of the herbicide treatments on plasmalemma fatty acids from plants grown at 15 C differed from that of the mitochondria (Table 2). Whereas in the mitochondria H-22234 increased linolenic acid and decreased palmitic acid, in the plasmalemma the reverse was true. Other herbicide treatments causing increases in palmitic acid and subsequent decreases in linoleic or linolenic acids were vernolate and alachlor plus R-29148. Of interest was the relative stability in the level of stearic and oleic acids in the plasmalemma in spite of the various herbicide treatments employed.

In general, treatment of soybean plants grown at 30 C showed enhancement of linolenic acid accumulation and an inhibition of palmitic acid production in the mitochondrial phospholipids as a consequence of herbicide treatment (Table 3). H-22234 plus R-29148 increased linolenic acid and decreased palmitic acid. In contrast, H-22234 without R-29148 shifted the fatty acid accumulation from linolenic acid to linoleic acid. Likewise, treatment with alachlor plus R-29148 caused a similar shift in fatty acid composition. Compared to the controls, most of the treatments reduced the saturated fatty acids and increased the amount of linoleic and linolenic acids.

At 30 C, the fatty acid composition of the plasmalemma appeared to be less affected than the mitochondria from the same plants

(Table 4). The largest change in palmitic and linolenic acids was observed when plants were treated with H-22234 plus R-29148. A similar increase in unsaturated fatty acids was observed in plants treated with alachlor plus R-29148.

In order to assess overall shifts in fatty acid composition from a predominately unsaturated condition to one that is predominantly saturated, or vice versa, an unsaturation ratio (UR) was calculated as previously reported (23) as the percent weight contributed by linoleic plus linolenic acid divided by the percent weight contributed by palmitic acid. These particular fatty acids appeared to be most commonly affected by the herbicide treatments. Table 5 shows that UR was higher at the lower temperature than at the higher temperature regime as expected. A few herbicide treatments, however, produced the opposite effect. H-22234 plus R-29148 and alachlor plus R-29148 greatly increased the degree of unsaturation in the plasmalemma at the higher temperature compared to the same treatments at the lower temperature. In contrast, the control plants exhibited a decrease in UR with an increase in growth temperature. In the mitochondria, only H-22234 plus R-29148 greatly increased the degree of unsaturation at the higher temperature.

The UR values also demonstrate the effect of a herbicide-temperature-membrane interaction on the shift from palmitic to linoleic, and linolenic acids. At the lower temperature none of the treatments significantly increased the degree of fatty acid unsaturation in the plasmalemma. Several treatments, H-22234 and alacholor plus R-29148, actually reduced UR below that of the control plants, whereas at the higher temperature they increased UR. It is

Table 5. Unsaturation ratio (UR)[a] of mitochondria and plasmalemma phospholipid fatty acid composition as affected by herbicides and growth temperature.

Treatment	Herbicide rate (kg/ha)	Mitochondria[b]		Plasmalemma	
		15 C (UR)	30 C (UR)	15 C (UR)	30 C (UR)
Control	--	1.98 ab	1.33 ab	1.66 d	1.12 a
Trifluralin	0.56	2.62 bc	1.87 bcd	1.51 bcd	1.29 ab
Alachlor	2.24	2.07 bc	1.17 a	1.68 d	1.18 a
H-22234	2.24	2.55 bc	1.87 bcd	1.29 abc	1.54 c
Vernolate	3.36	2.02 ab	2.15 cd	1.11 a	1.21 a
Vernolate + R-29148	3.36 + 0.14	1.89 ab	1.58 abc	1.63 cd	1.50 bc
H-22234 + R-29148	2.24 + 0.14	1.08 a	2.19 d	1.50 bcd	2.00 d
Alachlor + R-29148	2.24 + 0.14	3.00 c	2.00 cd	1.20 abd	1.59 c

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[a] Unsaturation ratio is calculated as (C 18:2% + C 18:3%)/(C 16%)

[b] Means within a column followed by the same letter do not differ significantly at the 5% level by Duncan's Multiple Range Test.

unclear how such action occurs considering that chain elongation probably occurs before unsaturation (6). At the higher temperature, however, most of the herbicide treatments increased the unsaturated fatty acids to some degree, thus potentially imparting increased tolerance to chilling injury. At 30 C, H-22234, vernolate plus R-29148, H-22234 plus R-29148, and alachlor plus R-29148 significantly increased UR above the control level in the plasmalemma, whereas in the mitochondria a significant increase in UR was observed in plants treated with vernolate, H-22234 plus R-29148, or alachlor plus R-29148. Therefore, the membrane fractions did not respond similarly to all herbicide treatments. In addition, the mitochondrial phospholipid fatty acid composition generally tended to have a higher degree of unsaturation than the plasmalemma regardless of temperature. This observation corroborates a similar observation noted in a previous report (23).

The results of this study emphasize two important considerations that should be taken into account when considering the effect of herbicides on lipid composition. Reports from field and controlled environment studies document the influence of temperature on herbicide action (16, 19). The results of this investigation indicate that temperature influences the effects of herbicides on lipid composition. Furthermore, gross lipid analysis of root or shoot tissue (12, 28) ignores the possibility that various lipid sources may not respond similarly to temperature and herbicides. In the present study, the two membrane fractions, plasmalemma and mitochondria, responded differently to herbicide treatment at 15 C. Alteration of membrane lipid composition may play an important role in herbicide action. It is possible that many of the observed effects of herbicides (e.g. disruption of electron transport and/or inhibition of oxidative phosphorylation) may be manifestations of action at the membrane surface affecting cellular metabolism.

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CHAPTER 4

EFFECT OF CALCIUM AND NITROGEN ON SOYBEAN (GLYCINE MAX (L.) MERR.) ROOT FATTY ACID COMPOSITION AND UPTAKE OF LINURON

Abstract

The effect of calcium and nitrogen level on the uptake of linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) from nutrient solution by soybean (Glycine max (L.) Merr.) seedlings was determined and related to root membrane fatty acid composition. Calcium levels in the nutient solution were 0.4, 4.0, or 8.0 mM and nitrogen levels were 4.0, 16.0, or 32.0 mM with the pH adjusted to 6.5. As calcium concentration increased, linuron uptake decreased. The reverse was true for nitrogen. These changes were not related to transpiration.

Increasing calcium concentration increased palmitic acid and decreased linolenic acid resulting in a greater degree of saturation in the plasma membrane of the soybean roots. Nitrogen likewise increased the degree of saturation in the plasma membrane, but the effect was mainly seen in the balance between stearic and linoleic acids. In the mitochondria, however the trends were inconclusive. Large increase in linolenic acid was observed regardless of treatment, but the effect may have been an artifact.

INTRODUCTION

Enhanced membrane permeability to herbicides with a subsequent increase in the uptake and phytotoxicity of herbicides have been shown to occur in a number of species treated with inorganic ions such as phosphorous (13), magnesium (1), and copper (15). Similarly, calcium has been shown to increase membrane thickness (10), affect membrane permeability to other inorganic ions (8), and influence ATPase activity in membrane (7). Morre and Bracker (10) demonstrated alternate thickening and thinning of soybean plasma membranes when successively treated with $CaCl_2$ and IAA (indole-3-acetic acid), respectively. Membranes isolated in $CaCl_2$ showed a greater response to IAA than membranes isolated in water or in a medium containing coconut water, ethylene diamine tetraacetic acid (EDTA), and sucrose. Thinning of the plasma membrane by IAA was accompanied by an increase in microviscosity (3). This increase in viscosity may explain the effect of 2,4-D (2,4-dichlorophenoxyacetic acid) in decreasing membrane permeability to sucrose and mannitol in cells of Chlorella (18). Calcium which has been shown to increase membrane thickness (8) may in turn reduce membrane microviscosity. Hence an increase in permeability might be expected which may explain the increased effectiveness of 2,4-D on eurasian watermilfoil (Myriophyhlum spicatum L.) when $CaCl_2$ was added to the culture solution (14).

It is also possible that microviscosity changes in the plasma membranes may be accompanied by qualitative changes in membrane lipids. Kuiper et al. (6) examined the lipid composition of wheat (Triticum aestivum L.) and oat (Avena sativa L.) root membrane fractions (mitochondrial and microsomal) as well as whole roots in relation to mineral nutrition and determined that at high salt concentration (defined as a complete culture medium) wheat roots exhibited a higher total phospholipid, phosphatidylcholine and sulfolipid content. The reverse was true for oat roots. On the other hand, low salt (0.2 mM $CaSO_4$) enhanced glycolipid and sterol content in whole roots of both species, but decreased the amount of lipids in the microsomal fraction. Other fractions examined (cell wall and mitochondrial membranes) showed little change. Lipid response to different salt conditions as reported by Kuiper et al. (6), did not isolate the effect of calcium on lipid composition since under low salt conditions other nutrients were absent and the plants may have been under considerable stress.

Low manganese levels have been demonstrated to decrease water utilization efficiency in pea beans (*Phaseolus vulgaris* L.) and corn (5). Although the authors postulated that the effect may have been due to an inhibition of photosynthesis or other related processes, it is also conceivable that manganese interaction with membrane or cuticular lipids could alter membrane permeability and affect water utilization. Less surface wax was found on manganese-deficient cauliflower (*Brassica oleracea* L.) leaves.[1]

Nitrate has been observed to increase simazine (2-chloro-4,6-bis (ethylamino)-*s*-triazine) uptake in rye (*Secale cereale* L.), corn (*Zea mays* L.), and soybean, whereas ammonium did not (9). In the field, soybeans fertilized with ammonium sulfate were injured less

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[1] B.G. Ellis (personal communication).

from simazine than soybeans fertilized with $CaNO_3$.

The objective of the present investigation was to examine the effect of calcium and nitrogen on the lipid composition of cellular membranes in soybean roots, and to relate this to linuron uptake.

MATERIALS AND METHODS

^{14}C-Linuron uptake

'Hark' soybean seeds were planted in washed quartz sand in 14.5 cm by 10 cm styrofoam flats and moistened with one of the treatment solutions listed in Table 1. The solutions were made according to Hoagland solution No. 1 (4), varying only the amount of available calcium and nitrogen. All solutions were adjusted to pH 6.5. Seedlings were germinated at 30 C, 16 hr day (15 klux). When the first trifoliolate leaf was fully expanded, the coyledons were excised. Uniform seedlings were then transferred to jars containing 100 ml of culture solution and acclimated for 2 days.

Treatment was performed by adding 100 ml of 10^{-6} M linuron containing 0.05 uCi of ^{14}C-linuron (specific activity 1.7 uCi/mmole) in nutrient solution. Plants were exposed to the treatment solution for 12 hr at which time they were separated into shoots and roots. The volume of solution remaining at the end of the treatment period was measured and the calculated difference was assumed to be equal to the amount taken up and/or transpired. The oven-dried plant material was weighed and combusted by the Schoeninger combustion method of Wang and Willis (17), and the radioactivity determined by liquid scintillation spectrometry. The data are the mean of two experiments with five replications per experiment.

Table 1. Modified calcium and nitrogen levels in Hoagland solution No. 1 utilized in present study. Other nutrient levels were constant for all treatments and were in accordance with Hoagland and Arnon (4). Solutions were adjusted to pH 6.5.

Treatment	Calcium (mM)	Nitrogen (mM)
Low Ca	0.4	16.0
Normal Ca/N	4.0	16.0
High Ca	8.0	16.0
Low N	4.0	4.0
High N	4.0	32.0

Membrane composition study

Seeds of soybean were planted in quartz sand in 946-ml wax-coated paper cups containing drainage holes. The cups were moistened with one of the treatment solutions listed in Table 1. Seeds were germinated at 30 C and 16-hr day in controlled environment chambers. Illumination was provided by incandescent and fluorescent bulbs with a light intensity of 15 klux. The plants were watered daily. The seedlings were grown to the second trifoliolate stage at which time they were harvested. The data presented are the mean of two experiments with three replications each.

Fatty acid analysis

Separation of membrane fractions, extraction and analysis of the phospholipids were performed as previously described (11).

RESULTS

^{14}C-linuron uptake study

The level of calcium in the nutrient solution had a significant effect on linuron uptake (Table 2). As calcium concentration increased from 0.4 mM to 8.0 mM, linuron concentration in the roots was significantly reduced by 28% (from 17086 to 12318 dpm/g root dry weight). In the shoots, decreasing linuron content was observed with increasing calcium. Water uptake did not account for the effects observed since a greater amount of label was found in those plants taking up the least (Table 2).

Increasing nitrogen concentration from mM to 32 mM increased the amount of label in the roots but had little effect in the leaves. There was no difference in the amount of solution transpired between nitrogen treatments.

Table 2. Effect of calcium and nitrogen levels on the uptake of ^{14}C-linuron by soybean seedlings grown in nutrient solution containing 10^{-6} M linuron.

Treatment (mM)	Dpm/g root weight[a]	Dpm/g shoot weight	Dpm/g leaf area[b]	Ml solution remaining[c]
Calcium				
0.4	17086 c	9037 a	4468 a	92.3 a
4.0	14904 b	7751 a	3936 ab	90.0 a
8.0	12318 a	8535 a	3417 ab	88.2 a
Nitrogen				
4.0	11790 a	7032 a	3019 a	92.1 a
16.0	14904 b	7751 a	3936 ab	90.0 a
32.0	16301 bc	5913 a	3180 a	93.0 a

[a] Means within a column followed by the same letter do not differ significantly at the 5% level by Duncan's Multiple Range Test.

[b] Leaf area calculated as weight of leaf tracing cut from paper.

[c] Treatments initiated with 100 ml of solution.

Table 3. Effect of calcium and nitrogen levels on the fatty acid composition of soybean root plasmalemma phospholipids.

Treatment	Fatty acid composition (%)					Unsaturation Ratio[a,b]
(mM)	C 16	C 18	C 18:1	C 18:2	C 18:3	
Calcium						
0.4	29.5	11.1	7.0	29.7	22.9	1.78 c
4.0	32.1	21.6	6.2	24.4	15.9	1.26 ab
8.0	38.7	11.1	6.6	33.1	10.0	1.11 a
Nitrogen						
4.0	29.3	15.8	10.1	29.0	15.9	1.53 bc
16.0	32.1	21.6	6.2	24.4	15.9	1.26 ab
32.0	30.0	25.4	13.7	16.4	14.7	1.04 a

[a] Unsaturation ratio (UR) = (C 18:2% + C 18:3%)/(C 16%)

[b] Means followed by the same letter do not differ significantly at the 5% level by Duncan's Multiple Range Test.

Membrane composition study

An increased percentage of saturated fatty acids and a decreased percentage of unsaturated fatty acids were found in the soybean root membrane fraction with increasing calcium and nitrogen (Table 3). Calcium had the greatest effect on palmitic and linolenic acids. As calcium concentration was increased, linolenic acid decreased and palmitic acid increased. Nitrogen, however, had the greatest effect on stearic and linoleic acid levels. As nitrogen concentration increased, a decrease was seen in the amount of linoleic acid and an increase observed in stearic acid; only slight shifts were observed in palmitic and linolenic acids.

As a measure of overall shifts toward greater saturation or unsaturation, an unsaturation ratio was calculated as the percent weight contributed by linoleic plus linolenic acids divided by the percent weight contributed by palmitic acid. The unsaturation ratio (Table 3) clearly demonstrates a shift toward greater saturation with higher levels of calcium or nitrogen.

The effect of calcium or nitrogen on the fatty acid composition of the mitochondria was inconclusive (Table 4). There was little difference in the fatty acid composition between treatments, although an increase in the degree of saturation was evident as nitrogen concentration was increased. Linolenic acid content was much higher in the mitochondria than in the plasmalemma. Unsaturation ratios for the mitochondria were about four times greater than those for the plasmalemma. Previous studies (11, 12) reported the level of linolenic acid in the mitochondria to be about 25% and the unsaturation ratio approximately 2.15.

Table 4. Effect of calcium and nitrogen levels on fatty acid composition of soybean root mitochondrial phospholipids.

Treatment	Fatty acid composition (%)					Unsaturation Ratio[a,b]
(mM)	C 16	C 18	C 18:1	C 18:2	C 18:3	
Calcium						
0.4	10.7	2.6	2.2	21.1	63.4	7.90 a
4.0	7.7	0.5	1.8	15.2	74.9	11.70 a
8.0	11.4	1.4	2.5	22.2	62.4	7.42 a
Nitrogen						
4.0	5.3	3.6	1.3	10.3	79.4	16.93 a
16.0	7.7	0.5	1.8	15.2	74.9	11.70 a
32.0	8.4	4.7	3.8	12.5	70.8	9.92 a

[a] Unsaturation ratio (UR) = (C 18:2% + C 18:3%)/(C 16%)

[b] Means followed by the same letter do not differ significantly at the 5% level by Duncan's Multiple Range Test.

Increasing nitrogen concentration had little effect on the fatty acid composition of the mitochondria. Linolenic acid levels were also high yielding unsaturation ratios that were much greater than the plasmalemma. Palmitic acid levels were also quite low compared to previous reports (11, 12).

DISCUSSION

Uptake of linuron by soybean was dependent upon the amount of available calcium and, to a less extent, the amount of nitrogen available to the plant. Greatest linuron uptake occurred with the lowest concentration of calcium tested. Calcium has been reported to increase membrane thickness with increasing calcium concentration (10). Morre and Bracker (10) demonstrated increases in membrane thickness as calcium was increased from 0.0 to 0.5 M at which point additional calcium did not cause further increases in membrane thickness. The levels of calcium utilized in the present investigation correspond to the low range of calcium concentrations within which Morre and Bracker were observing a response in membrane thickness. Therefore, it is possible that the low rate of calcium utilized in this study produced plants with a thinner membrane than the higher rates of calcium resulting in greater permeability to linuron. Helgerson et al. (3) noted that IAA increased membrane viscosity as is reduced membrane thickness. Calcium, on the other hand, might decrease membrane viscosity since it rapidly increases membrane thickness (10), even at very low rates. Decreased membrane viscosity may enhance the penetration of linuron.

A physical effect by calcium or any other ion on the membrane may cause qualitative and/or quantitative changes in its lipid composition.

The effect can occur through a direct interaction with membrane enzymes, or through a perturbation of the membrane thus disrupting enzymic activities. In either case, lipid composition is altered. Treatment with the low rate of calcium produced plants with the low rate of calcium produced plants with the highest degree of unsaturation in the plasmalemma (Table 3). As calcium concentration was increased, however, the degree of unsaturation decreased. A similar effect was observed with nitrogen.

In the mitochondria high levels of linolenic acid were found regardless of treatment (Table 4). This is in contrast to previous reports (11, 12) where lower levels of linolenic acid and higher levels of palmitic acid were observed. The difference between the studies could be accounted for by the watering procedure. In the present study nutrient solution was added daily instead of a single fertilization as in the previous studies. Although the values were seemingly quite high, they were consistent in all treatments and replications.

Altered lipid composition due to nutritional level has also been reported by Kuiper et al (6). Low salt (0.2 mM $CaSO_4$) enhanced phospholipid content in oats. But the increase in phospholipid content may have also been accompanied by an increase in the percent of linolenic acid. A greater degree of unsaturation permits a membrane to retain its fluidity at low temperatures, but also influences membrane compaction (16). As the degree of unsaturation increases, membrane compaction decreases. The result may be increased membrane permeability (16). The increased uptake of linuron observed in soybeans treated with the low rate of calcium may be due to a combination of altered membrane composition and membrane structure.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Controlled environment studies were initiated to determine the influence of temperature on soybean root membrane lipid composition as affected by temperature shift and agricultural chemicals (ie., herbicides, antidote and ion concentration).

Mitochondrial and plasmalemma membrane fractions exhibited a rapid response to shifts in temperature between 15 and 30 C. As temperature was increased, the degree of saturated fatty acids was enhanced in both membrane fractions. The fatty acid composition of both membrane fractions was similar, however, the mitochondrial phospholipids were generally more unsaturated than the plasmalemma. As temperature was decreased, the reverse occurred; however, the lipid composition of the mitochondrial membrane responded more quickly than the plasmalemma. It was thought that mitochondria were metabolically more active at the lower temperature than the plasmalemma and hence would exhibit a quicker response.

The unsaturation ratio (taken as the percent weight contributed by linoleic acid plus linolenic acid divided by the percent contributed by palmitic acid) clearly demonstrated a significant increase in unsaturation in both membrane fractions within 48 hours, often within 24 hours, when the temperature was lowered from 30 to 22 C or from 22 to 15C.

Pre-emergence treatment of soybeans with either 2.24 kg/ha alachlor, 0.56 kg/ha trifluralin, 2.24 kg/ha H-22234, 3.36 kg/ha vernolate, or a combination of 0.14 kg/ha R-29148 and 2.24 kg/ha H-22234, 2.24 kg/ha alachlor or 3.36 kg/ha vernolate resulted in increased resistance to chilling injury. Analysis of the fatty acid composition of the membrane fractions demonstrated an increased degree of unsaturation in both membranes which would account for the lack of chilling injury. The mitochondria and plasmalemma did not always respond similarly to the same herbicide-temperature treatment. At 15 C, H-22234 increased the degree of unsaturation in the mitochondria, but decreased it in the plasmalemma.

Low calcium concentration (0.4 mM) enhanced the uptake of linuron in soybean seedlings grown in nutrient solution at pH 6.5. As calcium level was increased, linuron uptake decreased. The reverse was true for nitrogen.

Linuron uptake may be related to membrane lipid composition. Increasing calcium level decreased the degree of unsaturation in the plasmalemma and caused a shift from linolenic acid to palmitic acid. Nitrogen also decreased the degree of unsaturation, however, a fatty acid shift was observed from linoleic to stearic acid. In the mitochondria, the shifts were not clear. Enhanced levels of linolenic acid were observed but it was not clear whether the effect was due to treatment or analysis.

In conclusion, many of the observed effects of herbicides on plants may ultimately be related to herbicide interaction with membranes, in particular the plasmalemma. Every herbicide must pass through the plasmalemma to reach its site of action. This may occur as a diffusion of the herbicide through the membrane (which will be a function of the lipid composition of the membrane, its physical state, and the lipid solubility of the herbicide), or as an interaction of the herbicide with the membrane (probably through a binding mechanism). In the latter case membrane-bound enzymes and/or cellular metabolism are affected as a result of an alteration in membrane permeability. The results of this study clearly demonstrate a herbicide-induced alteration of membrane lipid composition, the consequence of which may relate to modification of chilling resistance, uptake of other herbicides or ions, or uptake of the same herbicide. Furthermore, the rapidity of lipid compositional response to altered temperature may explain the increased phytotoxicity of some herbicides in the field with high or low temperatures.